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The interaction between the first and last intron nucleotides in the second step of pre-mRNA splicing is independent of other conserved intron nucleotides

Brian L.Ruis, Wendy J.Kivens and Paul G.Siliciano*
Department of Biochemistry and Institute of Human Genetics, University of Minnesota Medical School, 4–225 Millard Hall, 435 Delaware Street SE, Minneapolis, MN 55455, USA

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ABSTRACT

Virtually all pre-mRNA introns begin with the sequence /GU and end with AG/ (where / indicates a border between an exon and an intron). We have previously shown that the G residues at the first and last positions of the yeast actin intron interact during the second step of splicing. In this work, we ask if other highly conserved intron nucleotides also take part in this /G–G/ interaction. Of special interest is the penultimate intron nucleotide (AG/), which is important for the second step of splicing and is in proximity to other conserved intron nucleotides. Therefore, we tested interactions of the penultimate intron nucleotide with the second intron nucleotide (/GU) and with the branch site nucleotide. We also tested two models that predict interactions between sets of three conserved intron nucleotides. In addition, we used random mutagenesis and genetic selection to search for interactions between nucleotides in the pre-mRNA. We find no evidence for other interactions between intron nucleotides besides the interaction between the first and last intron nucleotides.

INTRODUCTION

Introns are removed from pre-mRNAs by a two-step process (reviewed in 1). In the first step, the 2′ OH of the internal branch site adenosine residue attacks the 5′ splice site, creating the lariat intermediate and liberating the 5′ exon. The second step involves attack of the 3′ OH of the 5′ exon at the 3′ splice site, generating mature mRNA and releasing the lariat intron. Because group II self-splicing introns employ a very similar mechanism, it is expected that the recognition and removal of pre-mRNA introns will also be carried out by RNA molecules (2).

In organisms as diverse as yeast and humans, nuclear pre-mRNA introns are nearly always bordered by sequences /GU at the 5′ splice site and AG/ at the 3′ splice site. Also highly conserved are the fifth nucleotide of the intron (a G in both systems) and the region surrounding the branch site (UACUAAC in yeast, YNYURAC in mammals; the branch site adenosine is underlined). These sequences are required for splicing, as mutations in them cause blocks to step I or step II of splicing (3–5).

Five small nuclear ribonucleoprotein particles (snRNPs) and a set of soluble proteins are also required for splicing (1). These *trans*-acting factors recognize splice sites in the highly ordered process of spliceosome assembly. Initial recognition of the 5′ splice site and branch site relies primarily on binding of the U1 and U2 snRNPs, respectively. Both of these recognition events occur at least in part by base pairing between the snRNAs and the intron sequences (6–11). In contrast, the initial recognition of the 3′ splice site is complex, requiring at least seven proteins in mammalian systems (12, also see ref. 1).

Within spliceosomes, an intricate series of snRNP–snRNP and snRNP–intron interactions brings the intron into a catalytically active configuration. In addition to the U1–5′ splice site and U2-branch site interactions, the U5 snRNA base pairs with exon sequences at both 5′ and 3′ splice sites (13,14). Similarly, the U6 snRNA forms a dynamic series of interactions with other RNAs, base pairing first with the U4 snRNA (15), and subsequently with the U2 snRNA (16–18) and the 5′ splice site (19–22). *Schizosaccharomyces pombe* (23), but not *Saccharomyces cerevisiae* (24), U1 snRNA can base pair with the 3′ splice site during the first, but not the second, step of splicing.

While several interactions leading to the recognition of the 5′ splice site and the branch site have been identified, the specific events required for 3′ splice site selection are not clearly defined. In *S.cerevisiae*, spliceosome assembly (5,25), and even the first step of splicing (26), can take place in the absence of an intact 3′ splice site. These results argue that the recognition of the 3′ splice site takes place after an active spliceosome has assembled.

We have recently found evidence for an interaction between the first and last nucleotides of the yeast actin intron that is involved in the recognition of the 3′ splice site (27). This /G–G/ interaction is essential for the second step of splicing. While mutation of the first G to an A or the last G to a C blocks the second step of splicing, these splicing defects are suppressed in the /**a**–**c**/ *cis* double mutant (mutant nucleotides are indicated

*To whom correspondence should be addressed

by bold lower case letters). This reciprocal suppression suggests that the double mutant restores an essential interaction that is destroyed by each single mutation.

This suppression is allele-specific; while the /**a**−**c**/ double mutant restores splicing to approximately 10% of wild-type levels, /**a**−**a**/ and /**a**−**u**/ introns splice very poorly. In addition, this suppression is position-specific because combinations of mutations at other intron positions do not restore splicing (27). A recent study of similar mutations in the yeast *RP51a* intron finds the identical results (28). This allele- and position-specific reciprocal suppression indicates that specific pairs of nucleotides are required at the first and last nucleotides of the intron. Based on this data, we have proposed that the first and last nucleotides interact during the second step of splicing (27).

In this work, we ask if the /G−G/ interaction is accompanied by other RNA−RNA interactions among neighboring intron nucleotides. Strong candidates for participation in the /G−G/ interaction are those few nucleotides that are highly conserved in yeast and mammalian introns: the second, fifth, branch site, and penultimate intron nucleotides. Intriguingly, these nucleotides are likely to be in close proximity to one another in the lariat intermediate (Figure 1). One appealing model predicts that the /G−G/ interaction extends to include a similar interaction between the neighboring second and penultimate intron nucleotides. Consistent with this suggestion, both the second and penultimate intron nucleotides are important for the second step of splicing. We tested interactions between the second and penultimate intron nucleotides by constructing double mutant introns and looking for another nucleotide pair that can substitute for the /GU−AG/ pair. We also tested an alternate model that predicts an interaction between the penultimate and branch site nucleotides, and two models that predict simultaneous interaction of three intron nucleotides. In a separate approach, we used random mutagenesis of the 5′ and 3′ splice sites in an unbiased search for interacting intron nucleotides. We find no evidence of interactions between intron nucleotides aside from the first and last nucleotides.

MATERIALS AND METHODS

Mutant construction

Mutations were constructed in the intron of the yeast actin-lacZ (5) and actin-*CUP1* (29) reporter genes using the Transformer oligonucleotide-directed mutagenesis kit (Clontech). Double mutant introns were constructed using standard subcloning techniques including PCR and restriction fragment swaps.

Splicing analysis

The actin-lacZ and actin-*CUP1* constructs were introduced into yeast strains YJC59 and I4, respectively, by lithium acetate transformation (30). β-galactosidase levels (31) were determined on at least four independent transformants and the results averaged. The standard error of the mean was less than 20%. Copper growth assays were performed as described (29). RNA preparation and primer extension of the actin-*CUP1* transcripts were as described (32) using the exon 2 primer CUP-mer (5′-TGATTTTTGGCATTGTTC-3′). The U4 snRNA primer (5′-AGGTATTCCAAAAATTCCCTAC-3′) was used as an internal RNA loading control.

Random mutagenesis of the 5′ and 3′ splice sites

Random mutations were incorporated into the 5′ and 3′ splice sites of the actin-*CUP1* /**a**−**c**/ intron by PCR amplification using 'doped' oligonucleotides, which contained a mixture of nucleotides at specific sites. The ratio of nucleotides in the mixture was chosen to maximize the likelihood of obtaining single mutations in each splice site. The 5′SS doped oligonucleotide (5′-CTGAATGAGATCT**atg/atatgttc**TAGCGCTTG-3′) was synthesized with a low level of degeneracy (97.3% correct nucleotide, 2.7% mixture of the three incorrect nucleotides) at positions −3 to +8 of the 5′ splice site (bold type). The 3′SS doped oligonucleotide (5′-GAACCCGGTACCCAacGt/GtaaaCATATAATATAGC-3′) was synthesized with a low level of degeneracy (95.8% correct nucleotide, 4.2% mixture of the three incorrect nucleotides) at the last five positions upstream and the first four positions downstream of the 3′ splice site except the last intron nucleotide and the second exon nucleotide. The last intron nucleotide and the second exon nucleotide were not allowed to vary in order to avoid creation of a wild−type 3′ splice site. These levels of doping gave a 22% chance of a single mutation, a 3% chance of two mutations, and a 74% chance of no mutation in each oligonucleotide (calculated as in ref. 29). Sequence analysis of unselected clones revealed that the 5′ and 3′ splice sites were mutagenized at the expected rates.

These oligos were used in a standard PCR reaction to amplify the yeast actin intron. The resulting pool of PCR products should represent all possible combinations of mutations at the doped sites. This pool of mutant introns was cloned into the A1C303BK vector to recreate a set of intact actin-*CUP1* genes. Twenty-five thousand *Escherichia coli* transformants were pooled and harvested to make the randomized library. The pool of mutant introns represented 82% of all possible mutant splice site combinations. The library was then transformed into copper sensitive yeast strain I4 (29) by electroporation (33). Thirty-four thousand I4 yeast transformants were selected on −Trp drop-out plates. All yeast plates were made with Phytagar (GIBCO Laboratories) as described (29). The yeast transformants were replica plated to 0.10 mM $CuSO_4$ −Trp media. The parental actin-*CUP1* plasmid with the /**a**−**c**/ intron does not allow the I4 strain to grow on this copper concentration. Forty-four colonies that exhibited resistance to 0.10 mM $CuSO_4$ were picked and retested. The actin-*CUP1* plasmids were recovered from 22 colonies and the splice sites were sequenced. The CUP-mer oligonucleotide was used to sequence the 3′ end of the intron and the branch site. The 5′ ACTIN-SEQ oligonucleotide (5′-TCGAGCAATTGGGACCGTGC-3′) was used to sequence the 5′ splice site.

RESULTS

During the second step of splicing, the first and last intron nucleotides interact. The close proximity of other conserved intron nucleotides suggests that they may also participate in this interaction. Here we test a series of models predicting interactions between neighboring conserved nucleotides in the 5′ splice site, branch site, and 3′ splice site.

To detect interactions between intron nucleotides, we made specific point mutations at candidate positions in the intron. If two intron positions interact, mutation of one nucleotide is expected to disrupt the interaction and disturb splicing. However, specific combinations of point mutations at the interacting sites

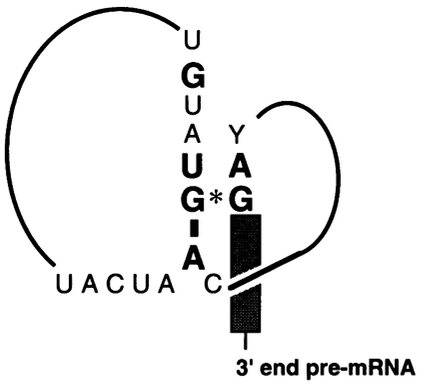

Figure 1. The lariat intermediate. The lariat intermediate of a yeast pre-mRNA is shown. The 5′ splice site is joined to the branch site in a 2′−5′ phosphodiester bond (thick vertical bar). The 3′ splice site is drawn alongside the 5′ splice site with the interaction between the first and last G nucleotides shown as an asterisk. The 3′ exon is shown as a shaded box. It is not known if the first and last G nucleotides interact directly or indirectly. Interactions among the highly conserved intron nucleotides (first, second, fifth, branch site, penultimate, and last; bold capital letters) are tested in this work. In this diagram, the 5′ splice site and 3′ splice site are drawn antiparallel to each other, placing the penultimate nucleotide near the second intron nucleotide. A parallel arrangement of the 5′ and 3′ splice sites is also possible, in which the 3′ exon points up. That model places the penultimate nucleotide in proximity to the branch site. The U6 RNA is known to be associated with the 5′ splice site of the lariat intermediate but is omitted for clarity, as are the free 5′ exon and the U2 and U5 snRNAs.

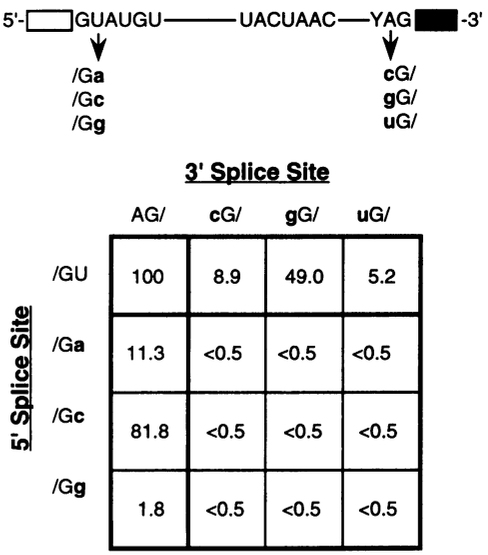

Figure 2. Double mutants between the second and penultimate intron nucleotides. A schematic of the actin intron shows the mutations made at the second and penultimate nucleotides. The table shows the β-galactosidase activities of the single and double mutants. Mutant nucleotides are indicated by bold lower case letters. We note that the β-galactosidase activities of penultimate intron mutants reported here are substantially higher than those reported for the analogous mutations in the yeast *RP51a* intron (28). Primer extension analysis of the same mutations in the actin-CUP1 gene (Figure 3) shows levels of mature mRNA commensurate with the β-galactosidase activities. This discrepancy may be caused by differences between the actin and *RP51a* introns and by differences in the expression plasmids used.

are predicted restore the interaction and relieve the splicing defect. As with the /G−G/ interaction, any pair of interacting nucleotides will be identified by reciprocal and position- and allele-specific suppression of splicing defects.

We constructed mutations in the intron of the actin-lacZ and actin-*CUP1* reporter genes by oligonucleotide-directed mutagenesis. Efficient splicing of the actin intron is required for lacZ or *CUP1* expression. Splicing of the mutant reporter genes was examined by plate and liquid lacZ assays of the actin-lacZ constructs, and by copper resistance and primer extension assays of the actin-*CUP1* constructs.

A test for interaction between the second and penultimate nucleotides

The second and penultimate nucleotides are logical candidates to be involved in the /G−G/ interaction as they are immediate neighbors to the first and last guanines (Figure 1). Like the first and last intron nucleotides, the second and penultimate nucleotides are virtually invariant in all introns. Furthermore, mutations in the second and penultimate nucleotides can block the second step of splicing, just as mutations in the first and last.

We tested interactions between the second (/GU) and penultimate (AG/) intron nucleotides by making introns with single and double mutations at these positions (Figure 2). As assayed by lacZ activity, these mutations cause splicing defects varying in severity from minimal (/Gc, 82%) to severe (/Gg, 1.8%). Primer extension analysis of the actin-*CUP1* transcripts bearing these intron mutations reveals accumulation of lariat intermediate, indicating a block to step II of splicing (Figure 3 and data not shown). Transversion mutations at the penultimate nucleotide, cG/ and uG/, make a small amount of correctly sized mature mRNA. In addition, both these mutants make a slightly larger spliced product using a cryptic 3′ splice site (a UG

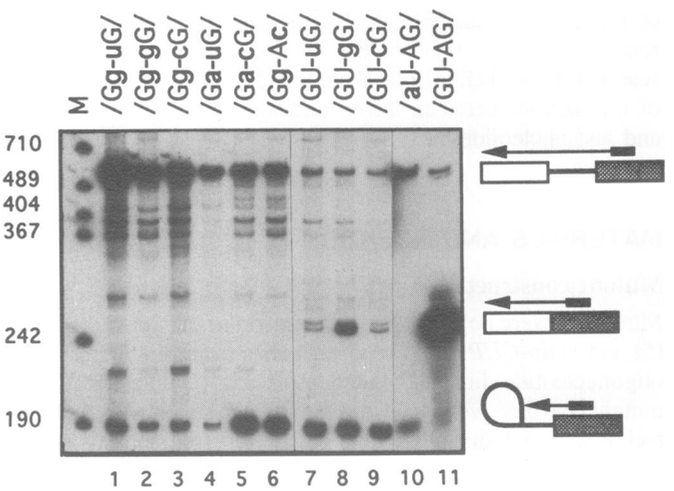

Figure 3. Primer extension analysis of mutant introns. RNA isolated from yeast strains carrying plasmids with mutant actin-*CUP1* genes was analyzed by primer extension. The primer anneals in the second exon and the positions of primer extension products from precursor mRNA, mature mRNA, and lariat intermediate are shown at the right. The sequence of the 5′ and 3′ splice site of each intron is shown above each lane. Five representative second-penultimate double mutants are shown in lanes 1−5. The /Gg−Ac/ mutant in lane 6 provides a control for position specificity. Single mutants in the penultimate position are shown in lanes 7−9. The uG/ and cG/ mutants use a cryptic 3′ splice site five nucleotides upstream of the correct 3′ splice site (lanes 7 and 9). The /a intron shown in lane 10 provides the correct position for primer extension products from unspliced precursor and lariat intermediate. Lane 11, wild-type intron. M, molecular weight markers.

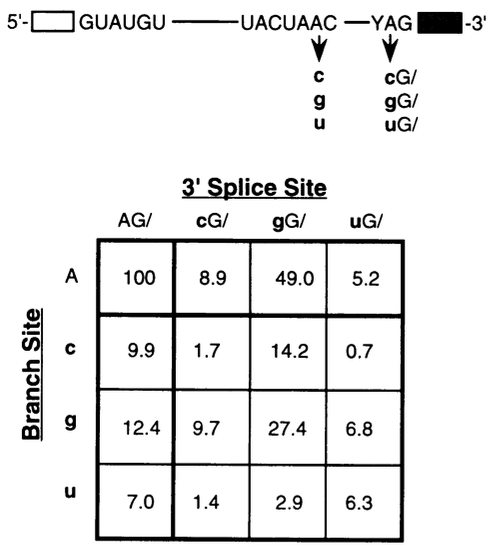

Figure 4. Double mutants between the branch site and penultimate nucleotides. A schematic of the actin intron shows the mutations made at the branch site and penultimate nucleotides. The table shows the lacZ activities of single and double mutants. Mutant nucleotides are indicated by bold lower case letters.

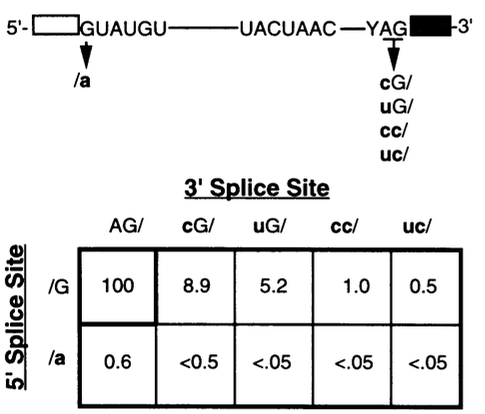

Figure 5. Triple mutants of the first, penultimate, and last intron nucleotides. A schematic of the actin intron shows the mutations made at the first, penultimate, and last nucleotides. The table shows the lacZ activities of various combinations of mutations. Mutant nucleotides are indicated by bold lower case letters.

dinucleotide 5 nucleotides upstream of the correct 3′ splice site; Figure 3, lanes 7 and 9, and ref. 27).

To test for interactions between these nucleotides, we constructed a matrix of all possible double mutants between the second and penultimate positions (Figure 2). We first assayed β-galactosidase activity from the actin-lacZ reporter constructs. No combination of mutations at the second and penultimate positions restored splicing activity (Figure 2). Primer extension analysis of actin-*CUP1* transcripts shows that all the double mutants have a partial block to step I, causing accumulation of unspliced precursor (Figure 3, lanes 1–5). Despite this block, these mutants accumulate sufficient lariat intermediate to determine the efficiency of step II. Importantly, all these double mutants have a complete block to step II and fail to accumulate mature mRNA (Figure 3, lanes 1–5). Thus, no reciprocal suppression between mutations at the second and penultimate intron nucleotides is observed. In fact, most of the double mutants are much more severe than either of the single mutants. This negative effect is especially striking in the case of the /G**c**–**g**G/ intron because each single mutation has only a minimal effect upon splicing efficiency.

Biochemical and genetic experiments demonstrate that the U6 snRNA interacts with both the 5′ and 3′ splice sites in the second step of splicing (21,22). Thus, the efficiency of the second-penultimate double mutants may be limited by the failure of an interaction with the U6 snRNA. We therefore assayed the second-penultimate double mutants in the presence of suppressor alleles of U6 (provided by C.Lesser and C.Guthrie) that are predicted to restore these interactions (data not shown). As before, we observed no suppression of the splicing defects and find no evidence of interaction between the second and penultimate nucleotides.

A test for interaction between the branch site and penultimate nucleotides

The branch site nucleotide is joined to the first intron nucleotide during the first step of splicing. Thus, the branch site nucleotide and other nucleotides of the UACUAAC box could be in proximity to the /G–G/ interaction prior to the second step of splicing (Figure 1). This proximity might allow the branch site or one of its neighboring nucleotides to interact with the penultimate nucleotide of the intron.

We tested for interactions between these nucleotides by making mutations in actin intron nucleotides 258 (UACUAAC), 259 (the branch site nucleotide, UACUAAC) and 260 (UACUAAC). Because mutation of positions 258 and 260 did not cause a strong block to step II, we concluded that they are not involved in an interaction important for step II and did not study them further. We constructed a matrix of double mutants between the branch site and the penultimate nucleotides (Figure 4). Some double mutants are much more severe than either single mutant (e.g. BS**c**–**c**G/) while other double mutants are not (e.g. BS**u**–**u**G/). The BS**g**–**g**G/ double mutant splices better than the BSg single mutant, but not as well as the **g**G/ mutant. However, in no case did we observe reciprocal suppression of branch site and penultimate nucleotide mutants that would indicate an interaction between these positions.

A test for interaction between the first, penultimate and last nucleotides

The first nucleotide of the intron could interact simultaneously with more than one nucleotide at the 3′ splice site. Two lines of evidence lead to this proposition. First, such an axial interaction has been suggested for the G-binding pocket of group I self-splicing introns (34). According to this hypothesis, the exogenous guanine makes hydrogen bonds with multiple neighboring nucleotides in the P7 helix. Second, in group II self-splicing introns, the first and penultimate nucleotides of the intron interact during the second step of splicing (35). Like mRNA introns, group II introns have a G at the first position and an A at the penultimate position.

In the case of pre-mRNA introns, the first intron nucleotide might interact with both the penultimate and last intron nucleotides simultaneously. The first and penultimate intron nucleotides do not interact strongly (27), but the penultimate nucleotide could

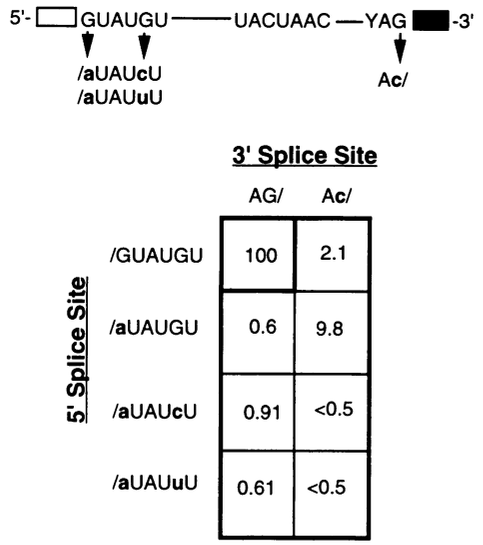

Figure 6. Triple mutants of the first, fifth, and last intron nucleotides. A schematic of the actin intron shows the mutations made at the first, fifth, and last nucleotides. The table shows the lacZ activities of various combinations of mutations. Mutant nucleotides are indicated by bold lower case letters.

make a minor contribution to the /G−G/ interaction. According to this model, the splicing efficiency of the /**a**−**c**/ intron may be limited by the presence of the incorrect nucleotide at the penultimate position.

We tested this model by making a series of triple mutants that vary the penultimate nucleotide in the /**a**−**c**/ intron (Figure 5). The lower splicing efficiency (9.8%, ref. 27) of the /**a**−**c**/ intron makes it a sensitive reporter for subtle improvements that would not be detectable in a wild−type intron. The splicing efficiency of the /**a**−**cc**/ and /**a**−**uc**/ triple mutants introns (Figure 5) is much less than the 9.8% splicing efficiency of the /**a**−**c**/ intron (27). We conclude that the penultimate nucleotide is not involved in the /G−G/ interaction. Chanfreau *et al.* also tested the /**a**−**cc**/ mutation in the yeast *RP51a* intron, and found no suppression of splicing defects (28).

A test for interaction between the first, fifth and last nucleotides

In metazoans, rare variant introns are found in which the first nucleotide is an A and the last a C (36,37). Intriguingly, these introns contain a further deviation from consensus splice site sequences by having a G to C transversion at position 5 of the 5′ splice site (/**a**UAAGU to /**a**UAucc). The G residue at position 5 is highly conserved in yeast and mammalian introns and is important for both steps in splicing (38). This covariation of the first, fifth, and last intron nucleotides suggests that position 5 might also take part in the /G−G/ interaction.

To address this question, we constructed a matrix of introns with point mutations at the first, fifth, and last nucleotides (Figure 6). Because 5′ splice sites with two mutations (at the first and fifth) block the first step of splicing, we examined these introns in the presence of suppressor alleles of U1 snRNA, designed to restore base pairing to the mutant 5′ splice site. Even in the presence of U1 suppressor alleles on high copy plasmids, only 10% of the actin-*CUP1* transcripts complete step I (data not shown). This reduced amount of lariat intermediate makes it more difficult to asses the efficiency of step II in the triple mutant introns. However, approximately 10% of the lariat intermediate from /**a**−**c**/ mutant introns completes step II (27). Therefore, if the position 5 mutation does not alter the step II efficiency, the triple mutant introns would make approximately 1% mature mRNA. This would be readily detectable by our lacZ and primer extension assays. Moreover, if, as this model predicts, mutations in intron nucleotide 5 increased the efficiency of the /**a**−**c**/ interaction, this level would be even greater and more easily detectable. Instead, we observe no lacZ activity (Figure 6) and no mature mRNA (data not shown), indicating that alteration of position 5 did not improve the /**a**−**c**/ interaction. Thus, we find no indication that intron position 5 participates in the /G−G/ interaction.

Random mutagenesis of the 5′ and 3′ splice sites

In a complementary approach, we looked for interactions between intron nucleotides by random mutagenesis of the regions around the 5′ and 3′ splice sites. Again, we chose the /**a**−**c**/ actin-*CUP1* reporter gene as a starting point because mutations that improve the /**a**−**c**/ interaction should be easily identifiable. This strategy makes no predictions about which intron nucleotides are involved.

The 5′ and 3′ splice sites were mutagenized using a PCR strategy in which oligonucleotides corresponding to the splice sites were synthesized to contain a small percentage of the incorrect nucleotides at specific positions (see Materials and Methods). Included for mutagenesis were the last three nucleotides of the 5′ exon, the first eight nucleotides of the intron, the last five nucleotides of the intron (except the last nucleotide), and the first four nucleotides of the 3′ exon (except the second exon nucleotide). The last nucleotide of the intron and the second nucleotide of the 3′ exon were not allowed to vary in order to minimize creation of **ag** dinucleotides, which would function as 3′ splice sites and cause a high background of false positives. With these exceptions, all conserved sequences at the 5′ and 3′ splice sites, as well as neighboring exon sequences, were allowed to vary.

The PCR products were cloned into the actin-*CUP1* construct to generate a pool of mutant introns. We tested this pool of mutant introns for increased splicing efficiency by selecting for those mutant introns conferring resistance to a higher copper concentration than the /**a**−**c**/ intron. Plasmids giving rise to higher copper resistance were recovered, retested, and sequenced to identify the new mutation(s). We believe our mutagenesis and selection procedure worked as expected because we recovered mutant introns that conferred increased copper resistance. Sequence analysis revealed that in these cases, the degenerate oligonucleotides had created wild-type splice sites that allow in-frame expression of *CUP1*. However, we found no set of mutations in conserved intron sequences that improved splicing of the /**a**−**c**/ intron.

DISCUSSION

The second step of splicing requires an interaction between the first and last nucleotides of the intron. Because of its proximity to other highly conserved intron nucleotides, we asked if this interaction might include these additional intron nucleotides. Through directed and random searches, we find no evidence that

additional intron nucleotides are involved in the /G−G/ interaction.

Some interactions might not be detectable by our experiments. For example, our double mutant strategy can only identify interactions if another nucleotide pair can substitute for the conserved intron nucleotides. It is possible that some interactions cannot be recreated by any other combination of A, C, G, and U residues. Synthetic pre-mRNAs containing nucleotide analogs at specific sites will allow additional nucleotides to be tested using *in vitro* splicing systems (39).

While we find no evidence for additional *cis*-acting sequences, the /G−G/ interaction could involve a *trans*-acting factor. Such a factor could participate directly in the interaction or could function indirectly to assist, stabilize, or proofread the interaction. The snRNAs, especially the U5 snRNA, which base pairs with exon sequences at both splice sites (13,14), are excellent candidates for such roles. Alternatively, a protein splicing factor could be involved. Candidates for such proteins include the products of the *PRP* genes (pre-mRNA processing), several of which are required exclusively for the second step (40−44). We will use genetic screens for increased splicing of /**a**−**c**/ introns to identify other molecules involved in the /G−G/ interaction.

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