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Effects on mRNA Splicing of Mutations in the 3′ Region of the *Saccharomyces cerevisiae* Actin Intron

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Point mutations, deletions, and a sequence context change were introduced at positions 3′ to the internal conserved TACTAAC sequence of the *Saccharomyces cerevisiae* actin intron. In vivo analysis of yeast mRNA splicing suggests that, in contrast to the importance of the polypyrimidine tract in metazoan introns, specific sequences in this region are not required for efficient excision of a yeast intron. However, a double point mutation near the 3′ junction (GG/AC) does severely inhibit splicing. Although this mutagenesis of the 3′ junction, as well as deletion of most nucleotides between the TACTAAC and the 3′ junction, caused only a slight accumulation of primary transcript, the observed accumulation of lariat intermediate by these mutants demonstrates the significance of this region for a step(s) in the splicing process after lariat formation.

Nuclear mRNA splicing in both *Saccharomyces cerevisiae* and higher eucaryotes is a two-stage process; the intermediates are exon 1 and a lariat intron contiguous with exon 2 (the lariat intermediate) (17). Steps in stage 1 include assembly of an RNA-protein complex on the primary transcript (the spliceosome) (3, 10, 12, 29), cleavage at the 5′ exon-intron junction, and subsequent formation of a 2′-5′ phosphodiester bond between the phosphate at the 5′ end of the intron and the ribose 2′ hydroxyl of an A nucleotide within the intron. Stage 2 processes include cleavage at the 3′ intron-exon junction and ligation of the two exons to produce mature mRNA. A free intron in a branched configuration is an additional product (8, 26, 32, 36, 40).

Efficient splicing in yeast requires the integrity of the conserved junction sequences, 5′ GTAPyGT. . . .AG 3′ and the internal conserved sequence (ICS), TACTAAC (9, 11, 14, 15, 19, 20, 25, 27, 30, 39). However, the presence of these is not sufficient; these conserved sequences have been found in close proximity and in the correct juxtaposition in at least two transcripts, but are not recognized as defining an intron during posttranscriptional RNA processing (A. Newman and G. Taylor, personal communications). Analysis of intron mutations has demonstrated that there are sequence context and spacing constraints for efficient splicing. Changes in intron sequences adjacent to the 5′ junction and ICS can dramatically effect the efficiency of splicing (28), and insertions at various positions within the yeast actin gene have demonstrated that splicing efficiency decreases as the distance increases either between the 5′ cap structure and the intron, between the 5′ junction and the ICS, or between the ICS and the 3′ junction (5, 18). Yeast introns are relatively small (50 to 700 nucleotides) and are present near the 5′ end of some primary transcripts.

Splicing of mRNA in higher eucaryotes and *S. cerevisiae* has many common characteristics; however, there are some distinctions. In contrast to the strong conservation of specific intron sequences in yeast, only the GT and AG dinucleotides at the 5′ and 3′ intron junctions, respectively, are invariant in higher eucaryotes (2, 24), indicating that the substrate nucleotide requirements for splicing in yeast are much more stringent. The branched nucleotide within a yeast intron lariat is always the penultimate nucleotide of the ICS (8, 21, 32), TACTAAC, even if this position is mutated to either a G or a C (9, 15, 39). Although the internal branch point in a higher eucaryotic intron is within a variant sequence with some similarity to the ICS of yeast (16, 36, 40), selection of the branch point is more critically dependent on its distance from the 3′ intron junction (18 to 37 nucleotides) and, in particular, requires a polypyrimidine tract adjacent to the 3′ junction (10, 31, 34, 35, 37). In contrast, the location of the TACTAAC sequence (and branch point) in yeast introns varies between 9 and 138 nucleotides from the 3′ junction (20, 30; P. Schatz, personal communication); the first PyAG downstream of the ICS is the 3′ junction. Data from an in vitro splicing analysis of truncated transcripts suggest that, unlike the case in higher eucaryotes, sequences downstream of the ICS are not required for first stage processes in yeast (38).

Here we examine the importance of the yeast 3′ intron region, i.e., sequences between the ICS and 3′ junction, by generating several mutations in this region of the yeast actin intron. This analysis demonstrates that splicing occurs efficiently in vivo despite mutations, including several deletions, in this region. However, a deletion of most nucleotides in this region did inhibit splicing, particularly the second stage. A double point mutation near the 3′ junction had only a minor effect on stage one but had a strong inhibitory effect on stage two.

MATERIALS AND METHODS

Bacterial and yeast strains and plasmids. *Escherichia coli* K-12 strain MC1000 [Δ(*lacIPOZYA*)X79 *galU galK rpsL*] was the bacterial host (4). *S. cerevisiae* PY-88 (α *leu2-3,-122* His′) was obtained from P. Sadowski. Yeast strain YF302 (α *leu2-3,112 ura1 rna2-1* Ade′ His′) is a segregant of a cross between strain *ts*368 (**a** *ade1 ade2 his7 lys2 tyr1 ura1 gal1 rna2-1*) from the Yeast Stock Center and LL20 (α *leu2-3,-122 his3-11,-15 can1*) from L. Lau. Growth and transformation of *E. coli* and *S. cerevisiae* were done as described previously (22, 23). Growth under conditions of thymidine kinase (TK) selection was as described by Himmelfarb et al. (13).

Plasmid pLF43 is a yeast-bacterial shuttle vector that contains a fusion between the 5′ portion of yeast actin, herpes simplex virus (HSV) TK, and the 3′ portion of yeast *tcm1*, in addition to the pBR322 *E. coli* origin of replication, the β-lactamase gene, the yeast 2μm origin of replication,

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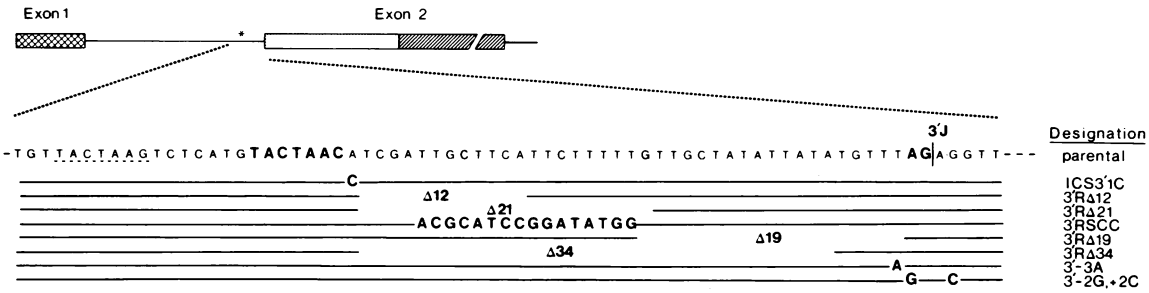

FIG. 1. Mutations in the 3' intron region of an actin-HSV TK fusion gene. Actin intron mutations were generated by oligonucleotide-directed mutagenesis by methods described previously (9). These mutations lie within an actin intron in a fusion of the 5' portion of yeast actin to the HSV TK gene carried on the yeast bacterial shuttle vector, pLF43 (9). Symbols: ▨, exon 1 actin RNA; ▩, actin portion of exon 2;——, actin intron (*, position of the ICS TACTAAC); ▨, TK portion of exon 2; ▬ (at the 3' end of exon 2), *tcm1* RNA. The ICS, TACTAAC, of the intron is indicated in bold type, and the cryptic TACTAAG sequence seven nucleotides upstream is underlined. The mutations are described in the text.

and the yeast *LEU2* gene. Its construction has previously been reported (9).

Construction of mutants. All mutations were generated by oligonucleotide mutagenesis on a fragment of pLF43 inserted into bacteriophage M13mp9 and identified as described previously (9). Shane Climie synthesized the oligonucleotides, which ranged in size from 17 to 36, by phosphoramidite chemistry (1) with an Applied Biosystems DNA synthesizer.

RNA purification and analysis. RNA was extracted and analyzed by blot hybridization and primer extension as described previously (9).

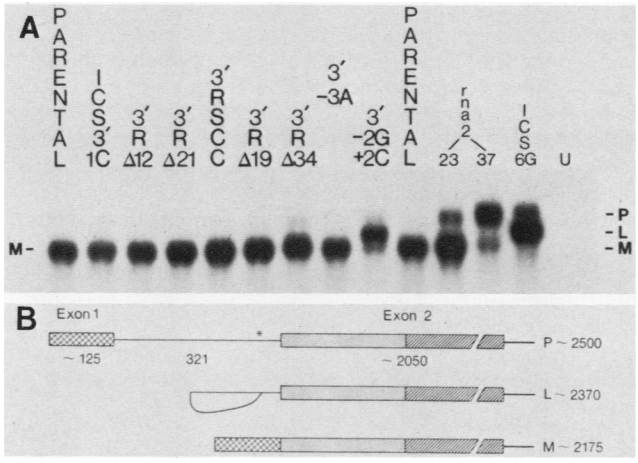

FIG. 2. Blot hybridization analysis of actin-TK fusion RNA from cells carrying plasmids with intron mutations. A, Total RNA was extracted from strain PY-88 that had been transformed with the parental or mutant plasmids. RNA was electrophoresed, the gel was blotted, and the filter was probed with a randomly primed TK-specific fragment (1.7-kilobase *Bgl*II-*Pvu*II fragment). Abbreviations: P and M, primary transcript and mature mRNA, respectively; L, lariat intermediate. Lanes: parental, RNA from PY-88 transformed with the unmutated plasmid; rna2 23 and 37, RNA from the *rna2* strain YF302 that was transformed with an actin-TK fusion plasmid and grown at 23 and 37°C, respectively (at the latter temperature, an *rna2* strain accumulates mostly primary transcript [33]); ICS-6G, RNA from an intron mutant that accumulates mostly lariat intermediate (9); U, RNA from an untransformed strain. Approximately equal amounts of RNA were loaded in each lane (exceptions are rna2 samples). B, The expected transcript sizes (excluding polyadenylation) are indicated for primary transcript (P), mature mRNA (M), and the lariat intermediate (L). The remaining symbols and designations are as in Fig. 1.

RESULTS

To investigate the importance of yeast intron sequences downstream from the ICS, we generated several mutations in this region of the actin intron that is in a previously described yeast actin-HSV TK fusion gene (Fig. 1) carried on a yeast-bacterial shuttle vector (9). All mutations were made by oligonucleotide-directed mutagenesis on a single-stranded template, screened by colony hybridization, and verified by dideoxy sequencing (9). DNA fragments containing mutations in the 3' intron region were then exchanged for the parental fragment of the plasmid (9).

Eight mutant plasmids were constructed (Fig. 1). Two of these are single point mutants, one of which contains an A-to-C transversion (ICS3'1C) immediately 3' to the conserved TACTAAC sequence; this position is often an A. The other contains a T-to-A transversion (3'–3A) at the conserved pyrimidine position that is three nucleotides upstream from the 3' junction. A double point mutant (3'–2G,+2C) that is mutated near the 3' junction contains a transition two nucleotides within the intron and a transversion two nucleotides outside of the intron; the second mutation was made to prevent an AG dinucleotide within exon 2 from being used as an alternative 3' junction. In addition, four deletions were constructed that range in size from 12 to 34 nucleotides (3'RΔ12, 3'RΔ19, 3'RΔ21, and 3'RΔ34). Finally, a sequence context change was made (3'RSCC) that maintains the normal spacing between the actin ICS and 3' junction but replaces a pyrimidine-rich segment with a randomly generated sequence. Yeasts were transformed with a 2μm-based plasmid carrying either the parental or a mutated actin-TK fusion, and the in vivo effects on splicing were examined.

First, the effects of these mutations on TK-dependent growth were determined. Although we have not examined RNA from cells grown under TK-dependent growth conditions, our previous characterization of 12 intron mutants suggests that correct excision of the actin intron from the actin-TK fusion primary transcript is required for production of TK activity, as defined by a TK-selective growth assay (9). We observed that most of the 3' region mutants had no effect on TK-dependent growth. The exception is mutant 3'−2G,+2C, which was slightly inhibited (data not shown).

Total RNA was prepared from the transformants and was first analyzed by blot hybridization with a radioactive TK

fragment as probe. Most of the mutations appear to have no effect on splicing. Mutants ICS3′1C, 3′RΔ12, 3′RΔ19, 3′RΔ21, 3′RSCC, and 3′-3A accumulated only mature fusion mRNA at a steady-state level comparable to that in the parental RNA sample. For two mutants, 3′−2G,+2C and 3′RΔ34, other RNA species were observed in addition to mature transcript. In particular, mutant 3′−2G,+2C accumulated an RNA species that migrated between the primary transcript and mature mRNA. RNA with this same migration pattern was observed for mutant ICS-6G (Fig. 2A); previous characterization of the major RNA from mutant ICS-6G demonstrated that it is lariat intermediate (9). Further analysis (see below) indicated that the major RNA species from mutant 3′−2G,+2C was also lariat intermediate. In addition, a small amount of primary transcript was observed in the RNA of mutants 3′−2G,+2C and 3′RΔ34 that was not observed in the RNA of the other 3′ region mutants or the parental transformant (Fig. 2A).

Total RNA from the mutant and parental transformants was also used for primer extension analysis with a primer that hybridizes in the TK portion of the fusion RNA. Note that a cDNA synthesized from the lariat intermediate is blocked from further extension at the branch point (8, 32, 36). In agreement with the observations from the blot hybridization analysis, the relative intensities of cDNA bands that corresponded to primary transcript (P), lariat intermediate (L), and mature mRNA (M) for most mutants were comparable to the relative cDNA band intensities observed for parental RNA (Fig. 3A). The exceptions again were mutants 3′−2G,+2C and 3′RΔ34; RNA from these two mutants produced the most intense L and P cDNA bands but the least intense M cDNA bands. A comparison of the L and M cDNA products from these two mutants demonstrates that lariat intermediate accumulates relative to mature mRNA. We assume that the small amount of lariat intermediate accumulated by mutant 3′RΔ34 is masked by the major accumulation of mature mRNA in the blot hybridization shown in Fig. 2A. Although less obvious, a comparison of P cDNA products in Fig. 3 suggests that there is a slight accumulation of primary transcript for mutants 3′−2G,+2C and 3′RΔ34.

To identify the lariat branch point of the mutant lariat intermediates, primer-extension reactions performed for Fig. 3 were also coelectrophoresed with dideoxy sequencing reactions from the same, end-labeled TK primer (Fig. 4). The L cDNA of all mutant RNAs and parental RNA was blocked from further extension at the same position, the last nucleotide of the ICS (TACTAAC), suggesting that the 2′ branch is made from the ribose moiety of the final A (TACTAAC) (9, 32); nuclease analysis of a branched intron indicates that this A is the internal branch point (8, 21). Since some of the mutants contain deletions within the region flanked by the site of primer hybridization and the lariat branch point, the L cDNA of these mutants is shortened by the size of the deletion (Fig. 4A).

A TACTAAG sequence that is 7 nucleotides 5′ to the ICS (Fig. 1) is apparently not used in any of the mutants for formation of an alternative branch, since cDNAs are not observed that terminate at the expected position 14 nucleotides upstream from the L cDNAs in Fig. 4 (see also the sequence for this region in Fig. 1). Previously we demonstrated that the same sequence at the normal ICS position still allows fairly efficient splicing (9), whereas others have shown that this upstream cryptic TACTAAG is used for branch formation, albeit inefficiently, only if the actin ICS is deleted (6) or contains a C-to-A transversion at the third

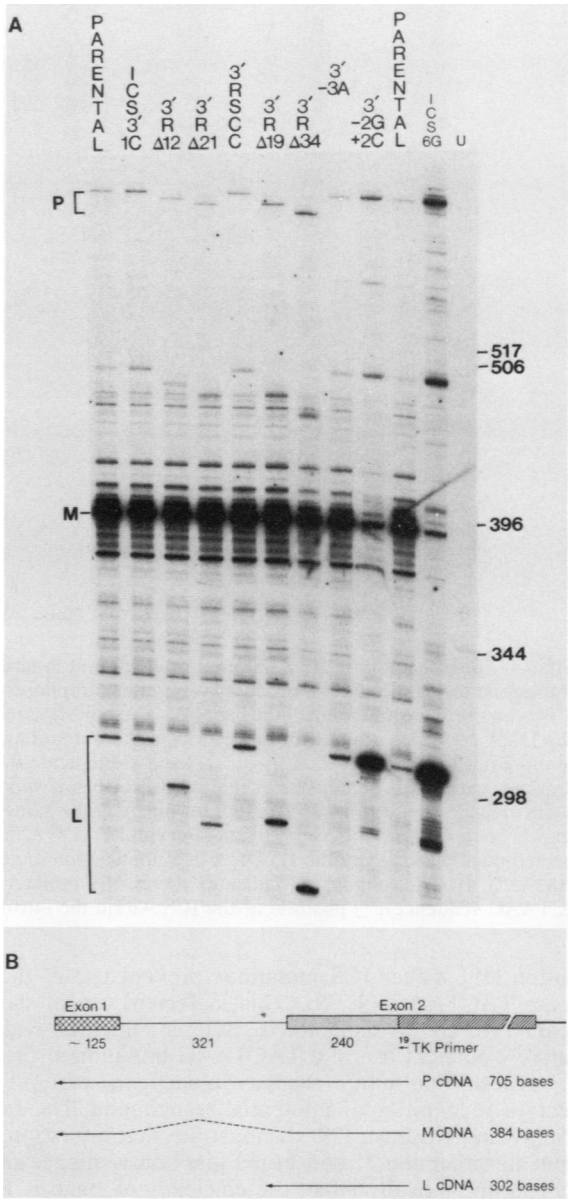

FIG. 3. Analysis of reverse transcriptase products synthesized from RNA using a TK-specific primer. A, Primer extensions were performed on total RNA with a primer that hybridizes to a TK sequence immediately downstream from the actin-TK fusion junction (TK primer in B). The numbers on the right refer to the positions of radiolabeled, pBR322 *Hin*fI restriction fragments. Abbreviations: P, M, and L, expected bands that correspond to cDNA products from primary transcript, mature mRNA, and lariat intermediate, respectively. Lanes: parental, RNA from PY-88 transformed with the unmutated plasmid; ICS-6G, RNA from an intron mutant that accumulates mostly lariat intermediate (9); U, RNA from an untransformed strain. In addition to the expected cDNA products, premature termination products originating from mainly primary and mature transcript are also observed. Approximately equal amounts of RNA were used as template for reverse transcriptase. B, The lengths of expected cDNA product derived from primary transcript (P), lariat intermediate (L), and mature mRNA (M) are indicated. The remaining symbols and designations are as in Fig. 1.

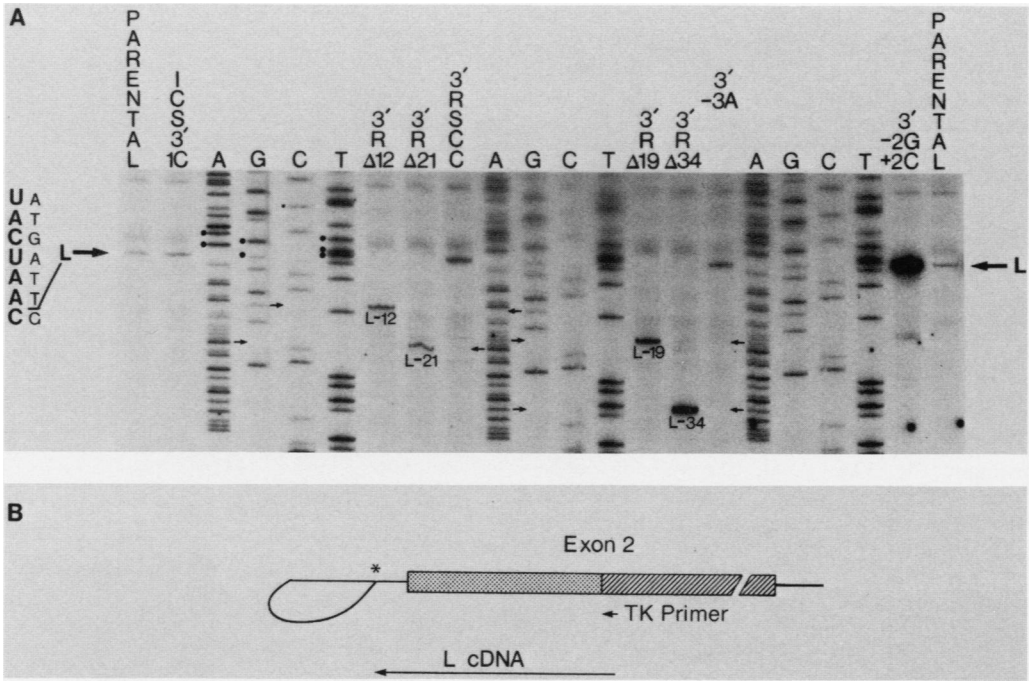

FIG. 4. Identification of the 2′ internal branch point in lariat intermediates. A, Primer extension reactions were carried out as indicated in the legend to Fig. 3, and the products were coelectrophoresed with end-labeled products of dideoxy sequencing reactions (lanes A, G, C, and T) with the same TK primer (see panel B). The bold-lettered sequence to the left represents the RNA template; the complementary strand is the DNA sequence which can be read from the autoradiogram (●). L indicates the expected position of a cDNA derived from normal parental lariat intermediate. L-12, -19, -21, and -34 indicate the L cDNAs from the deletion mutants; a comparison of the sequencing lanes and primer extensions demonstrates that each L cDNA is shortened by the exact size of the deletion (small arrows originating from dideoxy sequencing lanes point to L cDNAs that terminate at the same position). This figure demonstrates that the cryptic TACTAAG is not used as a branch point by any of these mutants (a cryptic L cDNA in the deletion mutants would be 14 nucleotides longer than that observed; the expected position for a cryptic L cDNA in a nondeletion mutant is indicated by a small square adjacent to a band in the C sequencing lane on the left). B, The long arrow indicates the cDNA product derived from lariat intermediate (it terminates at the final nucleotide of the TACTAAC sequence); *, position of the ICS within the intron. The remaining designations are as in Fig. 1.

position (39). Other ICS mutations prevent use of the upstream TACTAAG (9, 39). This inefficient use of the upstream TACTAAG sequence (6, 39), as compared with the relatively efficient use of a TACTAAG mutation at the ICS (9), suggests that subtle changes in sequence context may effect the recognition of a potential branchpoint. The data of Pikielny and Rosbash (28) demonstrate that alterations adjacent to either the 5′ conserved junction sequence or the ICS can dramatically effect the efficiency of stage 1. However, the analysis described in this paper demonstrates that certain sequence context changes (and deletions) near the ICS have no apparent effect on splicing (Fig. 1 through 3).

DISCUSSION

Several mutations were made downstream of the TACTAAC ICS in the actin intron of a yeast actin-HSV TK fusion gene. Most of the mutations in this region, those between the ICS and the 3′ junction, had no measurable effect on in vivo splicing. The two exceptions were mutant 3′−2G,+2C, which contains two point mutations near the 3′ junction, and mutant 3′RΔ34, which contains only eight nucleotides (instead of 42 nucleotides) between the ICS and the 3′ junction. For these, particularly mutant 3′−2G,+2C, lariat intermediate RNA accumulates relative to mature mRNA (Fig. 2 and 3). In addition, both of these mutants accumulate a small amount of primary transcript (Fig. 2 and 3). These observations indicate that stage 2, cleavage at the 3′ junction and ligation of exon 1 to exon 2, is less efficient in these two mutants and suggest that stage 1, cleavage at the 5′ junction and formation of the 2′-5′ branch, is only slightly hindered. Our criteria for demonstrating an effect on stages 2 and 1 are based on a two-step kinetic model that we described previously (9), which is an extension of a splicing scheme originally proposed by Pikielny and Rosbash (28). Briefly, the steady-state level of mature transcript relative to lariat intermediate mirrors the efficiency of stage 2 processes, whereas the steady-state level of mature transcript relative to pre-mRNA mirrors the efficiency of stage 1 processes. The validity of the ratio of mature transcript to pre-mRNA as a measure of stage 1 efficiency is dependent on certain assumptions (9). Therefore, in the present discussion, our conclusions with respect to an effect on stage 1 are based solely on an absolute increase in the level of primary transcript.

Several lines of evidence now demonstrate that the mechanism by which the branch point in a mammalian intron is chosen differs from that for a yeast intron. Although 3′ junction cleavage does not occur until stage 2 of splicing, in vitro analyses of higher eucaryotic splicing have demonstrated that 5′ junction cleavage and branch formation are dependent on prior recognition of the 3′ consensus splice site (Py_NAG), particularly the polypyrimidine region, by a spliceosome component (10, 31, 34). For yeast, in contrast, Rymond and Rosbash (38) have shown in vitro that efficient stage 1 splicing processes can still occur in the absence of

exon 2, the 3' junction, and the final 18 nucleotides of the yeast rp51Δ2 intron, demonstrating that the 3' AG junction is not required for efficient production of the lariat intermediate. The primary transcript used for this in vitro study, however, retained approximately 37 nucleotides downstream of the TACTAAC sequence, including a pyrimidine tract of six nucleotides. Our analysis suggests that efficient stage 1 and 2 processes do not require any specific actin intron sequences between the TACTAAC and AG conserved sequences; an exception may be the TT dinucleotide that is two and three nucleotides upstream from the 3' conserved junction sequence, since these nucleotides are present in all mutants. Although a polypyrimidine tract in the 3' region is probably dispensable, we are aware of data that suggest that a pyrimidine-rich sequence upstream from the ICS is necessary for efficient splicing (A. Newman, personal communication).

In contrast to higher eucaryotes and as originally proposed by Langford and Gallwitz (19), recognition of the 3' junction in yeast probably occurs after recognition of the TACTAAC sequence. Although we and others (38) conclude that sequences downstream of the ICS are not important for the bond cleavage and formation events in stage 1, two mutations that we have examined, 3'−2G+,+2C and 3'RΔ34, do slightly hinder stage 1 processes. We suggest that these two mutations cause a slight inhibition by interfering with normal assembly or altering a conformation within the complex. Although the data of Cellini et al. (5) suggest that stage 1 processes in yeast remain efficient even when 66 or 123 nucleotides of pBR322 sequences are inserted immediately downstream of the actin TACTAAC sequence, a truncation of the 66-nucleotide insertion mutant (leaving 38 nucleotides of foreign sequences downstream from the ICS) did result in a strong inhibition of stage 1 in vitro. We suggest that the above truncation, rather than missing a required substrate sequence, may simply contain an inhibitory sequence that is masked in the 66-nucleotide insertion mutant. Our results and those of Rymond and Rosbash (38) demonstrate that intron sequences downstream of the ICS are not required for formation of the lariat intermediate.

S. cerevisiae and higher eucaryotes use different mechanisms for the selection of the eventual branch point and the 3' junction. Therefore, it is not surprising that the observed ranges in spacing between these sequences may differ for lower and higher eucaryotes. Previous studies indicate that an A lying between 18 and 37 nucleotides upstream from the 3' junction is used for formation of a lariat branch point in higher eucaryotes (31, 35). Selection of the 3' junction in yeast is less constrained; examination of yeast introns sequenced to date indicates that distances between the TACTAAC and AG sequences range from 7 to 136 nucleotides (20, 30; P. Schatz, personal communication). However, since relative splicing efficiencies for yeast genes have not been reported, we cannot exclude the possibility that variations in splicing efficiency could reflect variations in this spacing.

Since the selection of the AG that defines the 3' junction appears to occur after lariat formation in yeast (38), it is not surprising that changes in spacing that affect splicing also interfere with stage 2 processes in particular. A 66- or a 123-nucleotide insertion (5) or a 34-nucleotide deletion (mutant 3'RΔ34; 8 nucleotides remaining) in this region of the actin intron causes an accumulation of the lariat intermediate, but little or no increase in primary transcript levels. Mutational analysis of the conserved intron sequences has demonstrated that the integrity of the first and second nucleotides of the 5' consensus sequence, GTATGT, and the branch point, TACTAAC, are also particularly important for efficient use of lariat intermediate (9, 25) as is the conserved 3' junction dinucleotide (mutant 3'−2G,+2C in Fig. 1 through 3; (39).

These observations indicate that after the formation of the lariat intermediate, recognition of the substrate RNA by components of the yeast spliceosome must change in some manner such that spacing and certain conserved nucleotides now become more significant. Two possibilities, as previously proposed (5), are that a conformational change within the spliceosome may occur or additional factors may assemble in the spliceosome. Although these alternatives are not mutually exclusive, the latter is strengthened by the observation in vitro that only stage 1 processes occur in specific extract fractions, but full splicing can be reconstituted if an additional fraction is added (7).

Although we have not identified the 3' junction used for splicing in mutant 3'−2G,+2C, both the size of the mature transcript (Fig. 3) in this mutant and the positive phenotype observed under TK-dependent growth conditions (data not shown) suggest that the GG dinucleotide is recognized as a 3' junction, albeit less efficiently. In contrast, a comparable mutation in a human β globin gene prevents 3' junction cleavage entirely (31). Mutant 3'RSCC also contains two GG dinucleotides, neither of which is used as a junction; presumably these cannot compete with an AG dinucleotide for recognition by a component of the spliceosome. Since mutant 3'−2G,+2C contains a transversion two nucleotides inside exon 2, we cannot exclude the possibility that this specific mutation might also contribute to the in vivo effects on splicing. A similar double point mutation has been made by others at the 3' junction of the actin intron, AC-AC, and this junction is used very inefficiently (39).

Two mutations (ICS3'1C and 3'−3A) that we introduced are transversions at intron positions that are either highly conserved (TACTAACA) or completely conserved (*Py*AG/3'). We could detect no effect on splicing for either of these mutations (Fig. 2 and 3). This is an unexpected result for the conserved pyrimidine adjacent to the conserved 3' AG sequence. However, observations similar to this have been made for transversions at the first and second positions of the ICS, TACTAAC (9, 14). As has been proposed previously (28), presumably a mutation at these conserved positions confers a disadvantage on yeast in a natural habitat, and selective pressures would maintain only cells with optimally spliced introns.

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