Conformational Switches Involved in Orchestrating the Successive Steps of Group I RNA Splicing†

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ABSTRACT: Group I introns possess a conserved guanosine residue at their 3′ end, termed ω G, that, in the case of the *Tetrahymena* pre-rRNA, is a major determinant of the second step of splicing. We examined the role of ω G in self-splicing of the 249-residue group I intron of the *Anabaena* PCC7120 $tRNA^{leu}$ precursor. Contrary to observations with the *Tetrahymena* pre-rRNA intron, a mutation that places an adenosine residue at the ω position did not have a severe effect on the second step of splicing; neither 3′ splice-site selection nor the rate of the second step was altered. The first step of splicing, however, was now readily reversed. This unexpected effect also resulted from a mutation that altered the nucleoside specificity of the intronic guanosine-binding site. The theme common to these mutations is that reversal of the first step of splicing results when there is not a strong interaction between the guanosine-binding site and the ω residue. This suggests that a major role of ω G is to compete with the exogenous guanosine molecule added to the intron in the first step of splicing for the single guanosine-binding site of the intron. From these data, we are able to extend the mechanism for the self-splicing reaction of this intron by proposing two distinct conformational changes between the first and second steps of splicing. The first of these is the exchange of the exogenous nucleoside for the ω nucleoside. This is the equilibrium that we can perturb by mutations at either the ω position or the guanosine-binding site. An additional conformational change then fully activates the intron for the second step of splicing.

The $tRNA^{leu}$ precursor of *Anabaena* PCC7120 contains a 249-residue group I intron (Figure 1A) inserted between the wobble and second bases of the anticodon (Kuhsel et al., 1990; Xu et al., 1990). It is a member of a rapidly growing family of small, self-splicing introns found in a wide variety of tRNAs. This intron is of particular interest for structural work and for determining minimal core requirements because, in spite of its small size compared to the well-studied *Tetrahymena* large ribosomal subunit (LSU)[1] rRNA intron at 413 nucleotides, it retains efficient splicing activity in the absence of accessory proteins and under physiological magnesium concentrations (Zaug et al., 1993). A multiple-turnover ribozyme has also been constructed from this intron, and a kinetic framework for its endonucleolytic cleavage of small RNA substrates has been developed (Zaug et al., 1994).

All group I introns excise themselves and ligate the surrounding exons in two sequential transesterification steps (Figure 1B). In the first step, an exogenous guanosine molecule is bound by the intron; the 3′-hydroxyl group of this molecule attacks at the 5′ splice-site, leaving the guanosine residue attached to the intron and a free 3′-hydroxyl on the 5′ exon. During the second step of splicing, a conserved guanosine residue at the 3′ terminus of the intron (ω G) is bound by the intron, and the 5′ exon then acts as a nucleophile, ligating the exons and releasing the linear intron. This reaction is chemically equivalent to the reverse of the first step of splicing (Inoue et al., 1986). Mutations at a single base pair within the core of the intron alter the nucleotide specificity for both the exogenous nucleoside and the ω position (Michel et al., 1989; Been & Perrotta, 1991). Thus, the exogenous guanosine and the ω G are thought to be bound by a single active site. This necessitates a conformational change after the first step of splicing and prior to the second step, in which the exogenous guanosine at the 5′ end of the intron dissociates from the active site and ω G is bound.

The *Anabaena* intron also exhibits hydrolysis at the 3′ splice-site which results in circularization of the intron (Inoue et al., 1986; Zaug et al., 1993). This unproductive hydrolysis reaction is likely initiated when ω G becomes bound by the G-binding site prior to the first step of splicing (van der Horst & Inoue, 1993).

Except for its propensity to circularize in the absence of guanosine, the *Anabaena* intron is an excellent candidate for structural studies. To prevent this side reaction, we made mutations at the ω position of the *Anabaena* intron (G249) and observed how these affected both the second step of splicing and the circularization reaction. These mutant introns lost the ability to circularize readily but unexpectedly retained the ability to splice. Thus, ω G is not the primary determinant for reactivity at the 3′ splice-site or for the occurrence of the second step of splicing. In the absence of an ω G residue, however, we found that the first step of splicing was severely affected, becoming readily reversible.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; k_{cat}, maximum rate of the first step of splicing at saturating nucleotide substrate; Hepes, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; LSU, large ribosomal subunit; Tris, tris(hydroxymethyl)aminomethane.

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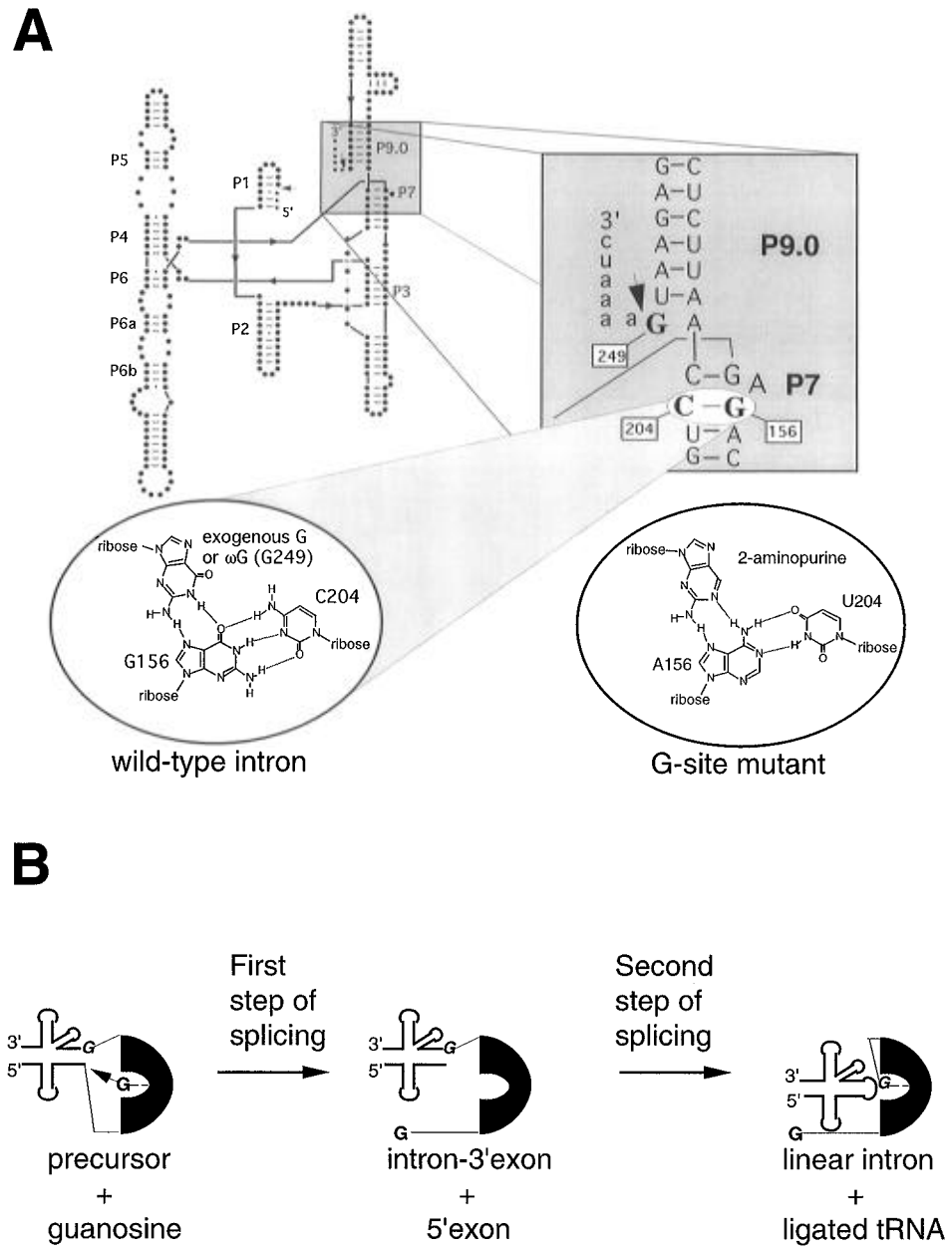

FIGURE 1: (A) *Anabaena* intron and its G-binding site. The secondary structure of the *Anabaena* $tRNA^{leu}$ intron is drawn according to Cech et al. (1994). Each dot represents a single nucleotide, and the splice-sites are indicated by arrows. Highlighted by the shaded box and shown in detail is the P7/P9 junction which includes the guanosine-binding site and ωG (G249). Drawn in the white oval is the interaction between the G-binding site and the exogenous guanosine nucleophile or ωG. Note that the three nucleotides are not planar as drawn in this schematic (Yarus et al., 1991a). On the right is a diagram of the G-site mutant and its interaction with 2-aminopurine. (B) Group I intron splicing. Splicing proceeds by two sequential transesterification steps. In the first step, a guanosine molecule attacks the 5′ splice-site, becoming covalently attached to the intron. In the second step, the 5′ exon attacks at the 3′ splice-site, ligating the two exons and releasing the linear intron.

A strong interaction between the nucleotide in the ω position and the G-binding site appears to be required to prevent the efficient reversal of the first step of splicing. This role requires guanosine at the 3′ terminus of the intron, at least in the case of the *Anabaena* intron, and may contribute to the conservation of guanosine at this position in the group I introns.

MATERIALS AND METHODS

Mutagenesis. Mutations at position 249 of the *Anabaena* intron were constructed by oligonucleotide-directed mutagenesis using the method of Kunkel et al. (1987). A double mutant, G156A:C204U, was constructed by the method of Deng and Nickoloff (1992) using a selection oligonucleotide that deleted a unique *Hin*dIII site on the plasmid. Briefly, the mutagenic DNA oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer, and phosphorylated using ATP and T4 polynucleotide kinase. The oligonucleotides were annealed to deoxyuridine-containing, single-stranded circular DNA derived from the plasmid pAtRNA-1 or double-stranded, alkali-denatured pAtRNA-1 (Zaug et al., 1993). T4 DNA polymerase, T4 DNA ligase, and dNTPs were added to synthesize the second strands. The plasmid containing the double mutation was selected for by *Hin*dIII digestion and was passaged through *Escherichia coli* BHM71−18 *mut* S^- cells. The resultant double-stranded DNA was used to transform competent *E. coli*, strain XL-1 Blue, using a Bio-Rad gene pulser set to 1.8 kV and 400 Ω.

Plasmids that contained the mutations were identified by restriction endonuclease digestion and sequencing.

Transcription of Precursor RNAs. Transcription reactions typically contained 4 mM ATP, CTP, GTP, and UTP, 40 mM Tris, pH 7.5, 5 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 20−50 mg/mL plasmid DNA linearized with *Ear*I, and T7 RNA polymerase purified from *E. coli* strain BL21 pAR1219 (Davanloo et al., 1984). For uniformly labeled RNA, 50 μCi of [α-^{32}P]ATP (New England Nuclear) was also included. Reactions were incubated at 37 °C for 2−8 h and quenched by the addition of stop buffer containing 50 mM EDTA, 95% formamide or 10 M urea, bromophenol blue, xylene cyanol, and 0.1 × TBE. TBE is 0.1 M Tris base, 0.83 M boric acid, and 1 mM EDTA. RNA was purified by gel electrophoresis on a 6−8% polyacrylamide gel [all polyacrylamide gels contain 7 M urea, 1.0 × TBE, 29:1 acrylamide:bis(acrylamide)]. Full-length precursor RNA was identified by UV-shadowing or autoradiography. To gel-purify RNAs, the bands containing the material were excised and crushed, and the RNA was eluted into TEN (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 250 mM NaCl). Following removal of the polyacrylamide by filtration, the RNA was recovered by ethanol precipitation and dissolved in a minimal amount of H_2O. The concentration of unlabeled RNA was determined by the UV absorbance.

Splicing Reactions. Uniformly ^{32}P-labeled precursor was preincubated in splicing buffer (25 mM NaHepes, pH 7.5, and 15 mM $MgCl_2$) at 50 °C for 15 min. Following equilibration at 32 °C for 2 min, splicing was initiated by the addition of guanosine, adenosine, or 2-aminopurine ribonucleoside in splicing buffer. Guanosine was purchased from Sigma and used without further purification. Adenosine and 2-aminopurine ribonucleoside were purchased from Sigma and R. I. Chemical, Inc., respectively, and purified by preparative thin-layer chromatography on silica plates (the mobile phase consisted of 90% 1-propanol/10% NH_4OH), where guanosine remains near the origin (McConnell & Cech, 1995). At the indicated times after the addition of nucleoside, portions were removed, and the splicing reactions were stopped by the addition of stop buffer. The RNA products were then separated on 6% polyacrylamide gels. The gels were dried and exposed to phosphor storage screens, which were imaged and quantitated using a Molecular Dynamics Phosphorimager. The data were initially plotted as log F vs t, where F = fraction precursor remaining = p/total counts; p = counts in precursor; total counts equals counts in the precursor + counts in intron−3′ exon + counts in linear intron + counts in tRNA. The kinetics of the splicing reaction were biphasic with over 90% of the RNA reacting quickly and 5−10% reacting slowly. To correct for the slowly reacting fraction such that only the more active fraction was analyzed, the data were then normalized: fraction of precursor (normalized) = $(F - F_{slow})/(1 - F_{slow})$. When the ωA intron spliced, an additional, guanosine-dependent side reaction occurred in which the intron−3′ exon intermediate cleaved a cryptic 5′ splice-site within the 3′ exon, circularized and subsequently linearized the circle. To account for these products, the counts in bands corresponding to these circular and linear products were included in the total counts. Rate constants were obtained by fitting the normalized data to an exponential curve (KaleidaGraph v. 3.0, Abelbeck Software). To obtain values for k_{cat} and K_M, the observed rate constants were plotted as a function of nucleoside concentration. These data were then fitted to the Michaelis−Menten equation to obtain values for the constants (KaleidaGraph v. 3.0, Abelbeck Software).

Sequencing the Ligated Exons. The tRNA product was generated by initiating a splicing reaction as described above with 50 μg of unlabeled precursor RNA and 2 mM guanosine in 50 μL total volume. Reactions were continued at 32 °C for 15 min, quenched with 50 μL of stop buffer, and gel-purified (as above) on an 8% polyacrylamide gel. The tRNA was resuspended in H_2O to a concentration of approximately 1 μg/μL. The DNA primer for reverse transcription (AEP64−20) is complementary to residues 44−64 of the tRNA. AEP64−20 (0.83 pmol) was 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and annealed to ~1 μg of tRNA in 0.5 M Tris-HCl, pH 8.3, 0.6 M NaCl, and 0.01 M DTT by heating the mixture briefly to 95 °C and subsequently cooling to room temperature. The annealed mixture was divided into five aliquots to which dideoxyA, -C, -G, or -T or no dideoxynucleotide was added. Reverse transcription was initiated by the addition of AMV reverse transcriptase (Life Sciences) and dNTPs (Pharmacia). The reactions proceeded at 60 °C for 15 min and were stopped by the addition of an equal volume of stop buffer. The products were separated on an 8% polyacrylamide sequencing gel and analyzed by autoradiography.

Identification of 5′ Nucleotide. The nucleotide at the 5′ end of the linear intron product was identified in the following manner. Unlabeled precursor RNA, 10 μg, was preincubated in splicing buffer as for a splicing reaction. Either adenosine or guanosine was added to a final concentration of 2.5 mM or 25 μM, respectively. Reactions were incubated at 32 °C for 2 h. T4 polynucleotide kinase, [γ-^{32}P]ATP, and kinase buffer were then added to the reaction to phosphorylate the 5′ end of the linear intron splicing product (the only high molecular weight species with a free 5′-hydroxyl group). The reaction was quenched by the addition of an equal volume of stop buffer, and the products were resolved on a 6% polyacrylamide gel. The radiolabeled product was identified by autoradiography and purified from the gel matrix as described above. The RNA was digested to completion with nucleases P1 (in water), T1, U2, and T2 (in 0.83 M urea, 2.5 mM sodium citrate, and 0.125 mM EDTA), and the products were separated on a 24% polyacrylamide gel.

Isolation and Reaction of Splicing Intermediates. The splicing intermediate, intron−3′ exon, for the wild-type and mutant introns was accumulated by preincubating radiolabeled precursor RNA in splicing buffer for 15 min at 50 °C, and equilibrating at 32 °C for 2 min. The RNA was reacted with saturating nucleoside (1 mM guanosine for the wild-type and ωA introns, 5 mM 2-aminopurine ribonucleoside for the G-binding site mutant) in splicing buffer for 1 min, and the reaction was stopped by the addition of EDTA to 50 mM. RNA was separated from the nucleoside, EDTA, and magnesium using a Quick Spin G-25 column (Boehringer Mannheim). In this manner, 60−80% of the RNA was isolated as the intron−3′ exon splicing intermediate. The intermediates were then reannealed in splicing buffer at 50 °C for 5 min. In the reactions of the ωA and G-site mutants, this procedure generated a higher molecular weight RNA that comigrates with the precursor tRNA. Therefore, 1 mM guanosine or 5 mM 2-aminopurine ribonucleoside was added

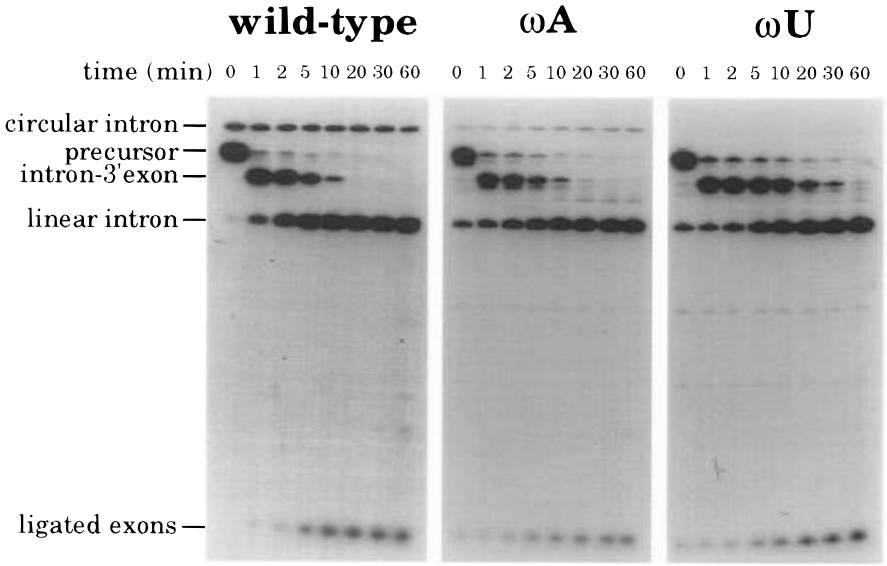

FIGURE 2: ω mutants of the *Anabaena* intron can splice. Splicing of uniformly ^{32}P-labeled precursor tRNAs. The precursor tRNAs containing either the wild-type, ωA, or ωU intron were preincubated in 15 mM $MgCl_2$, 25 mM Hepes, pH 7.5, for 15 min at 50 °C to renature the RNA, and the reaction mixtures were equilibrated at 32 °C for 2 min prior to the addition of nucleoside. Splicing was then initiated by the addition of guanosine to a final concentration of 2 mM. Portions of the reaction mixture were removed at the indicated times and stopped, and the products were analyzed on a 6% polyacrylamide gel. In some preparations, small amounts of linear intron and ligated exon products appeared in the $t = 0$ lanes. Thus, the starting point for the reaction was not 100% precursor, and this can be seen in subsequent plots of the data. Minor bands migrating slightly faster than the precursor and intron−3′ exons were not characterized. As indicated by the formation of linear intron and tRNA products, the ω mutants were capable of performing the second step of splicing.

to the ωA and G-site product RNAs, respectively, and these reactions were incubated for 60 min at 32 °C. The products were analyzed by polyacrylamide gel electrophoresis as described for splicing reactions.

Pulse−Chase Reactions with ^{32}P-Labeled Guanosine. About 50 pmol of unlabeled precursor RNA was preincubated in 4 μL of splicing buffer for 15 min at 50 °C. The reaction was initiated by the addition of 1 μL of [α-^{32}P]-GTP (3 pmol). After 1 min, 5 μL of 2 mM guanosine in splicing buffer was added to the reaction to dilute the unincorporated labeled GTP. At the indicated times, aliquots were removed and added to stop buffer. Radioactivity incorporated into high molecular weight products was analyzed as above for the splicing reactions.

RESULTS

ω Mutations Have Little Effect on the Rate of the Second Step of Splicing. The self-splicing construct of the *Anabaena* pre-tRNA^{leu} contains a 249-residue intron with a full-length 85-residue tRNA^{leu}. The 5′ and 3′ exons are 34 and 51 residues, respectively (Zaug et al., 1993). This precursor is purified under denaturing conditions and is renatured by heating to 50 °C in the presence of magnesium ion. During renaturation, a circular form of the intron is produced, at levels up to 10% of the total RNA, by site-specific hydrolysis at the 3′ splice-site followed by attack at the 5′ splice-site by ωG (G249 in the *Anabaena* intron) (Zaug et al., 1993). This results in five distinct products of the preincubation: 5′ exon, 3′ exon, circular intron, linear intron (because the circle is labile), and the intact precursor. We wanted a more homogeneous system in which to perform structural studies. Therefore, to prevent this side reaction, ωG of the *Anabaena* intron was mutated into an adenosine (designated ωA) or a uridine (ωU). We anticipated that, in addition to reducing the circularization reaction, these mutations would inhibit the second step of splicing.

The mutants were initially evaluated under conditions identical to those used in prior work on the *Anabaena* intron. As expected, formation of the circular intron during the preincubation at 50 °C was drastically reduced as compared to the wild-type intron (Figure 2). Even at long timepoints of the splicing reaction, only small amounts of the circular form of the intron (typically <1% of the products) were observed. Surprisingly, the second step of splicing was not severely affected by the mutations. Upon reaction of the mutants with saturating guanosine concentrations (1−2 mM), the linear intron product appeared with the same rate constant as with the wild-type RNA (0.3 min^{-1}). Also, tRNA product was formed with a rate constant comparable to that observed with the wild-type pre-tRNA. Thus, the reaction was splicing and not merely hydrolysis at the splice-sites. Dilution of the nucleophile after the first minutes of the splicing reaction did not speed the second step of splicing, indicating that exon-ligation was not inhibited by high concentrations of free guanosine, even with the ω mutants (data not shown).

Missplicing by the ωU Mutant. When the products of the wild-type, ωA, and ωU splicing reactions were run side-by-side on a denaturing gel, it was apparent that the ligated tRNA produced by the ωU mutant was slightly smaller than the tRNA generated by the wild-type and ωA introns. When ligated exons were gel-purified and sequenced as described under Materials and Methods, it was confirmed that tRNA generated by the ωU mutant is missing a single nucleotide at the 5′ end of the 3′ exon (data not shown). This meant that the ωU mutant was using the first adenosine residue in the 3′ exon as the terminal residue in the intron, becoming, effectively, an ωA intron. Because the ωU mutant mis-spliced in this manner, it was not investigated further.

Splicing of the Wild-Type Intron with Adenosine Is Poor. The precursor RNA appeared to splice readily with adenosine at the ω position and shifted to an adenosine when a uridine was in the ω position. This suggested that the G-binding

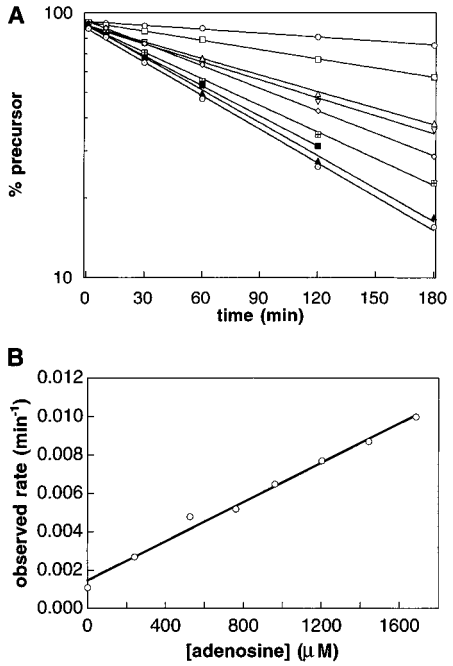

FIGURE 3: (A) Slow reaction of the wild-type pre-tRNA with adenosine. Semi-log plot showing the disappearance of precursor with time at the indicated adenosine concentrations. Splicing reactions were performed at 32 °C under standard conditions (15 mM $MgCl_2$, 25 mM Hepes, pH 7.5). Splicing with 240 μM (□), 530 μM (△), 760 μM (▽), 960 μM (◇), 1200μM (plus sign inscribed in open square), 1400 μM (■), 1700 μM (▲), or 1900 μM (⊙) adenosine. The reaction of the RNA with adenosine is slow, but accelerated as compared to the loss of precursor in the absence of nucleoside (○). (B) k_{cat}/K_M determination for the wild-type *Anabaena* intron splicing with adenosine. Values of k_{obs} for the first step of splicing were determined by fitting the plots in (A) to exponential curves. The dependence on adenosine concentrations in this range is linear, and the slope of this line gives a k_{cat}/K_M of 5 M^{-1} min^{-1}.

site of the intron had some ability to bind and recognize adenosine. The reaction of the precursor with exogenous adenosine as the nucleophile was therefore investigated. Several precautions were taken to ensure that we were observing splicing with adenosine and not a small amount of guanosine contaminant. The nucleoside was purified prior to splicing (see Materials and Methods), and, after the splicing reaction, the nucleotide at the 5′ end of the linear intron product was positively identified as adenosine (data not shown; see Materials and Methods). This demonstrated that the slow reaction of the precursor with this nucleoside was not due to contamination by a small amount of guanosine. Because the first residue in the intron was also an adenosine, this experiment does not rule out the possibility that the adenosine was somehow acting to increase the rate of site-specific hydrolysis at the 5′ splice-site.

Cleavage at the 5′ splice-site in the presence of adenosine was slow with a k_{cat}/K_M of only 5 M^{-1} min^{-1} (Figure 3A, B). This value is 10^4-fold lower than the k_{cat}/K_M for the reaction of the *Anabaena* intron with guanosine. These results indicated that the wild-type *Anabaena* intron has little specificity for adenosine.

ω Mutations Affect the First Step of Splicing. The reaction of the ωA mutant was followed over a wider range (10 μM−2 mM) of guanosine concentrations. The kinetic profile of the first step of the reaction was drastically altered compared to that of the wild-type intron. There was an initial burst of disappearance of precursor that occurred with the same rate constant as that of the wild-type RNA (Figure 4A). The amplitude of this burst phase increased with guanosine concentration (Figure 4B). Following this initial burst was a slow phase characteristic of the ωA mutant with a rate that was also guanosine-dependent (Figure 4B). At saturating guanosine (1−2 mM), the burst amplitude was >90%, causing the reaction to appear like that of the wild-type intron.

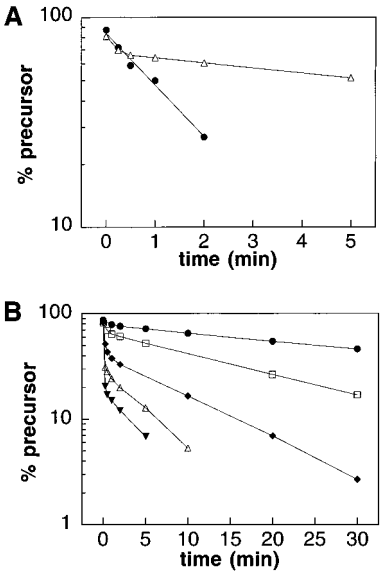

FIGURE 4: (A) Splicing by the ωA mutant shows biphasic kinetics. Disappearance of precursor during reaction with 25 μM guanosine is plotted for the ωA mutant (△) and for the wild-type intron (●). Characteristic of the ωA mutant is a burst phase that reacts at the same rate as the wild-type followed by a leveling off of activity. (B) Burst is guanosine-dependent. Disappearance of precursor as the ωA intron splices with 10 μM (●), 25 μM (□), 50 μM (◆), 100 μM (△), or 500 μM (▼) guanosine.

A G-Binding Site Mutant Behaves Like the ωA Mutant. The ω position interacts with the G-binding site of the intron during the second step of splicing. Therefore, it seemed possible that the effects on the first step of the splicing reaction were caused by the disruption of this interaction. If this were true, then changing the nucleotide specificity of the G-binding site of the intron while retaining the guanosine residue at the ω position should result in an intron with characteristics similar to those of the ω mutants. Such an intron was created by changing the G:C base pair at the G-binding site into an A:U base pair. The double mutant G156A:C204U (Figure 1) is referred to in this paper as the G-site mutant.

This G-site mutant reacted very poorly in the absence of nucleoside or with guanosine but was active using 2-aminopurine ribonucleoside (Figure 1) as previously demonstrated with the *Tetrahymena* intron (Michel et al., 1989; Yarus et al., 1991b; Legault et al., 1992). The reaction of the *Anabaena* G-site mutant with 1 mM guanosine occurred with a rate constant of 0.007 min^{-1}, only 7-fold greater than the rate constant for hydrolysis (0.001 min^{-1}). In contrast, the reaction with 1 mM 2-aminopurine ribonucleoside as the nucleophile was quite rapid, progressing with a rate constant of 2.5 min^{-1} (Figure 5A). Again, the reaction profile of the first step of splicing revealed nucleoside-dependent burst and slow phases (Figure 5B). The reaction profiles of splicing of the G-site mutant with 2-aminopurine ribonucleoside were very similar to those of the ωA mutant with guanosine,

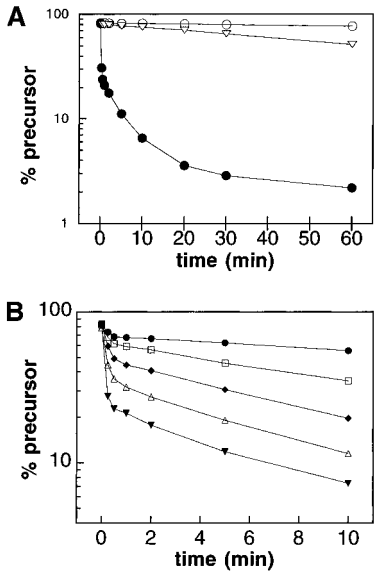

FIGURE 5: (A) G-site mutant has specificity for 2-aminopurine ribonucleoside. The reactions of the G-site mutant with (○) no nucleoside, (▽) 1 mM guanosine, and (●) 1 mM 2-aminopurine ribonucleoside are shown. The intron reacts very poorly in the absence of nucleoside cofactor or with guanosine, but the reaction is quite rapid with 2-aminopurine ribonucleoside. (B) Kinetic profile of the G-site mutant resembles that of the ωA mutant. Reaction of the precursor in the presence of 50 μM (●), 100 μM (□), 200 μM (◆), 500 μM (△), and 1 mM (▼) 2-aminopurine ribonucleoside reveals that this mutant also exhibits a nucleoside-dependent burst phase in the first step of splicing. In these reactions, the rate of the disappearance of precursor cannot be described by a simple exponential; thus, the lines connecting the data points do not represent a curve fit.

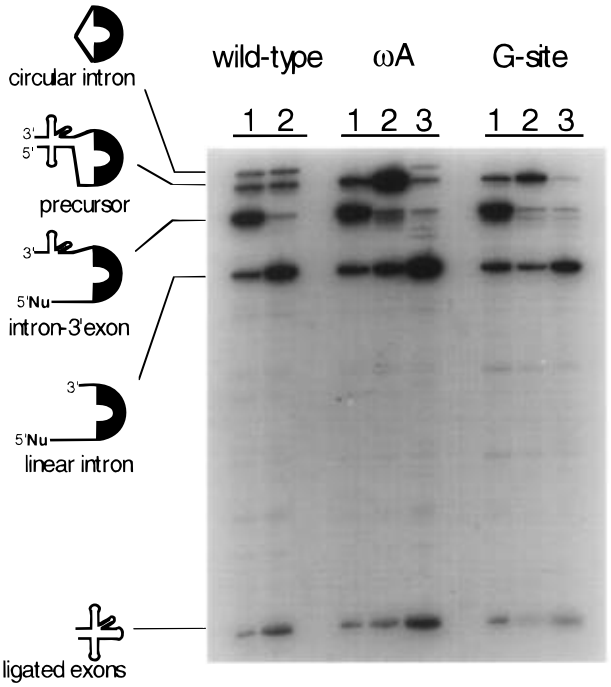

FIGURE 6: Reverse splicing of isolated splicing intermediates. The first lane in each group (lanes marked 1) is the starting sample of the intron−3′ exon intermediate isolated as described in the text. This material was renatured by heating the RNA with 15 mM $MgCl_2$ at 50 °C for 5 min, and the products of this reaction are shown in lane 2 of each group. With the ωA and G-site mutants, a higher molecular weight product was formed during this procedure. To determine if this was intact precursor, saturating concentrations of guanosine or 2-aminopurine ribonucleoside were added to the ωA or G-site samples respectively. After 60 min at 32 °C, all of the higher molecular weight material had spliced to form expected linear intron and tRNA products (lanes 3).

presumably for the same reason: the active site does not bind the ω nucleotide well.

It was possible to estimate the kinetic parameters k_{cat} and K_M for the G-site mutant reacting with 2-aminopurine ribonucleoside by plotting the rate constant for the burst phase against the nucleoside concentration (not shown). The estimate of k_{cat}, ~7 min^{-1}, is approximately equal to the k_{cat} determined for wild-type intron reacting with guanosine (Zaug et al., 1993) while the K_M, ~1000 μM, is 4-fold higher. The best estimate of k_{cat}/K_M comes from the slope of the linear portion of this graph; the value of 4100 M^{-1} min^{-1} is in reasonable agreement with 7000 M^{-1} min^{-1} obtained by dividing the estimate of k_{cat} by the estimate of K_M. These values are about 10-fold lower than those for the wild-type intron reacting with its native substrate, guanosine (Zaug et al., 1993). Similarly, in the case of the *Tetrahymena* intron, the same comparison gives k_{cat}/K_M to be ~20-fold lower for the G-site mutant reacting with its preferred nucleophile (Michel et al., 1989; Legault et al., 1992).

ωA and G-Site Mutant Introns Undergo a Reversal of the First Step of Splicing. Burst and slow phases observed in the profile of the first step of splicing can indicate that this step is readily reversed. To directly test if reversal of the first step of splicing can take place, the intron−3′ exon splicing intermediate was accumulated by reacting the precursor RNA with saturating nucleoside for 1 min and then stopping the reaction by the addition of EDTA. Because the second step of splicing is slow relative to the first step, this preparation resulted in a sample that largely consisted of the intermediate (Figure 6, lanes 1). The nucleoside, magnesium ion, and EDTA were separated from the RNA (as described under Materials in Methods) which was then renatured in the absence of nucleoside. When treated in this manner, the intermediate from the wild-type intron resumed splicing to form the linear intron and tRNA products. In contrast, the intermediate from the two mutants (ωA and G-site) religated with the 5′ exon to form the precursor, a reversal of the first step of splicing (Figure 6, lanes 2). When saturating concentrations of nucleoside were then added to the products of the reverse reactions, the splicing reactions proceeded to completion, and the expected linear intron and tRNA products were observed (Figure 6, lanes 3). This demonstrated that the higher molecular weight species formed during the reverse reaction was intact precursor.

Measuring the Rate of Reverse Splicing. We wished to measure the rate of reverse splicing without denaturing the RNA as required to isolate the splicing intermediate. One means of doing this is to perform a pulse−chase experiment to determine the fate of the radiolabeled nucleotide that becomes covalently attached to the intron in the first step of splicing. Unlabeled precursor RNA was reacted with [α-^{32}P]GTP for 1 min. This procedure radiolabeled the intron−3′ exon splicing intermediate and a small amount of linear intron product. Unlabeled guanosine was then added in great excess, and the fate of the radiolabeled splicing intermediate was observed. If the reverse reaction occurs, the radiolabeled guanosine will be released, and the religated precursor will react with the unlabeled nucleoside and will no longer be observable.

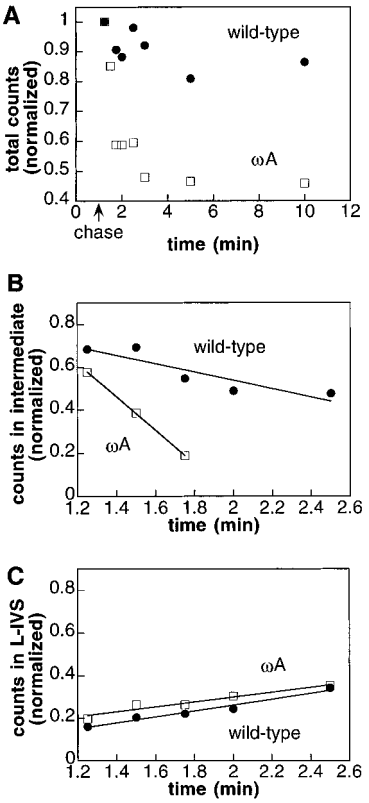

FIGURE 7: Reverse splicing monitored by guanosine loss. Unlabeled precursor RNA was pulsed with [α-^{32}P]GTP for 1 min, followed by a chase with unlabeled guanosine. Radiolabeled GTP is incorporated into the intron−3′ exon intermediate and linear intron product by the splicing reaction. (A) Guanosine radiolabel incorporated into high molecular weight products. The number of counts observable in high molecular weight products following the addition of unlabeled guanosine is plotted. The points are normalized to the amount of counts at the first timepoint, and there is no correction for errors in loading. Reproducibly, >80% of the label is retained in the wild-type splicing products following the chase but <50% is retained by the ωA mutant. (B) Loss of radioactivity from the intron−3′ exon intermediate. The number of counts in the intron−3′ exon, normalized to the total number of counts in all RNA species in the first timepoint shown, is shown on a linear plot. The intermediate of the ωA mutant disappears at a rate 4−5-fold faster than that of the wild-type RNA. (C) Accumulation of radiolabeled product. The linear intron is produced at the same rate by both the wild-type and ωA introns.

When this experiment was performed with the wild-type intron, the radiolabeled intermediate was nearly quantitatively converted into linear intron. Reproducibly, >80% of radiolabeled guanosine was retained in high molecular weight species in the presence of the unlabeled guanosine (Figure 7A). With the ωA mutant, however, the amount of radioactivity incorporated into high molecular weight products declined following the addition of cold guanosine, indicating that the reverse reaction occurred to a significant extent.

With the *Anabaena* RNA, the second step of splicing is rate-determining for the overall splicing reaction, and this allows the rate of the second step of splicing to be measured by monitoring the loss of intermediate and the accumulation of linear intron product. With the wild-type RNA, the rate constant for the second step of splicing is 0.3 min^{-1} (Zaug et al., 1993). When this pulse−chase experiment was performed with the ωA mutant, radiolabeled intermediate disappeared 4−5 fold faster (1.2−1.5 min^{-1}) than with the wild-type intron. In contrast, the linear intron products of the wild-type and ωA RNAs accumulated with the same rate constants which were, within error, the same as for the disappearance of wild-type intermediate (Figure 7B,C). This suggested that the rate of the second step of splicing was not altered by the ωA mutation, but the reverse of the first step of splicing was rapid with the intermediate being converted back into the precursor with a minimum rate constant of ∼0.9 min^{-1} (calculated as 1.2 min^{-1} to 0.3 min^{-1}).

DISCUSSION

ωG Is Not a Major Determinant of the Second Step of Splicing. In the context of the *Tetrahymena* intron, mutations at the conserved 3′-terminal guanosine residue severely reduce the rate of the second step of splicing (Price & Cech, 1988; Been & Perrotta, 1991; Suh & Waring, 1993). In contrast, we found that changing the identity of the ω position of the *Anabaena* intron had little effect on the rate of the second step. Unlike the first step of splicing of the *Anabaena* intron, the rate of the second step shows little pH-dependence (A. J. Zaug and T. R. Cech, unpublished results), suggesting that it is rate-limited by a conformational change occurring with a first-order rate constant of 0.3 min^{-1}. We conclude that ωG is not a major determinant of this conformational change. Nor is ωG necessary for efficient 3′ splice-site reactivity, since ωA also functions well.[2] ωG is surrounded by many structural elements, including P7, P9.0, P10, the anticodon stem of the tRNA, and P1. These helices, and perhaps additional interactions that have not yet been identified, are likely to be the dominant factors that serve to activate the intron for the second step of splicing.

The effects of both adenosine and uridine at the ω position were investigated. While an adenosine substitution spliced normally, a uridine residue was excluded from the active site in favor of a downstream adenosine residue. One possible explanation involved the *Anabaena* intron being capable of specifically interacting with adenosine as well as guanosine, a phenomenon not observed with other group I introns (Inoue et al., 1986; Yarus et al., 1991b). This possibility was investigated by examining the reaction of the intron with free adenosine as the exogenous nucleoside. While this reaction was faster than the rate of hydrolysis, it was still very slow and required high concentrations of the nucleoside (the k_{cat}/K_m was reduced 10^4-fold relative to the reaction with guanosine). This suggests that the ability of the ωA intron to splice normally and the missplicing by the ωU intron may be attributed to an ability to use purines over pyrimidines at the ω position rather than a high affinity for adenosine.

Reversibility (Unsplicing). When we examined how the ωA intron behaved in the first step of splicing, it was apparent that this intron was actually severely affected by the mutation in a manner that we had not predicted. An initial burst of activity occurred with the same rate constant as that for wild-type intron, but this was followed by a slower phase that was atypical of the *Anabaena* intron. It seemed likely that the characteristics of the ωA mutant were due to a disruption of the interaction between the ω position and the guanosine-binding site of the intron. To investigate this

[2] Any effect that this mutation may have on the chemical step of the exon-ligation reaction would be hidden by the rate-limiting step (although a drop in this activity to below 0.3 min^{-1} would have been observed).

possibility, the other half of the interaction was probed by constructing an intron with an altered nucleoside-binding site. This mutation, in which the G:C base pair at the G-binding site is converted into an A:U base pair, is known to confer specificity for 2-aminopurine ribonucleoside on group I introns (Michel et al., 1989). We found the reaction of the G-site mutant with 2-aminopurine ribonucleoside to be very similar to that of the ωA intron with guanosine; burst and slow phases were observed in the kinetic profile of the first step of splicing while the second step occurred with the same rate constant as that for the wild-type RNA.

There are several possible reasons for the altered kinetic profile of the first step of splicing, but the explanation that best describes the data is an unusually high rate of reversal of the first step of splicing in the ωA and G-site introns. In this model, the size of the burst phase corresponds to the portion of the reaction prior to the appearance of significant concentrations of intermediate, and the rate constant for this reaction is that of the wild-type intron. With the two mutants, a significant rate of the reverse reaction results in a slower apparent rate constant once the splicing intermediate is present, and therefore they exhibit a slow phase. We were able to observe directly the religation of the intron−3′ exon intermediate with the 5′ exon in the case of the mutant introns. In contrast, with the wild-type intron, this reverse reaction was inconsequential, and the isolated intermediate was rapidly converted into the linear intron and spliced tRNA products.

Development of a Mechanism. Group I introns use a single guanosine-binding site in both the first and second steps of splicing (Been & Perrotta, 1991). The exogenous guanosine that becomes covalently attached to the intron in the first step must exit the active site to allow the ωG to bind for the second step. In this pathway, after the first step of splicing and prior to the second step, a conformational change must occur in which the exogenous guanosine exits the G-binding site and the nucleotide at the ω position enters it. The second step of splicing shows little pH-dependence, suggesting it is, in fact, rate-limited by a conformational change (A. J. Zaug and T. R. Cech, unpublished results); in principle, this might or might not be the exchange of guanosine residues at the G-binding site.

We believe that a single conformational change between the first and second steps of splicing cannot explain the data presented in this paper. At least two conformational changes are required for the following reasons. (1) The rate-limiting conformational change prior to the second step of splicing appeared to be unaffected in the ωA and the G-site mutations. This is inconsistent with this rate constant being governed by an interaction between the ω position and the G-binding site. (2) If there is only a single conformational change that converts the exogenous G-bound state to the ω-bound state, then it can be assigned the previously observed rate constant of 0.3 min^{-1}. As this rate constant was not affected by the mutations, the exogenous G-bound state would be equally long-lived in the wild-type and mutant introns. The rapidity with which the mutants revert from splicing intermediate to intact precursor would then be due to a change in the rate of the chemical step of this reaction. However, a direct effect on the chemistry of the reverse of the first step of splicing is unlikely: the ω nucleotide is not directly involved in the first step, and we showed that distinct mutations at the ω position and G-binding site exhibited reversibility. Furthermore, the rate constant for the chemical step of the forward reaction was unaffected by the ωA mutation. Therefore, to account for the differences between the wild-type and mutant introns, a second conformational change is postulated, and it is this change that involves somehow the exchange of the exogenous guanosine for the ω position.

Now, two conformational changes have been proposed to occur between the first and second steps of splicing: one that is rate-limiting for the second step and one that involves the exchange of guanosine residues in the active site. Which comes first? If the rate-limiting step is first, that would imply that the state in which the exogenous guanosine occupies the G-binding site is as long-lived in wild-type intron as the mutants. Again, since the mutants had a fast rate of reverse splicing as compared to the wild-type and probably catalyzed the chemical steps equally well, this scenario does not seem likely. The alternative order provides a better fit to the data. In this order, binding of the ω nucleotide and the guanosine ligated to the 5′ end of the intron compete for the G-binding site prior to the rate-limiting step. If binding of these two nucleotides is in rapid equilibrium, then the intermediate will have two steady-state conformations—one with ωG bound and one with the exogenous guanosine bound. The relative population of these two states will be determined by their relative affinities for the G-binding site and thus influence directly the partitioning of the intermediate between products (linear intron + tRNA) and reactants (precursor + guanosine).

The resulting mechanism is illustrated in Figure 8, and a summary of the kinetic parameters is given in Table 1. K_1 is the binding constant for guanosine and is taken to be 240 μM. This is the K_M previously determined for the wild-type intron reacting with guanosine, and it appears to be equal to the K_d for guanosine (Zaug et al., 1993). Also, when kinetic simulations of the data are performed, they determine the K_d to be around 170 μM, which is, within error, equal to K_M. The rate of the catalytic step of the first step of splicing, k_{+2}, is estimated to be 10−15 min^{-1} from the rate of the burst phase at saturating guanosine and the k_{cat} determined by Zaug et al. (1993). The rate of the reverse of the first step of splicing, k_{-2}, must be greater than or equal to 0.9 min^{-1}, the rate of guanosine loss by the ωA mutant. Following the first step of splicing is the conformational change that removes the exogenous guanosine from the G-binding site and allows the ω position access to the G-binding site (its equilibrium constant is given by K_3). Although we have no evidence regarding what is happening physically during this conformational change, the G-binding site must be at least partially occluded after this first conformational change, perhaps even by the ω nucleotide, since exogenous guanosine does not bind to these intermediates and inhibit the second step of splicing [as also described by Zaug et al., (1993)]. Finally, there is the conformational change (described by K_4) that allows the intron to proceed through the second step of splicing. The forward rate constant for this conformational change, k_{+4}, which is rate-limiting for the second step of splicing, is determined to be 0.3 min^{-1}. The reversal of this conformational change is estimated to be small with respect to k_{+5}, the rate constant for chemistry of the second step of splicing, which is also assumed to be irreversible.

The kinetic data on the ωA mutant at every guanosine concentration investigated could be fit to this mechanism

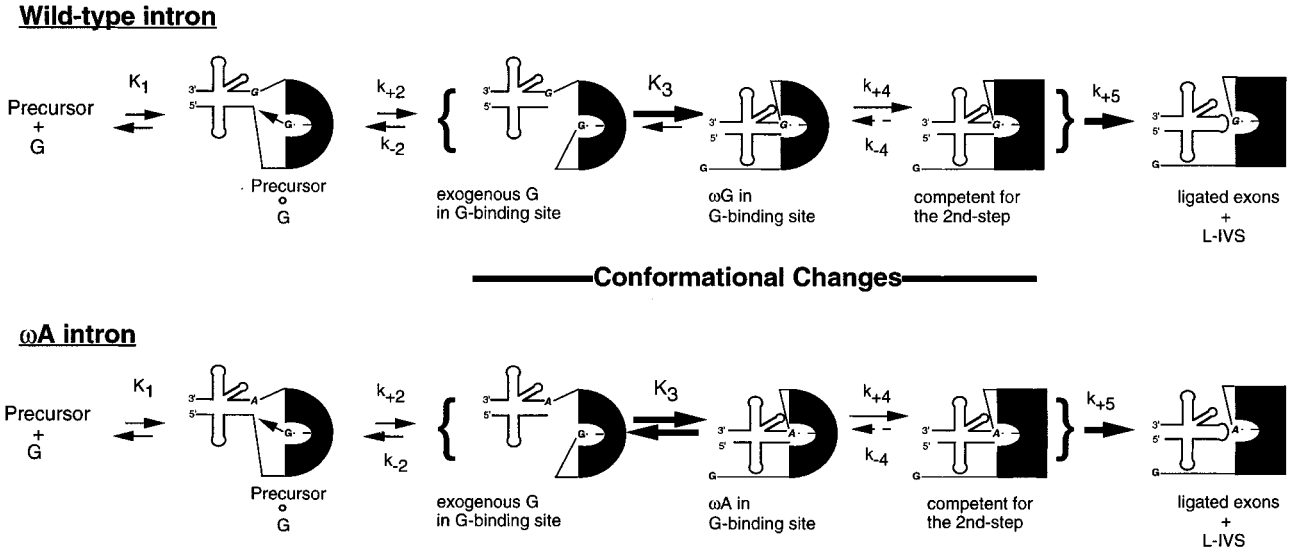

FIGURE 8: A mechanism. The proposed reaction scheme is shown pictorially, and available rate information is given for the wild-type (top) and ωA mutant (bottom). The different conformational states of the splicing intermediate are enclosed within braces.

Table 1: Kinetic Parameters for Self-Splicing of the *Anabaena* $tRNA^{leu}$ Precursor[a]

	WT	ωA	G-site
exogenous nucleoside	guanosine	guanosine	2-aminopurine ribonucleoside
$K_M = K_1$ (μM)	240[b]	240	~1000
$k_{cat} = k_{+2}$ (min^{-1})	15[b]	15	~7
k_{cat}/K_M (M^{-1} min^{-1})	4.6×10^4 [b]	4.6×10^4	$\sim 4 \times 10^3$
k_{-2} (min^{-1})	≥0.9	≥0.9	nd
K_3	>1	≥1	nd
k_{+3} (min^{-1})	>0.9		
k_{+4} (min^{-1})	0.3	0.3	nd
k_{-4} (min^{-1})	slow	slow	nd
k_{+5} (min^{-1})	fast	fast	nd

[a] All constants were determined under the following conditions: 15 mM $MgCl_2$, 25 mM Hepes, pH 7.5 and at 32 °C. [b] From Zaug et al. (1993).

(data not shown). It is a satisfying picture of the reaction because by variation of K_3 it is possible to shift from simulations that mimic the ωA intron to simulations that mimic the wild-type intron. Physically this makes sense because K_3 determines the relative concentrations of intermediate with exogenous nucleoside bound and that with ω nucleoside bound.

Implications of This Mechanism. In addition to the identification of two distinct steps following the first step of splicing and prior to the second step, the current work has implications for the question of why ωG is a conserved nucleotide. Previous studies on ω mutants of group I introns led to the conclusion that the residue is conserved because residues other than guanosine would not be positioned for attack of the 5′ exon by the G-binding site during the second step of splicing. We found this not to be true, at least in the case of the *Anabaena* intron, as ωA can substitute. In this system, the role of ωG is to drive the splicing reaction in the forward direction. It does this by competing for the G-binding site with the exogenous guanosine that becomes attached to 5′ end of the intron during the first step of splicing, and thereby inhibiting reversal of the first step. This model explains why a high rate of reversal of the first step of splicing is observed in both the ωA and the G-binding site mutants, and it incorporates the observation that there is little effect on the second step of splicing.

Whether these observations are unique to the *Anabaena* intron or general features of group I RNA splicing remains to be determined. Several observations suggest that at least part of the proposed mechanism will be general. A related intron found in the $tRNA^{ile}$ from the purple bacterium *Azoarcus* exhibits guanosine-dependent burst and lag phases in its splicing profile, suggesting reversibility of the first step of splicing even in the wild-type RNA (Tanner & Cech, 1996; and unpublished data). When hydrolysis rather than guanosine attack initiates the splicing reaction, the reaction occurs with a single first-order rate constant. This is consistent with the reaction no longer being reversible in the absence of competition between ωG and an exogenous G at the 5′-end of the intron. The *Azoarcus* intron has been shown to be remarkably thermostable (Tanner & Cech, 1996). Perhaps, in achieving thermostability, this intron has compromised its ability to undergo the first conformational change. Additionally, van der Horst and Inoue (1989) observe that an ωA mutant of the *Tetrahymena* intron will catalyze a reaction analogous to exon ligation in which a dinucleotide 5′ exon analog attacks at the 3′ splice-site, releasing a trinucleotide product of the transesterification reaction. This result apparently differs with observations made with self-splicing constructs where the exon-ligation step is severely inhibited by the ωA mutation (Been & Perrotta, 1991; Suh & Waring, 1993). Clearly, this is an area which needs further investigation.

The reversibility of the first step of splicing, as exhibited by the mutant introns described here, is expected to increase the accuracy of 5′ splice-site selection by allowing the system to come to equilibrium before committing to the second chemical step. Such a scheme has been described by Chin and Pyle (1995) in the context of a ribozyme derived from a group II intron. This has ramifications for trans-splicing applications of ribozymes (Sullenger & Cech, 1994). If mutations at the ω position confer greater specificity to the first step of splicing yet still allow the second step of splicing to proceed with accuracy, such a strategy could be used to produce trans-splicing constructs that are less promiscuous *in vivo*.

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