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REPORT

Deletion of a conserved dinucleotide inhibits the second step of group II intron splicing

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ABSTRACT

Few point mutations have been described that specifically inhibit the second step of group II intron splicing. Furthermore, the effects of such mutations are typically not apparent unless the mutations are studied in the context of a substrate that harbors a very short 5' exon. Truncation of the 5' exon slows the second step of splicing. Once the second step has been slowed, the effects of point mutations can be seen. We report the unexpected observation that the deletion of a conserved GA dinucleotide dramatically inhibits the second step of splicing, even when the mutation is studied in the context of a full-length substrate. In contrast, we find that this mutation does not significantly affect the first step of splicing, unless the mutation is studied in combination with a second point mutation that is known to inhibit the first step. Even in that context, the effect of the GA deletion mutation on the first step is modest. These observations, together with the inferred location of the GA dinucleotide in the three-dimensional structure of the intron, suggest that this dinucleotide plays a particularly important role in the second step of splicing.

Keywords: catalytic RNA; group II intron; ribozyme**INTRODUCTION**

Group II introns are comprised of a series of six helical domains, referred to as domains 1–6. Deletion analysis has demonstrated that, of these domains, only domains 1 and 5 are absolutely required for catalysis (Koch et al., 1992). Nonetheless, a few highly conserved residues are located outside of those domains. When group IIA and group IIB introns are compared, four conserved residues are seen outside of domains 1 and 5 (Michel et al., 1989). Given their high degree of conservation, it is likely that these residues are important for one or both steps of splicing. Two of these residues are located in the joining region that separates the 5' exon from domain 1, and two are located in the joining region that separates domain 2 from domain 3 [J(23)]. The second pair of residues, a conserved GA dinucleotide (i.e., G588 A589) located in J(23), were the subject of this investigation.

The conserved GA dinucleotide was first implicated as having an important role in splicing by Chanfreau

and Jacquier (1996), who showed that modification of the adenosine residue (A589) with diethylpyrocarbonate interferes strongly and specifically with the second step of splicing. In their hands, however, mutation of the adenosine residue affected both steps of splicing. Thus, the nature of the alteration (modification vs. mutation) at position A589 impacted the effect on splicing.

Peebles and coworkers (Peebles et al., 1995; Podar et al., 1998) have reported that both of the residues of the GA dinucleotide can be crosslinked to residues within or near the base of the domain 5 helix, where catalytically important residues are known to reside. This observation, like the Chanfreau and Jacquier study, provides strong evidence that the GA dinucleotide may play an important catalytic role in splicing, but does not dissect its effects on the individual splicing steps.

We report the analysis of splicing substrates from which the GA dinucleotide has been deleted. We have studied this mutation (Δ GA) alone and in combination with a domain 5 point mutation (D5 G3U) that is known to slow the first step of splicing (Boulanger et al., 1995; Peebles et al., 1995). Furthermore, modification of D5 G3 slows both the first and second steps of splicing (Chanfreau & Jacquier, 1994). The effects exerted by the Δ GA mutation, the D5 G3U mutation, and the double

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mutation (DM) were studied in three different contexts: in a full-length splicing substrate that allowed assessment of overall effects on splicing, and in two different modified substrates that isolated effects on either the first or second steps of the splicing reaction. We find that deletion of the GA dinucleotide has a profound negative effect on the second step of splicing, but has very little effect on the first step. Deletion of the GA dinucleotide does modestly reduce the efficiency of the first step of the reaction if that step is already slowed by the D5 G3U mutation; such deletion can completely abolish the second step of the reaction when that step has been reduced by the D5 G3U mutation.

These findings indicate that the conserved GA dinucleotide plays a critical role in splicing and is particularly important for the second step of the reaction.

RESULTS AND DISCUSSION

Model reactions that isolate individual steps of splicing

The reactions catalyzed by a self-splicing group II intron *in vitro* are depicted in Figure 1. Figure 1A is the complete two-step splicing reaction. Figure 1B is a hydrolysis pathway that is also observed when the intron is incubated under splicing conditions *in vitro*. In the hydrolysis pathway, the bond between the 5' exon and

the intron is broken by a water molecule rather than by the 2' branch point hydroxyl; subsequent steps are identical to those observed in the two-step transesterification reaction except that the intron is released in a linear form rather than as a lariat. Mutational studies have indicated that intron elements that are required for splicing by transesterification are also required for hydrolysis (Podar et al., 1995). Figure 1B also shows that the excised intron can catalyze cleavage of the ligated exons at their ligation point; this reaction is referred to as spliced exon reopening (SER) (Jarrell et al., 1988b; Podar et al., 1995). Note that, although it is not indicated in the diagram, the lariat form of the intron can also catalyze SER (Jarrell et al., 1988b).

Early studies of the reactions catalyzed by group II introns *in vitro* demonstrated that certain salt conditions (i.e., those employing KCl) favor the hydrolysis reaction, whereas others (i.e., those employing $(\text{NH}_4)_2\text{SO}_4$) favor transesterification (Peebles et al., 1987; Jarrell et al., 1988b). In the present study, therefore, reactions containing KCl are referred to as "hydrolysis" reactions and are designated H0.5 or H1.5, depending on the amount of salt involved (i.e., 0.5 M or 1.5 M KCl). Analogously, ammonium sulfate reactions are referred to as "transesterification" reactions and are designated as T0.5 or T1.5.

We have studied our group II intron mutations not only in the context of the complete group II intron splicing

FIGURE 1. A: Splicing occurs by a two-step transesterification reaction. In this simple representation, the group II intron (line 1), which is comprised of six conserved structural domains, is represented as a black line labeled IVS 1–6. The first step of splicing yields the free 5' exon (E5) and the lariat intermediate, (IVS 1–6 LAR)E3. The second step yields ligated exons and an excised intron lariat. B: Intron-catalyzed hydrolysis reactions. Site-specific hydrolysis yields E5 and a linear intron containing intermediate (line 2). Exon ligation is followed by hydrolysis of the ligated exons (E5-E3) to yield the individual exons (line 4). C: A schematic representation of the group II intron secondary structure. The intron (black line) consists of six helical domains that radiate from a central wheel. The domains are numbered sequentially starting at the 5' end. The γ and γ' nucleotides are shown, along with the GA dinucleotide, D5 G3, and the branch "A" in domain 6.

reaction, but also in the context of certain model reactions designed to mimic the individual steps of splicing (see Figs. 3 and 4).

Effect of point mutations on the complete splicing reaction

We introduced each of the mutations discussed above, including the double mutation, into a complete splicing template containing both exons and a full-length intron (such templates are designated E5(IVS 1–6)E3), and assayed the ability of each template to catalyze the complete splicing reaction (see Fig. 1). Time course experiments were done under each of four reaction conditions. Representative time course data for each precursor RNA incubated under T0.5 conditions are shown in Figure 2.

As can be seen with reference to Figure 2, the wild-type control RNA (WT) reacts to yield ligated exons and the excised intron lariat as major products (Fig. 2A). Some free 5' and 3' exons are also seen. The free exons are products of SER; they are much more abundant when the RNA is incubated under either H0.5 or H1.5 conditions (Peebles et al., 1987; Jarrell et al., 1988b).

The D5 G3U mutation clearly reduces the efficiency of splicing (compare Fig. 2A and 2B). Furthermore, the mutation appears to primarily affect the first step of the reaction, as the mutation does not lead to the accumu-

lation of the products of the first step of the reaction: the free 5' exon, and the intron lariat plus 3' exon.

In contrast, the Δ GA mutation appears to primarily affect the second step of the reaction, as the mutant accumulates the products of the first step of the reaction. For reasons that remain unclear, the negative effects of the Δ GA mutation on the second step of splicing appear to be somewhat relieved under hydrolysis conditions, where less accumulation of the intron/E3 intermediate is observed (data not shown).

The double mutation (DM) has a more severe splicing defect than either single mutant (compare Fig. 2B, 2C, and 2D). As expected, the double mutant, like the Δ GA mutant, accumulates the products of the first step of the reaction. As with the Δ GA mutant, the defects of the double mutant are less dramatic under hydrolysis conditions.

These data confirm reports that D5 G3 is important for both steps of splicing. In contrast, we find that the Δ GA mutation has little effect on the first step of splicing and appears to primarily reduce the efficiency of the second step of splicing (as evidenced by the accumulation of the E5 and lariat/E3 intermediates).

To more fully dissect the effects of each of these mutations on the first and second steps of splicing, we analyzed their behavior in the model reactions that are designed to mimic either the first or the second step of the reaction in isolation.

FIGURE 2. Effect of point mutations on the complete splicing reaction. Representative time course data. **A:** Samples of WT RNA were incubated for 0, 1, 2, 4, 6, 10, 20, and 40 min (lanes 1–8, respectively) in T0.5 buffer (see Materials and Methods). **B:** Same as **A**, except the D5 G3U RNA was the substrate. **C:** Same as **A**, except the Δ GA RNA was the substrate. **D:** Same as **A**, except the DM RNA was the substrate.

Effect of point mutations on the first step of splicing

This experiment was designed to allow us to specifically study the effects of the point mutations on the first step of splicing. We studied the effects of the same set of point mutations described above on the reactivity of a series of RNAs that lack the 3' exon; the general composition of each RNA in this series is denoted as E5(IVS 1–6). Because these substrates lack the 3' exon, they can undergo the first, but not the second, step of splicing. The four precursor RNAs are diagrammed in Figure 3.

Preliminary time course experiments were done under each of the four reaction conditions discussed above. For purposes of comparison of relative splicing efficiency, we used H1.5 conditions for these experiments because, under the other three conditions, both the D5 G3U and DM RNAs splice very slowly. In particular, both substrates are nearly inactive in T1.5 buffer (data not shown).

The truncated WT reacts to yield the 5' exon plus liberated linear intron, and some lariat intron (Fig. 3A). The D5 G3U mutation clearly reduces the efficiency of the first step of splicing (Fig. 3B). In contrast, the Δ GA mutation does not appear to slow the first step of splicing. Instead, the major effect of the Δ GA mutation is to increase the ratio of lariat to linear intron indicating that

the mutant transcript is more likely to catalyze the first step of splicing by transesterification than by hydrolysis.

Consistent with the results obtained from our analysis of full-length precursors, these data show that D5 G3 is much more important for the first step of splicing than is the GA dinucleotide. However, analysis of splicing of the DM substrate indicates that the GA dinucleotide does have some effect on the first step of splicing, because the double mutant catalyzes E5 release less efficiently than does the D5 G3U mutant.

We note that an alternative explanation for the results presented in this section is that the D5 G3U mutation does not slow the first step of splicing, but instead acts to speed reversal of the first step. We ruled out that possibility by measuring the effects of the D5 G3U mutation and the Δ GA mutation on the reversal of the first step of splicing. Intron lariat was purified from the WT, D5 G3U, or Δ GA substrates. Samples of each lariat were mixed with saturating amounts of a 17-nt E5 RNA (Chin & Pyle, 1995) and the rate of the reverse reaction was measured under T0.5 conditions. We found that the D5 G3U mutation slowed the rate of reversal, whereas the Δ GA mutation had no effect on the rate of reversal (data not shown). We conclude that the D5 G3U mutation does not speed the rate of reversal of the first step of splicing. Instead, the D5 G3U mutation slows the first step of splicing, and also slows the reversal of the first step of splicing.

FIGURE 3. E5 release from the E5(IVS 1–6) series of precursor RNAs (modeling the first step of splicing); representative time course data. **A:** Samples of WT RNA were incubated for 0, 1, 2, 4, 6, 10, 20, and 40 min (lanes 1–8, respectively) in H1.5 buffer (see Materials and Methods). **B:** Same as **A**, except the D5 G3U RNA was the substrate. **C:** Same as **A**, except the Δ GA RNA was the substrate. **D:** Same as **A**, except the DM RNA was the substrate.

Effect of point mutations on the second step of splicing

This experiment was designed to allow us to specifically study the effects of the point mutations on the second step of the splicing reaction. The four precursor RNAs that were analyzed in this experiment are diagrammed in Figure 4. Each RNA includes the entire intron plus the 3' exon; the general composition of each RNA in this series is denoted as: (IVS 1–6)E3. Each precursor was mixed with saturating levels of free E5, and the rate of the second step of splicing (exon ligation) was measured.

We note that previous studies have shown that, if E5 is not included in these model second step reactions, accurate release of E3 does not occur (Jarrell et al., 1988b). Instead, the intron/3' exon RNA catalyzes a self-cleavage reaction that breaks E3 downstream of a sequence (5'-AUUUUC) (Jarrell et al., 1988b) that is identical to the last 6 nt of the 5' exon. Addition of E5 fully suppresses this cryptic cleavage when reactions are performed under transesterification conditions; under hydrolysis conditions, the extent of cryptic cleavage is reduced but not abolished. To avoid confounding our analyses by the occurrence of the cryptic cleavage reaction, we performed our model second-step reactions under transesterification conditions.

As expected, the WT reacts to yield ligated exons, some free 3' exon, and liberated linear intron (Fig. 4A). We note that cryptic cleavage is observed in the absence of added E5 (Fig. 4A, lane 8). Interestingly, both point mutations suppressed this cryptic cleavage (Fig. 4B and 4C, lanes 8), but the significance of this observation is not clear.

The D5 G3U mutation inhibited splicing very significantly. One explanation for our observation of a rather dramatic second-step effect in these reactions, when we saw little effect of the D5 G3U single-mutation in our complete splicing reactions, is that the present reactions employed a linear intermediate instead of a lariat. It is well known that the lariat structure is important for efficient exon ligation (Jacquier & Jacquesson-Breuleux, 1991). Thus, we speculate that if the present bimolecular splicing reaction were performed with the lariat version of the splicing intermediate, the effect of the D5 G3U mutation on the second step of splicing would be less apparent.

The Δ GA mutation dramatically inhibits the *trans* splicing reaction, and the DM abolishes splicing under transesterification conditions. Note, however, that the splicing defects of all four substrates (including DM) are partially rescued under hydrolysis conditions (data not shown).

For this set of experiments, we also confirmed that the D5 G3U and Δ GA mutations do not exert their ef-

FIGURE 4. *Trans* splicing of E5 plus the (IVS 1–6)E3 series of precursor RNAs (modeling the second step of splicing); representative time course data. **A:** Mixtures of WT RNA (45 nM) plus E5 RNA (150 nM) were incubated for 0, 1, 5, 10, 20, 40, and 60 min (lanes 1–7, respectively) in T1.5 buffer (see Materials and Methods). As a control, WT RNA was incubated for 60 min in the absence of E5 RNA (lane 8). **B:** Same as **A**, except the D5 G3U RNA was the substrate. **C:** Same as **A**, except the Δ GA RNA was the substrate, and the bimolecular reactions were incubated for 0, 1, 5, 10, 20, 60, and 120 min (lanes 1–7), and the control was incubated for 120 min (lane 8). **D:** Same as **C**, except the DM RNA was the substrate.

fects by increasing the reversal of the second step of splicing (data not shown).

Whether group II introns harbor one or two catalytic sites is a matter of debate. If group II introns harbor two distinct catalytic sites, then one might expect to find certain intron nucleotides that are essential for the first step of splicing and other nucleotides that are essential for the second step. This topic was reviewed by Michel and Ferat (1995), who concluded that no evidence exists indicating that any particular group II intron nucleotide participates in catalysis of only one of the two steps of splicing.

Although the fact remains that no intron nucleotide has been found to participate exclusively in only one of the two steps of splicing, our results with the Δ GA mutation are particularly striking in this regard. Point mutations that primarily affect the second step of group II intron splicing are rare. Furthermore, to see the effects of such mutations on the second step of splicing, it is typically necessary to study the mutations in the context of a precursor with a short (13 nt) 5' exon (Jacquier & Michel, 1990; Jacquier & Jacquesson-Breuleux, 1991; Chanfreau & Jacquier, 1993). The short precursor is used because, even in the absence of point mutations, the second step of splicing is known to be slow when short transcripts are used. In the past, the effects of second-step specific mutations have only been seen in the context of the short precursor. In contrast, we find that even in the context of the full-length precursor, the Δ GA mutation dramatically slows the second step of splicing. Thus, to our knowledge, the Δ GA mutation has the most dramatic effect on the second step of splicing of any point mutation that has been studied.

Why does deletion of the GA dinucleotide specifically slow the second step of splicing? We have recently found that group II introns can be released by an alternative pathway that results in release of the intron as a true circle in which the first residue of the intron is joined to the last residue by a 2'–5' phosphodiester bond (H.L. Murray et al., submitted). We hypothesized that the Δ GA mutation slows the second step of splicing because it stimulates the use of the terminal uridine residue of the intron as an alternative branch site and that, when the alternative branch site is used, the second step of splicing is inhibited. However, primer extension analysis of the intron/E3 intermediate showed no evidence that the terminal uridine is used as an alternative branch site in the mutant substrate (data not shown).

As an alternative model, we considered the possibility that the Δ GA mutation inhibits the conformational change that has been shown to occur between the first and second steps of splicing. The conformational change was first described by Chanfreau and Jacquier (1996), who showed that mutations that block the conformational change also speed the reversal of the first step of splicing. As described above, we find that the Δ GA

mutation does not speed the reversal of the first step of splicing. Thus, it is unlikely that the mutation affects the conformational change.

It is unlikely that the GA dinucleotide is directly involved in catalyzing the second step of splicing, because the deletion of catalytically critical residues would be expected to create a total splicing block. Deletion of the GA dinucleotide does not abolish the second step of splicing. Thus, the GA dinucleotide is not required for catalysis. On the other hand, it is likely that the GA dinucleotide is positioned very near the catalytic core of the intron, at least during the second step of the reaction (see below).

Significantly, the GA dinucleotide is located immediately adjacent to the γ nucleotide, a nucleotide located in the J(23) sequence, which is known to base-pair with the terminal uridine of the intron (the γ' nucleotide). Like the GA dinucleotide, the γ - γ' pairing has been shown to be important for the second step of splicing (Michel & Jacquier, 1987; Jacquier & Michel, 1990; Jacquier & Jacquesson-Breuleux, 1991). Furthermore, the GA dinucleotide has been shown to cross-link to the bottom of domain 5, which is a highly conserved region of the intron that harbors residues that are very important for catalysis of the splicing reaction (Boulanger et al., 1995; Peebles et al., 1995; Podar et al., 1998). We speculate that, like the γ nucleotide, the GA dinucleotide interacts directly with bases near the 3' splice site, and that the interaction between those bases is particularly important for the second step of splicing. Experiments designed to test this hypothesis are in progress.

MATERIALS AND METHODS

Plasmids and constructions

Plasmid pJD20 (Jarrell et al., 1988a) was the template for the full-length precursor RNA, E5(IVS1–6)E3. Plasmids that were the templates for mutant precursor RNAs were derived from pJD20 via site-directed mutagenesis. These plasmids differ from pJD20 as follows; pJD20 (D5 G3U) contains a G3U conversion in domain 5, pJD20(Δ GA) contains a deletion of the dinucleotide GA between domains 2 and 3, and pJD20(DM) contains both G3U and Δ GA mutations. These plasmids were the templates for the mutant precursor RNAs, E5(D5 G3U)E3, E5(Δ GA)E3, and E5(DM)E3, respectively. Plasmids that were the templates for truncated and mutant truncated RNAs were constructed by subcloning fragments of the full-length clones into the pCRTMII vector using the TA Cloning Kit (Invitrogen). The appropriate DNA fragments from either pJD20, pJD20(D5 G3U), pJD20(Δ GA), or pJD20(DM) were amplified by PCR using specific primers. The plasmids that encoded the truncated RNAs, E5(IVS1–6) and (IVS1–6)E3, were designated pE5(IVS1–6) and p(IVS1–6)E3, respectively. The plasmids that encoded the mutant truncated RNA: E5(IVS1–6D5G3U), E5(IVS1–6 Δ GA), E5(IVS1–6DM), (IVS1–6D5G3U)E3, (IVS1–6 Δ GA)E3, and (IVS1–6DM)E3

were designated as, for example, pE5(IVS1–6D5G3U). The E5 RNA used in the bimolecular splicing experiments was generated by transcription in vitro of a template generated by PCR. To generate the template, pJD20 was cut with *Hind*III and the E5 encoding sequence was amplified as follows: the cut pJD20 DNA was combined with 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton-X, 1 mM MgCl₂, 0.2 mM each dNTP, 1 U *Taq* Polymerase (Promega), 0.016 U *Pfu* Polymerase (Stratagene), and 500 ng each primer in a total volume of 100 μ L. The conditions of amplification were, 94 °C denaturation (1 min), 57 °C, annealing (2 min), and 72 °C elongation (3 min), for a total of 30 cycles. The oligonucleotide sequence of the forward primer, 5'-ACG AAG CTT GTA ATA CGA CTC ACT ATA GGG, corresponded to nucleotides upstream of the 5' exon encoding a T7 polymerase recognition site. The oligonucleotide sequence of the reverse primer, 5'-GAA AAT GTC CCA CCA CGT AGT, corresponded to the last 21 nt of the exon. The amplified E5 sequence served as a template for transcription.

In vitro transcription and purification of RNA

All plasmids that were templates for RNAs containing both E5 and E3 were cut with *Hind*III. These plasmids include pJD20, pJD20(D5 G3U), pJD20(Δ GA), and pJD20(DM). The truncated plasmid p(IVS1–6)E3 and mutant truncated plasmid p(IVS1–6DM)E3 were also cut with *Hind*III. Plasmids pE5(IVS1–6), pE5(IVS1–6D5G3U), pE5(IVS1–6 Δ GA), and pE5(IVS1–6DM) were cut with *Eco*RV. When any of the pE5(IVS1–6) series plasmids is digested with *Eco*RV and transcribed in vitro, the natural 3' terminal base of the group II intron is the last base of the resulting run-off transcript. Plasmids p(IVS1–6D5G3U)E3 and p(IVS1–6 Δ GA)E3 were cut with both *Hind*III and *Eco*RV. Each transcription reaction contained 6 μ g of cut template DNA, 50 U of T7 Polymerase (Stratagene), and 30 μ Ci of α -³²P-UTP (NEN) in a final volume of 0.05–0.1 mL buffer containing 40 mM Tris-HCl, pH 7.6, 6 mM MgCl₂, 2 mM spermidine, and 5 mM NaCl, 10 mM DTT, 0.4 mM unlabeled UTP, and 0.5 mM each of unlabeled ATP, CTP, and GTP. The reaction mixture was incubated at 40 °C for 1 h. The RNA mixture was passed through a Sephadex G25 column to remove unincorporated nucleotides. The RNA was precipitated with ethanol, dried in a vacuum desiccator, and dissolved in 10 μ L water and an equal volume of gel loading buffer (200 mM EDTA, pH 8.0, formamide, xylene cyanol, and bromophenol blue). The samples were heated at 85 °C for 3 min, cooled in an ice water bath, and loaded onto a 4% acrylamide-8 M urea gel. Radioactive bands were located by autoradiography, excised with a razor blade, and eluted by shaking in buffer (0.5 M sodium acetate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1% SDS) at 37 °C overnight. RNA was precipitated with ethanol, dried in a vacuum desiccator, and dissolved in water.

In vitro splicing

RNA was diluted in water to 50,000 cpm/ μ L and 2 μ L were used for each splicing reaction. The RNA was dried in a vacuum desiccator and dissolved in 4 μ L of splicing buffer. The splicing buffer for hydrolysis conditions contained 0.04 M

Tris-Cl, pH 7.5, 0.1 M MgCl₂, and either 0.5 M or 1.5 M KCl. The splicing buffer for transesterification conditions contained 0.04 M Tris-Cl, pH 7.5, 0.1 M MgCl₂, and either 0.5 M or 1.5 M (NH₄)₂SO₄. The splicing reactions were performed at 45 °C for the length of time indicated in each experiment. Reactions were stopped by cooling in an ice water bath. Gel loading buffer was added, and each mixture was heat denatured and loaded onto a 4% acrylamide-8 M urea gel. The splicing reaction products were detected by autoradiography.

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