A Quantitative Analysis of the Effects of 5' Junction and TACTAAC Box Mutants and Mutant Combinations on Yeast mRNA Splicing

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Summary

We have introduced four point mutations into the 5' junction (GTATGT) and TACTAAC box of a yeast intron-containing gene coding for beta-galactosidase. To analyze quantitatively mutant combinations, we deliberately avoided nucleotides adjacent to the sites of cleavage (GTATGT) and lariat formation (TACTAAC) and chose positions with expected weak effects on splicing. All four mutants do not affect, or only marginally affect, gene expression. The levels of precursor RNA and intermediates indicate, however, that splicing efficiency is affected in all cases; the first stage of splicing, 5' cleavage and lariat formation, is reduced two to 15-fold in these four mutants. The mutants were combined to generate compensatory and noncompensatory double mutant combinations. No evidence for a specific base-pairing interaction between the 5' junction and TACTAAC box could be obtained. The results suggest that all conserved positions in the 5' junction and TACTAAC box play a role in splicing efficiency.

Introduction

The last two years have witnessed a dramatic increase in our understanding of mRNA splicing in eukaryotes. Perhaps most significant is the definition of a splicing pathway, due in large part to the development of active extracts from mammalian cells (Hernandez and Keller, 1983; Padgett et al., 1983; Krainer et al., 1984). Based upon the kinetics of splicing in vitro (Grabowski et al., 1984; Krainer et al., 1984) and the characterization of the RNA intermediates and products in vitro (Ruskin et al., 1984; Padgett et al., 1984) and in vivo (Domdey et al., 1984; Rodriguez et al., 1984; Zeitlin and Efstratiadis, 1984), a two-stage splicing pathway has been proposed for both yeast and higher eukaryotes. In the first stage, cleavage at the 5' junction takes place, generating two splicing intermediates, the 5' exon and an RNA species comprising the intron and 3' exon. The intron-3' exon intermediate is in the form of a lariat in which the guanosine at the 5' end of the intron is joined to a 2'-5' phosphodiester bond to an adenosine residue near the 3' end of the intron (referred to as the lariat intermediate or ICS). In the second stage, cleavage at the 3' junction and ligation of the exons occurs concomitant with intron excision (Ruskin et al., 1984; Padgett et al., 1984; Rodriguez et al., 1984; Domdey et al., 1984; Zeitlin and Efstratiadis, 1984; Konarska et al., 1985). The more recent development of a splicing-active extract from yeast has reinforced these conclusions (Newman et al., 1985).

Yeast introns contain three regions required for accurate and efficient splicing in vivo. The 5' end is included within a 5' splice site (5' SS) consensus sequence, GUAUGU. Although 5' splice junctions of higher eukaryotes show a large degree of variation around a consensus sequence (Mount, 1982), yeast 5' junctions are highly conserved as all yeast introns sequenced to date contain GUApGU, and the vast majority conform precisely to the consensus sequence (Langford et al., 1984; Teem et al., 1984). The TACTAAC box is absolutely conserved and is located near (20-50 nucleotides upstream of) the 3' junction (Langford and Gallwitz, 1983; Pikielny et al., 1983; Toom et al., 1984). The last adenosine of this sequence (TACTAAC) is the site at which the yeast lariat is formed, i.e., the 5' end of the intron is joined to this A by a 2'-5' phosphodiester bond (Rodriguez et al., 1984; Domdey et al., 1984). A weakly conserved functional analog of this sequence has been described in mammalian introns (Ruskin et al., 1984; Padgett et al., 1984; Zeitlin and Efstratiadis, 1984; Keller and Noon, 1984; Reed and Maniatis, 1985; Ruskin et al., 1985). The 3' splice site (3' SS) is included within a 3' junction consisting of a pyrimidine-rich stretch (albeit perhaps less dramatic than what is found in higher eukaryotes) followed by the 3'-terminal AG (Teem et al., 1984).

To investigate in more detail the role (or roles) of these regions, we report here the effects of introducing point mutations into specific sites of the 5' junction and the TACTAAC box of a well characterized intron-containing gene coding for beta-galactosidase. Previous characterization of this gene has demonstrated that the production of mature mRNA and beta-galactosidase activity is dependent upon proper splicing and, in fact, the presence of the 5' junction and the TACTAAC box (Teem and Rosbash, 1983; Pikielny et al., 1983). In this study, we deliberately ignored conserved nucleotides adjacent to the sites of cleavage to investigate the potentially weaker effects of other conserved positions. The mutant genes have been compared according to a scheme with which the steady-state RNA levels can be related to the splicing efficiency of the primary transcript (Pikielny and Rosbash, 1985). (We use the terms splicing efficiency and rate constant interchangeably.) In brief, the theory indicates that the M/P ratio (mRNA level:precursor RNA level), rather than the mRNA level alone, is a fairly accurate measure of splicing efficiency. Taking into account the two individual stages of splicing, an expanded view of this theory indicates that the steady-state level of three RNA species (precursor RNA, lariat-intermediate, and mRNA) will reflect the relative splicing efficiency of a mutant gene at both steps in splicing.

Finally, we have constructed and analyzed a number of double mutant combinations. These combinations have been designed in order to test an intramolecular pairing model proposed for yeast and subsequently expanded.
Results

Mutations in the 5' Consensus Sequence

Two point mutations have been introduced into the 5' consensus sequence (Figure 1). The gene 5'I contains a U to G transversion at position 6. This nucleotide change has almost no effect on the splicing reaction, as assayed by the effect on the steady-state levels of RNAs in vivo. First, the mRNA level is normal as is the beta-galactosidase activity. Second, the lariat intermediate level is normal, as the reverse transcriptase stop occurring in the TACTAAC sequence has the same intensity as from the wild-type gene. Third, the level of precursor RNA is increased less than twofold, the limit of detection with this assay (Figure 2A, Table 1, and data not shown). As previously described (Pikielny and Rosbash, 1985), an increase in precursor RNA level indicates that the gene 5'I undergoes the first splicing step at an efficiency slightly reduced relative to the wild-type, parent gene.

The second mutant gene, 5'I', contains a G to A transition at position 5 (Figure 1). The gene 5'I contains a U to G transversion at position 6. This nucleotide change, like 5'I, also exhibits no reproducible decrease in the level of mRNA, as assayed by RNA analysis or by measurement of beta-galactosidase activity. However, the level of precursor RNA is increased about 15-fold, indicating that the rate of the first step of the splicing reaction, 5' cleavage and lariat formation, is significantly retarded. We also detect a slight but reproducible increase (about 1.5-fold) in the amount of lariat intermediate, as measured by the reverse transcriptase stop at the TACTAAC box (Figure 2A). This result indicates that the rate of the second step of the splicing reaction, 3' cutting and exon ligation, is also slowed, although to a much lesser extent than the first step.

In addition to the 3' exon primer, we used an intron primer for the reverse transcription analysis. This primer gives rise to two classes of cDNA molecules. The longer are derived from the precursor molecules, and the shorter from 5' cut RNAs, i.e., the lariat-intermediate and the excised intron (Rodriguez et al., 1984). The 5' cut RNAs from the wild-type gene (and all mutant genes) generate two cDNA bands, one nucleotide apart (Figures 3A and 3B). Precise mapping of the two bands indicates that the lower one maps to the 5' splice site (Figure 3B). The upper band is more likely due to the addition of a nucleotide to the end of the reverse transcript (see Discussion). As the same pair of bands is visible in the two 5' mutant genes (Figure 3A), we infer that the proper 5' junction is used in both cases. Also visible in Figure 3A is the fact that the proper 5' cut cDNA bands from the 5'I gene are significantly more intense than the corresponding bands from the wild-type gene. This intensity increase is more pronounced than what is visible with the 3' exon primer, suggesting that the oxoiod intron is contributing to the lariat signal when assayed with the intron primer. A direct examination of the intron levels on Northern gels verifies this hypothesis (data not shown).

In RNA from the 5'I gene an additional pair of faint
Effect of Point Mutations in Yeast rp51A Intron

Figure 2. Primer Extension Analysis of the Mutant Transcripts Using a 3' Exon Primer

DB745 cells transformed with the wild-type HZ18 plasmid or with a plasmid carrying point mutations were grown as in Experimental Procedures. The oligonucleotide DB1, used as a primer, is homologous to rp51A 3' exon sequences contained in the fusion gene as indicated in Figure 1 and is the same primer used in Teem and Rosbash (1983). After synthesis, the cDNA products were analyzed by electrophoresis on a 6% acrylamide sequencing gel. The arrows denote cDNA molecules corresponding to (from top to bottom): unspliced fusion transcripts (P), spliced fusion transcripts (M), stop of the reverse transcriptase at the branchpoint of the lariat molecule (ICS), and spliced mRNAs from rp51A (rp51A). The name of the plasmid used is indicated above each lane.

bands is visible above the normal pair of 5' cut cDNA molecules (Figure 3A). The lowest band of the doublet maps three nucleotides upstream of the proper 5' splice site (Figure 3C; see Discussion). Presumably, these additional RNAs result from the use of a cryptic 5' splice site three nucleotides upstream of the normal GT pair (Teem and Rosbash, 1983). The result suggests that the position 5G is involved in the fidelity of the 5' cleavage. As no linear molecules and mRNA corresponding to the use of this cryptic site are detectable, we infer that it results in "frozen" lariat molecules, as previously observed by Parker and Guthrie (1985), and/or stable, excised introns which accumulate to a low level. The same mutation, introduced into the yeast actin intron, gives rise to qualitatively similar results. The effect of this mutant in the actin gene, however, is stronger as mRNA levels are significantly decreased and the level of aberrant, frozen lariat is higher than the results reported here (Parker and Guthrie, 1985).

Table 1. Estimation of the Splicing Efficiency of the Different Mutants

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Beta-galactosidase Activity</th>
<th>M/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HZ18</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>51</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>51I</td>
<td>1−0.75</td>
<td>0.1</td>
</tr>
<tr>
<td>31</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>31I</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>51-31</td>
<td>1−0.5</td>
<td>0.05</td>
</tr>
<tr>
<td>51-31I</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>51-31</td>
<td>0.005</td>
<td>0.00025</td>
</tr>
<tr>
<td>51-31I</td>
<td>0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>5'4</td>
<td>1−0.5</td>
<td>0.05</td>
</tr>
<tr>
<td>5'4-3'1</td>
<td>0.001</td>
<td>0.00005</td>
</tr>
<tr>
<td>5'4-3'1I</td>
<td>0.1</td>
<td>0.005</td>
</tr>
</tbody>
</table>

The beta-galactosidase activity was assayed as in Teem and Rosbash (1983). The numbers represent the relative activity normalized to the wild-type plasmid HZ18. Some variation occurred from experiment to experiment, so these values should be taken as averages. For 51I, 51-31, and 5'4 the activity was most often equal to wild-type but has, in some cases, been found to be slightly lower as indicated. The ratio M/P represents the ratio of mRNA level to precursor RNA level, normalized to the ratio observed for the wild-type plasmid HZ18. The quantitation of those two RNA species was done by densitometric analysis of different exposures of several primer extension experiments. Here again, some minor variations did occur for different cultures of the same mutant, so these values are ±20%. In the case where the mRNA level was too low to be measured by this method, it was derived from the enzymatic activity (by assuming that the beta-galactosidase activity was linearly proportional to the mRNA level).

Mutations in the ICS

Two point mutations have been introduced into the invariant TACTAAC sequence (ICS). The gene 31 contains an A to C transversion at position 2 (1GCCAAC). The gene 31I contains a C to T transition at position 3 (TATTAAC) (Figure 1). Both mutations have very similar effects. The level of mRNA is unchanged relative to wild-type. Also, the intensity of the reverse transcription stop at the TACTAAC sequence is identical to what is observed with RNA from the wild-type gene, indicating that the second step of the reaction is essentially unaffected. The level of precursor RNA, however, is increased in both cases. It is increased about fivefold from the 31 gene and about threelfold from the 31I gene (Figure 2A and Table 1). No qualitative change has been observed with either of these mutants. We conclude that both mutations modestly decrease the rate of the first splicing step and have no effect on the second step.

Analysis of Double Mutants

It has been previously proposed (Pikielny et al., 1983) that the 5' consensus (GUAUGU) and UACUAAC sequences are associated, at some step of the splicing reaction, with the help of base-pairing interactions between the two conserved blocks. The base pairs proposed are between the two GU pairs of the 5' consensus sequence and the two AC pairs of the TACTAAC sequence (see Figure 4). To test this hypothesis we combined the single point mutations in such a manner that they produced compensatory changes according to this model (Figure 4). Indeed, the
Figure 3. Analysis of the Transcripts by Reverse Transcription Using Intron Primers

(A) RNA was extracted from cells transformed with HZ18 or one of the mutant plasmids and used as a template to synthesize cDNAs using the intron primer RB17, homologous to sequences ~300 bp downstream of the 5' splice site (nucleotides 289 to 298 from the intron 5' end) as indicated in Figure 1. At the top of the gel, an mRNA-like pattern of bands is visible; they correspond to the different transcription start sites and thus represent the unspliced precursor transcripts (P). A doublet band, corresponding in size to the 5' end of the intron, is visible at the bottom of the gel (5' cut). An additional doublet is visible in the 5'11 mutant (5'11 cut). Other bands which appear between the precursor molecules and the 5' cut molecules almost certainly correspond to partial degradation products or random reverse transcriptase stops that occur when the precursor RNA accumulates to high levels.

(B) HZ18 RNA was analyzed by primer extension with another primer (RB27) located closer to the 5' end of the intron (nucleotides 21 to 30 from the intron 5' end; see Figure 1). The same kinased primer was used to sequence the fusion gene on the HZ18 plasmid after digestion with Bam HI and treatment with exonuclease III. The cDNAs and the sequence were analyzed in parallel on an 8% acrylamide sequencing gel. The letters adjacent to the gel indicate the sequence complementary to the sequence ladder and correspond to the RNA sequence. Only the portion of the gel around the 5'splice site is shown. The big arrow indicates the 5'splice site. The two small arrows point to the doublet corresponding to transcripts cleaved at the 5' splice site.

(C) The RB27 primer was used to synthesize cDNA using the 5'11 transcripts as templates. The products were analyzed on an 8% acrylamide sequencing gel in parallel with the HZ18 cDNAs. The two big arrows denote cDNA corresponding to transcripts cut at the normal 5' splice site while the two small arrows correspond to transcripts cut at the cryptic site in 5'11. P denotes the cDNAs representing the unspliced transcripts.

51–31 and 5'II–3'11 combinations form two pairs of compensatory changes. The results of each double mutant combination were thoroughly quantitated (from Figure 2B, other gels, and beta-galactosidase activity measurements). We included in this analysis an additional mutant, 54, which has a deletion of approximately 50 bp near the 5' end but leaves the 6 nucleotide 5' consensus sequence intact. This mutant, described in Pikielny and Rosbash (1985), causes a 20-fold increase in the level of precursor RNA and a twofold decrease in mRNA level, resulting in an overall decrease of 40-fold in splicing efficiency. Figure 4 and Table 1 summarize the results of this quantitative analysis. They show that the double mutant combinations have a lower splicing efficiency than either of the single mutations from which they were constructed. Thus, no overt compensation is visible.

In more quantitative terms, the effect of the double mutant combinations can be compared with the effect of the individual single mutations. The RNA analysis from the double mutants containing the 51 mutation confirms the results presented above on the effect of the individual 51 mutation. The splicing efficiency of the 51–311 double mutant is approximately twofold less than the 311 single mutant, and the splicing efficiency of the 51–31 double mutant is about fourfold less than the 31 single mutant (Table 1).

The effect of the 3'11 mutation is, to a first approximation, also additive to the effect of the 5' mutation with which it is combined, i.e., the overall splicing efficiency is reduced approximately three to tenfold by the 3'11 mutation when present in an otherwise wild-type intron or in any of the three 5' mutation-containing introns (Figure 4). The effect of the 31 mutant, however, is not so straightforward. It appears that the stronger the effect of the individual 5' muta-
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Figure 4. Base Pairing Model

(A) The diagram represents the base pairing between the 5' consensus sequence and the TACTAAC box as proposed by Pikielny et al. (1983). In addition, the point mutations introduced in these sequences are indicated.

(b) The table summarizes the effect of the two TACTAAC box point mutants when introduced into a wild-type gene (HZ18) or into genes carrying a potentially compensatory (c) or non-compensatory substitution or the 5'4 deletion.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>wt HZ18</th>
<th>wt HZ18</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'1 lowers 5'1</td>
<td>-5</td>
<td>-3</td>
</tr>
<tr>
<td>(c) 3'1 lowers 5'1</td>
<td>-20</td>
<td></td>
</tr>
<tr>
<td>3'1 lowers 5'11</td>
<td>-400</td>
<td></td>
</tr>
<tr>
<td>(c) 3'1 lowers 5'11</td>
<td>-10</td>
<td></td>
</tr>
<tr>
<td>3'1 lowers 5'4</td>
<td>-1000</td>
<td></td>
</tr>
<tr>
<td>3'1 lowers 5'4</td>
<td>-10</td>
<td></td>
</tr>
</tbody>
</table>

As described above, an increase in the level of lariat-intermediate (or, more properly, a decrease in the M/L ratio) indicates an effect on the second step of the splicing process. Only 5'11, of the four single mutations, has a small effect on the second step (Figure 2A). When the 5'11 mutation is combined with the 3'1 mutation, the first step of the splicing reaction is so strongly inhibited that the level of lariat intermediate is no longer measurable by primer extension (Figure 2B). It is, therefore, impossible to estimate the relative rate of the second step of the splicing reaction in this double mutant. When the 5'11 mutation is combined with its "compensatory counterpart," 3'1, the mRNA level is reduced approximately fivefold and the ICS level is identical to what is observed with only the 5'11 mutant (Figure 2B). We conclude that the 3'11 mutation not only fails to manifest any compensation of the 5'11 mutation, but also leads to a further twofold reduction in the rate of the second step as compared to 5'11 alone. As the 3'11 mutation alone has no effect on the second step of the splicing reaction, we surmise that, in combination with 5'11, it is enhancing the effect of 5'11 on 3' cleavage and ligation. In contrast to 5'11, the 5'1 mutation, when combined with either of the two TACTAAC mutants (the two remaining double mutant combinations), does not affect the second step of the splicing reaction since the M/L ratio remains identical to the wild-type in both cases (Figure 2B).

Discussion

In this report we present the effect of four point mutations in the highly conserved 5' junction and the absolutely conserved TACTAAC box of yeast introns (Teem et al., 1984; Langford et al., 1984). Three of the four mutants were inserted at completely conserved positions. Only the 5'1 mutation (GUAUGU-GUAUGG) occurs at a less stringently conserved position; of the 5' junctions sequenced to date all have a U at position 6 except S10-2, which has an A (Langford et al., 1984).

The positions and nature of the mutations were chosen with several objectives in mind. First, and most generally, we were interested in the reasons for the absolute conservation of some of these positions. Second, we wished to explore further some initial results in which two different TACTAAC box point mutations (TCCTAAC and TATTAAC) had strikingly different effects on splicing although, as mentioned above, both positions are equally and apparently absolutely conserved (Langford et al., 1984). Third, the mutations were chosen so that a particular intramolecular pairing model could be examined, i.e., so that potential compensatory and noncompensatory mutant pairs could be constructed (Pikielny et al., 1983).

Perhaps the most surprising feature of these results is that none of the four mutations significantly affect the level of mature mRNA or beta-galactosidase activity (Figure 2A and Table 1). These results are in marked contrast to the strong effects observed with mutations adjacent to the sites of 5' cleavage (GTATGT) and lariat formation (TACTAAC) (Newman et al., 1985). Either the nucleotide changes reported here have no effect on splicing (an unattractive possibility given their conservation) or they do not have sufficient impact to render splicing rate limiting for gene expression. As previously described, a quantitative examination of the levels of precursor RNA and intermediates distinguishes between these possibilities (Pikielny and Rosbash, 1985). Indeed, all four mutants cause an increase in precursor RNA levels (from two to 15-fold), from which we conclude that splicing efficiency is affected, albeit variably, in all four mutants. This universal effect on precursor RNA levels is not paralleled by a similar effect on the levels of lariat intermediate. Only 5'11 causes a small increase in this species; the other three mutants have no
effect. As a simple extrapolation of the steady state model indicates that M/P (mRNA:precursor ratio) reflects the efficiency of the first splicing step and that M/L (mRNA:latract intermediate ratio) reflects the efficiency of the second step, the data indicate that all four mutants manifest an effect on the first step. They also imply that the first step is at least 15-fold more efficient than necessary to produce wild-type levels of mRNA. These analyses do not distinguish among the various possibilities by which the first step could be affected, e.g., intron recognition, assembly into a splicing particle, or some difficulty with the catalytic step (or steps), 5' cleavage and lariat formation.

The conclusion that both 3'1 (TCTAAC) and 3'11 (TAT-IAAC) have a similar, mild impact on splicing differs significantly from conclusions previously drawn for these same two mutants introduced into actin gene-derived constructs (Langford et al., 1984). Although differences in the test gene could influence the results and conclusions, it is likely that the artificial construct used to examine the 3'1-like mutant in the actin constructs is responsible for the disparity in the results (Langford et al., 1984). This construct was markedly inefficient, rendering gene expression highly sensitive to the addition of a relatively mild TACTAAC mutation. Indeed, we observe a very similar, strong effect when the 3'1 mutation is added to the 5'4 deletion (Table 1, Figure 4, and see discussion below). In contrast, the 3'11-like mutation was introduced into a wild-type actin intron (Langford et al., 1984). Although this mutation was reported to have no effect, precursor RNA was not detectable; thus the assay was insensitive to modest effects on splicing. We suggest, therefore, that these two mutations will have similar mild effects when both are introduced into a wild-type actin intron and precursor RNA levels assayed.

Of the four mutants examined, only 5'11 (GUAUGU-GUAUAU) manifests an effect on the second stage of splicing as evidenced by a mild increase in the lariat intermediate level (and more significantly L/M levels). 5'11 is also the only mutant to manifest a qualitative effect on splicing as an additional 5' cleavage event apparently takes place. The absence of any detectable aberrant splicing events in 51 is in contrast to what has been observed with 5'11 position six mutants in mammalian genes (Treisman et al., 1985).

We experienced some difficulty in defining the precise location of the aberrant 5' cleavage in 5'11 as these additional cDNAs as well as the cDNAs derived from the normal 5' cleavage are doublets. Precise mapping of the wild-type doublet shows, somewhat unexpectedly, that it is the lower band that corresponds precisely to the proper 5' cleavage site; the upper band is one base too long (Figure 3B). Moreover, chemical sequencing of these two cDNAs shows that the shorter one has the expected sequence while the longer one has an extra nucleotide, apparently added without preference during cDNA extension (R. Raymond, personal communication). A similar artifact has been reported to occur during the reverse transcription of the 5' ends of capped molecules (Luse et al., 1981).

As these analyses define the lower cDNA band as corresponding to the proper 5' cleavage site, we assume that a similar artifact occurs at the 5' cryptic site in the 5'11 gene, thereby placing the aberrant cut directly upstream of an AT dinucleotide, three nucleotides upstream of the normal GT (Figure 4C). It is worth noting that the same mutation, when introduced into the yeast actin intron, has a similar qualitative effect. In this case an aberrant cut upstream of an AT pair, six nucleotides upstream of the normal 5' splice site, is also activated (Parker and Guthrie, 1985). This similarity reinforces the hypothesis that the mutant 5' junction retains some recognition specificity for the aberrant 5' cleavage that occurs nearby (Parker and Guthrie, 1985). As a position 1 G→A mutant (GUAGUG→AUAGUG) undergoes 5' cleavage at the proper location (Newman et al., 1985), it would appear that PuU is a reasonably efficient 5' cleavage site.

It is interesting to note that all the lariats with an A-A branch appear to be relatively stable. From the quantitative point of view, this implies that the aberrant lariats described here and elsewhere may be produced at extremely low rates but accumulate to relatively high levels because they are frozen. We have confirmed this hypothesis by performing in vivo pulse-chase experiments (data not shown). (As HZ18 and its mutant derivatives contain a galactose-inducible glucose-repressible upstream activator sequence, the GAL UAS [Quarrie et al., 1982, Teem and Rosbash, 1983], the gene 5'11 was "turned off" by subjecting the galactose UAS to glucose repression.) Thus, the ratio of aberrant to proper cDNA molecules over-represents the rate of aberrant cleavage relative to proper 5' cleavage. The fact that these aberrant A-A lariats are frozen also implies that the rate of the second step, 3' cleavage and ligation, is sensitive to some primary sequence changes of the 5' junction when attached to the lariat A of the TACTAAC box in a 2-5' phosphodiester bond (as previously noted, Parker and Guthrie, 1985; Newman et al., 1985). Similarly, the C-A change at position 5 reported here appears to have a direct effect on the second step, as evidenced by the increase in the ratio of proper lariat to mRNA levels. (We argue that in the case of 5'11 the amount of aberrant lariat is small compared to proper lariat; thus, the increase in ICS might reflect a genuine increase in the level of proper lariat.) Nevertheless, it is interesting to note that the effect of 5'11 is much more pronounced on the first step than the second and that the other three mutants apparently affect only the first step. These results contrast with a marked effect on the second step of a G to A transition at the 5' junction G (ATATGT) (Newman et al., 1985). The results of the double mutant analysis suggest that the 5' junction and TACTAAC mutants affect at least one common step in splicing as, in every case, the double mutants are less efficient than the corresponding single mutants. They also argue quite persuasively that the base-pairing interactions originally proposed between the 5' junction and the TACTAAC box do not play a major role in determining splicing efficiency. As possible compensatory changes have not been found by examining natural variation among different yeast introns, it is virtually certain that these two conserved sequences are involved in a primary sequence recognition process. Thus, the ex-
amination of this base-pairing hypothesis included an attempt to filter out or subtract primary sequence contributions from any potential intramolecular interaction. To this end, we quantitated the effect of an initial point mutation on a noncompensatory mutation. This was compared with the effect of the same initial point mutation on a noncompensatory point mutation or deletion (6') (Figure 4).

If the proposed intramolecular pairing is relevant and detectable, the effect of the initial substitution should be less pronounced when combined with a compensatory mutant than with a noncompensatory one, even if the splicing efficiency of every double mutant is less than either of the two corresponding single mutants. Although this prediction is verified in the case of 31−51 (compensatory) and 31−511 (noncompensatory), the even more dramatic effect of 31 on the deletion mutant 54' argues against a positive interpretation (Figure 4 and Table 1). At a qualitative level, we note that in the double mutant combinations containing 511, the aberrant 5′ cut characteristic of 511 is no longer detectable. While this result may indicate a role for the proposed pairing in splice site selection, it is more likely a trivial consequence of the very low overall rate of 5′ cleavage in these double mutant combinations.

How can we explain the fact that three of the four positions examined in this communication appear absolutely conserved yet mutations at these positions have no effect on the level of gene expression? While other interpretations are possible, we favor the notion that this apparent paradox is a result of uniform laboratory growth conditions. Even in the absence of variation, we note that in the double mutant combinations containing 511, the aberrant 5′ cut characteristic of 511 is no longer detectable. While this result may indicate a role for the proposed pairing in splice site selection, it is more likely a trivial consequence of the very low overall rate of 5′ cleavage in these double mutant combinations.

Experimental Procedures

Yeast Strains, Media, and Transformation

Procedures were as in Roosbeek et al. (1985) except that the in vitro transformation protocol was followed (Ito et al., 1983).

In vitro Mutagenesis and Plasmid Constructions

All the constructions were done using the HZ18 plasmid described in Teem and Rosbash (1983). For the 5' mutation, the Barn HI-Sal I restriction sites of the pEMBLS' plasmid were annealed to 0.5 pg of the single-stranded template by slow cooling from 80°C to 25°C in a 10 ml solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 50 mM NaCl, and 1 mM dithiothreitol. To this was added 30 ul of a solution containing each dNTP at 0.3 mM, 0.3 mM ATP, 12.5 mM Tris-HCl (pH 7.5), 0.25 mM EDTA, 2.5 units of the Klenow fragment of DNA polymerase I, and 3 units of T4 DNA ligase. The reaction was incubated for 12 hr at 15°C. An aliquot of the reaction was used directly to transform E. coli K12 JM101. Nitrocellulose colony lifts were made directly from the transformation plates (which were then rean-

cubated for an additional 6 hr), treated 5 min with 0.5 M NaOH, neutralized 2 x 1.0 min with 1 M Tris-HCl (pH 7.5) and 1 min with 0.5 M Tris HCl (pH 7.5), 16 M NaCl, then baked 2 hr at 80°C and prohybridized at 65°C for 2 hr in a solution containing 5 x Denhardt's solution (Denhardt, 1966), 0.5% SDS, 6 x SSC (0.9 M NaCl, 0.09 M sodium citrate), and 0.1 µg/ml sonicated, denatured salmon sperms RNA. Hybridization was performed at 25°C for 12 hr in a solution containing 5 x Denhardt's solution, 6 x SSC, 0.1% SDS, 250 µg/ml sonicated, denatured salmon sperm RNA, and 10 x cpm/ml of 32P-end-labeled mutagenic oligonucleotide. The filter was washed 3 x 15 min in 6 x SSC, 0.1% SDS at 45°C, and autoradiographed. Further washes were performed at increasing temperatures (4°C increments) in order to distinguish mutant and wild-type colonies. After washes, the HZ18 insert of the each mutatinal pEMBL plasmid was sequenced according to Sanger et al. (1977). The HZ18 mutated restriction fragment was then gel purified and used to replace, by in vitro recombination, the corresponding restriction fragment of the wild-type HZ18 plasmid.

RNA Extraction and Primer Extension

All RNA was extracted from cells harvested at OD600 of 0.4−0.6 as described (Roosbeek et al., 1985). Primer extensions were as described (Teem and Rosbash, 1983).

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