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Activation of a Cryptic TACTAAC Box in the *Saccharomyces cerevisiae* Actin Intron

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We constructed a translational fusion between the *Saccharomyces cerevisiae* actin gene and the *Escherichia coli* β-galactosidase structural gene such that expression of β-galactosidase activity required accurate splicing of the actin intron. Using this chimeric gene, we generated a series of internal deletions which removed the TACTAAC box or, in addition, TACTAAC-like sequences within the intron. Analysis of the fusion transcripts produced in these deletions allowed us to conclude that the TACTAAC-like sequence TACTAAG can substitute, albeit inefficiently, for the authentic TACTAAC box in the splicing process. These results indicate that the yeast splicing machinery can utilize a cryptic TACTAAC box, but there are requirements for primary sequence and proper position.

A key step in the splicing process is the definition of the precise boundaries of the intron by the splicing machinery. In the yeast *Saccharomyces cerevisiae*, a subset of nuclear genes encoding mRNAs contain intervening sequences (IVS) (34). Previous work has identified two regions within the intron which are required for splicing. The sequence at the 5′ intron-exon junction, although subject to substantial variation about a consensus sequence in metazoans, in yeasts is an essentially 100% conserved hexanucleotide (11). This apparent lack of variance in yeast splicing signals allowed the identification of an additional conserved sequence within the intron, the so-called TACTAAC box, a heptanucleotide found in all yeast introns 4 to 53 nucleotides upstream of the AG at the 3′ splice junction. The importance of these conserved sequences in the splicing process was demonstrated by the observation that deletion of either the 5′ consensus sequence (4) or the TACTAAC box (11, 12, 26) abolishes splicing and leads to the accumulation of full-length precursor (11, 12, 26).

While consensus sequences at the 5′ (GTAAGT) and 3′ [$(PY)_n$AG] intron-exon junctions had been recognized in metazoan introns (1, 21), there has been no evidence to suggest an analog of the TACTAAC box. This apparent difference has recently been verified with the development of extracts which carry out the splicing reaction in vitro. A detailed analysis of the transcripts in both mammalian and yeast extracts (22, 24), and more recently the characterization of intermediates in the splicing process in vivo (3, 28, 37), has shown that splicing of pre-mRNA in both systems occurs via a common intermediate. This intermediate has been termed a lariat, because the 5′ end of the intron is joined by a 2′-5′ phosphodiester bond to a site within the intron. The location of this 2′-5′ linkage, or branch (36), is within the TACTAAC box in yeasts (3). In mammalian cells, branch points are determined primarily by their location with respect to the 3′ splice site regardless of the sequence surrounding the branch point, and an obvious consensus sequence is less readily identifiable (9, 24, 27, 30, 31).

The novel nature of these intermediates argues that splicing must proceed by fundamentally similar mechanisms in yeasts and mammals. Yet a significant distinction between these two systems is revealed by the response of the splicing machinery to mutations in the 5′ splice junction. In striking contrast to mammalian cells, in which inactivation of a 5′ splice site almost invariably leads to the activation of an alternative, or cryptic, site (for a review, see reference 20), deletions or point mutations in the 5′ consensus sequence in yeast introns have so far failed to activate cryptic sites (4, 25). The failure of the yeast splicing machinery to use alternative 5′ splice sites has proven to be extremely useful, in that it has revealed the molecular consequences of the attempt to use a mutant junction (25). It suggests, moreover, that yeasts may be more stringent in the requirement for primary sequence of the splicing signals and perhaps the location of these signals within the intron as well.

One of the intriguing features of some yeast introns is the presence of TACTAAC-like sequences in the vicinity of the TACTAAC box. If these TACTAAC-like sequences do play any biologically important role, the splicing machinery must be able to recognize them at some level. As a first step, we asked whether a cryptic sequence in the yeast actin intron can function as a TACTAAC box. Our approach to this problem was to construct a translational fusion which allowed a simple quantitative assay for the efficiency of a splicing event. Using this fusion we constructed a series of deletions which removed the TACTAAC box or, in addition, TACTAAC-like sequences nearby. The results of these analyses allowed us to conclude that a TACTAAC-like sequence, TACTAAG, can be utilized by the yeast splicing machinery, albeit inefficiently.

MATERIALS AND METHODS

Enzymes and biochemicals. Restriction endonucleases, T4 DNA ligase, and DNA polymerase I (Klenow fragment) were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). ^{32}P-labeled nucleotides were purchased from either ICN Pharmaceuticals, Inc. (Irvine, Calif.) or New England Nuclear Corp. (Boston, Mass.). Avian myeloblastosis virus reverse transcriptase was obtained from Life Sciences, Inc. (St. Petersburg, Fla.). Oligonucleotides were prepared by triester synthesis on a solid support followed by purification by high-performance liquid chromatography.

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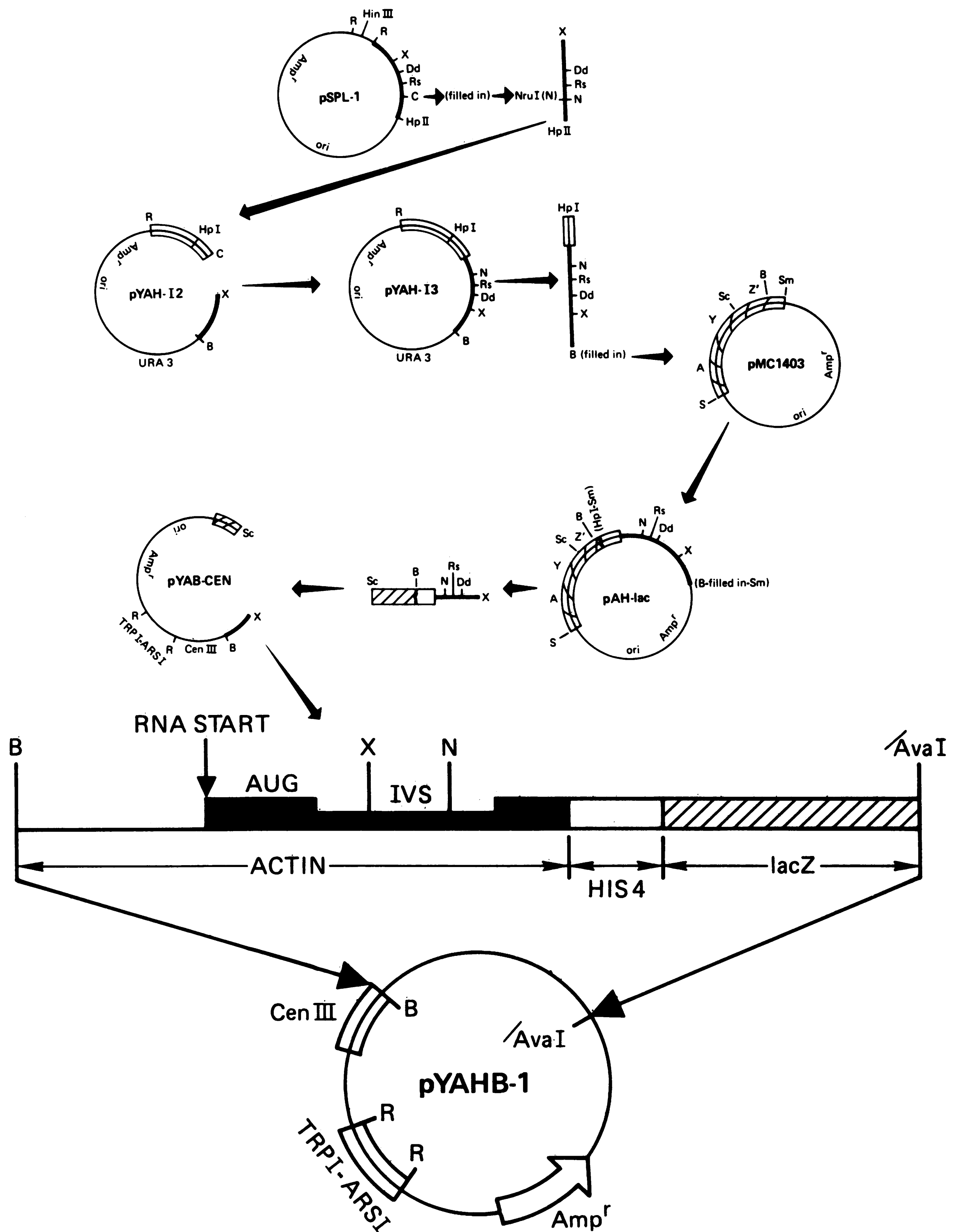
pSPL-1
Hin III
(filled in)
NruI (N)
pYAH-I2
pYAH-I3
URA 3
B (filled in)
pMC1403
pAH-lac
(B-filled in-Sm)
pYAB-CEN
TRPI-ARSI
Cen III
RNA START
AUG
IVS
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ACTIN
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lacZ
pYAHB-1
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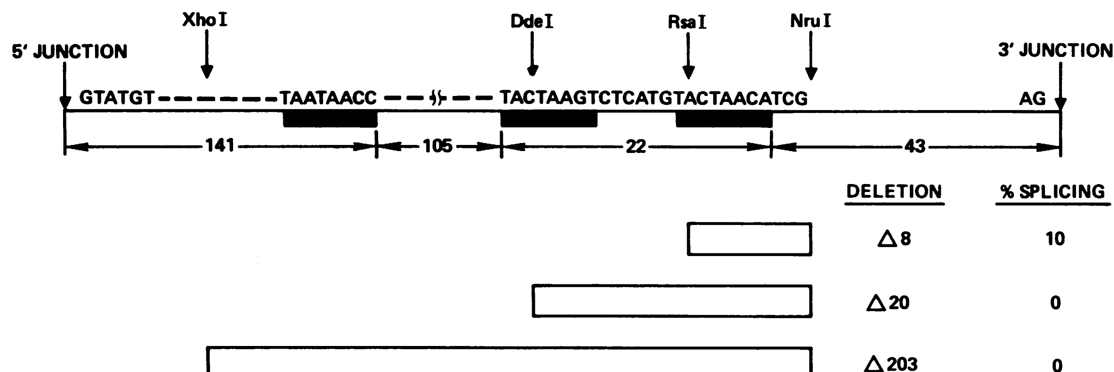

FIG. 2. The actin intron is shown with the relevant sequences. The 5′ and 3′ junctions are marked by the 5′ consensus sequence, GTATGT, and the 3′ AG. The TACTAAC box and the TACTAAC-like sequences are underlined with black boxes. The region deleted in each construct is shown as the open box beneath the intron. The nomenclature of deletion sizes is with respect to the wild-type actin intron. Restriction sites utilized for the construction of these deletions are shown above the intron. Distances, when appropriate, are shown beneath the intron.

Strains. *Escherichia coli* MC1061 (2) was used for routine manipulations and was provided by M. Casadaban. Yeast strains and their sources were as follows: NNY1, *MATα trp1 gal2 gal10 ura3-52 his3*; NNY1-*rna2*, *MATα trp1 ura3-52 his7 lys2 tyr1 gal1 rna2* (derived from a cross of NNY1 and *rna2-1* [obtained from Berkely Stock Center]; FC8-24D, *MATα trp1-1 leu2 his4-401 ura3-52 HOL* Δactin IVS (25).

Bacterial and yeast transformations. *E. coli* cells were transformed by the method of Kushner (10). *S. cerevisiae* strains were transformed either by the spheroplasting method with glusulase (6, 33) or by the lithium acetate procedure (7).

β-Galactosidase assays. Cells were grown in liquid minimal medium to an optical density at 600 nm of 0.5 to 1.8. Cells (0.1 to 1.0 ml) were pelleted, washed in 1.0 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol [pH 7.0]), and assayed as described previously (13).

RNA analysis. Preparation of RNA, kinasing of oligonucleotides, and primer extension experiments were all done as described by Domdey et al. (3).

Debranchase reactions were carried out by suspending 1 to 15 μg of RNA in 20 μl of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0)–20% (vol/vol) glycerol–100 mM KCl–0.2 mM EDTA. A crude debranchase preparation (5 μl; kindly supplied to us by B. Ruskin [30]) was added, and the samples were incubated at 30°C for 30 min. After incubation the RNA was diluted to 100 μl with water, extracted three times with phenol-chloroform, and ethanol precipitated.

Construction of pYAHB-1. An outline for the construction of pYAHB-1 is presented in Fig. 1. To construct pYAHB, pSPL-1 was cleaved with *Cla*I, and the overhang was filled in, resulting in a two-base insertion. This modification of the intron does not have a detectable effect on splicing (data not shown). The resulting plasmid, pSPL-2, was cleaved with *Xho*I and *Hpa*II, and the 287-base-pair fragment containing most of the intron and part of the 3′ exon was ligated into *Xho*I-*Cla*I-cut pYAH-I2 (25) to yield pYAH-I3. From this plasmid the *Bam*HI-*Hpa*I restriction fragment containing the entire actin region of the fusion and parts of the *HIS4* gene was inserted into the *Sma*I site of pMC1403 to create a *lacZ* fusion. Finally, this plasmid, pAH-lac, was cleaved with *Xho*I and *Sac*I, and the fragment containing the fusion was inserted into pYAB-cen to yield the final plasmid, pYAHB-1 (Fig. 1).

Deletion constructions. Deletions were constructed by first cleaving pYAHB-1 with *Xho*I and *Nru*I followed by insertion of various restriction fragments purified from pSPL-1. All fragments are internal portions of the actin intron. The following fragments were inserted for the following constructs: Δ8, *Xho*I-*Rsa*I; Δ20, *Xho*I-*Dde*I (*Dde*I overhang removed with S1 nuclease as described previously [16]; Δ203, no fragment inserted; Rep, *Xho*I-*Dde*I (*Dde*I overhang filled in with Klenow polymerase). The location of these sites is shown in Fig. 2. Deletion constructions were all sequenced by labeling the DNA at the *Hin*dIII site ca. 100 base pairs downstream of the modified region, followed by cleavage at a second site and purification of the end-labeled fragment. Sequences were then determined by the chemical method of Maxam and Gilbert (17).

RESULTS

Construction of spliced *lacZ* fusion. To investigate the contributions of various intron features to the efficiency of the splicing process, we desired a simple, quantitative assay for a splicing event. For this reason, we constructed a translational fusion between the yeast actin gene, which contains a 309-nucleotide IVS (5, 23), and the easily assayed *E. coli* β-galactosidase structural gene. This fusion, like the RP51-*lacZ* fusion reported elsewhere (35), is designed such that precise and efficient splicing of the IVS is required for

FIG. 1. Schematic diagram of YAHB fusion and the yeast centromere plasmid, pYAHB-1 (not to scale); for details of the construction see Materials and Methods. The features of the fusion are shown in greater detail above the plasmid map. Transcribed regions are shown as boxes; filled-in boxes correspond to actin sequences, open boxes correspond to *HIS4* sequences, and slashed boxes correspond to *lacZ* coding sequence. The fusion presumably initiates translation at the AUG normally used in the actin gene, 10 bases 5′ to the 5′ intron-exon junction. In this construction there is no defined transcriptional terminator; the transcripts terminate at a number of positions within the plasmid sequences (data not shown). Abbreviations for restriction endonuclease sites are: B, *Bam*HI; C, *Cla*I; Dd, *Dde*I; HinIII, *Hin*dIII; HpI; *Hpa*I; HpII, *Hpa*II; N, *Nru*I; R, *Eco*RI; Rs, *Rsa*I; S, *Sal*I; Sc, *Sac*I; and X, *Xho*I. ori, Origin.

TABLE 1. β-Galactosidase measurements of yeast cells harboring intact, deleted, or modified TACTAAC sequences[a]

Vector	Units	% pYAHB-1
pYAHB-1	108 (±34)	100
pYAHB-Δ8	9.6 (±6)	9
pYAHB-Δ20	0.2	0.2
pYAHB-Δ203	0.1	0.1
pYAHB-Rep	106 (±33)	98

[a] All measurements were carried out in transformants of the strain NNY1. For pYAHB-1, -Δ8, -Δ20, and -Rep, the means and standard deviations from seven independent measurements are presented. For the other construct, the value is derived from five independent measurements.

production of full levels of β-galactosidase activity. Failure to splice should prevent translation because of numerous stop codons within the intron. Inaccurate splicing should, in most cases, prevent functional expression by altering the reading frame. Finally, inefficient splicing will be reflected as a decrease in the level of β-galactosidase activity produced.

Constructed as described in detail in Materials and Methods, the fusion (Fig. 1) contained 364 base pairs of 5′ flanking sequence, the RNA start site, and the translational start of the actin gene. In addition to the *lacZ* coding sequence, the chimeric gene also included 454 codons from the yeast *HIS4* gene (see Materials and Methods). The *HIS4* sequences were included to facilitate exchange of intron mutants between the *lacZ* and a *HIS4* fusion reported elsewhere (25). We refer to this construct as the YAHB (yeast-actin-*HIS4*-β-galactosidase) fusion. For all the experiments described in this paper the YAHB fusion or its derivatives were maintained in yeasts on the centromere plasmid pYAHB-1 (Fig. 1). A useful feature of this plasmid was the presence of two unique restriction sites within the intron (Fig. 1). These sites, *Xho*I and *Nru*I, were located such that cleavage at both sites removed the internal portion of the intron without altering the 5′ and 3′ portions. Thus, the cassette nature of this plasmid allowed us to easily remove the internal region of the intron and replace it with a modified region for analysis.

Transformation of a yeast strain with this plasmid, pYAHB-1, resulted in the production of 108 U of β-galactosidase activity (Table 1). As we will show below, this activity is dependent on precise and efficient splicing of the actin intron.

Nearby sequence substitutes for TACTAAC box in splicing event. The actin intron has two TACTAAC-like sequences in addition to the TACTAAC box (Fig. 2). To test the role of these sequences, we constructed a series of deletions which first removed only the TACTAAC box and then either one or both of the TACTAAC-like sequences. These deletions were made by inserting different restriction fragments from within the actin intron into the *Xho*I-*Nru*I sites of pYAHB-1 (see Materials and Methods) (Fig. 2). We refer to a deletion by the number of bases removed with respect to the wild-type actin-IVS sequence. For instance, an eight-base-pair deletion with respect to the wild-type actin intron is designated Δ8; pYAHB-Δ8 is the corresponding plasmid name.

Plasmids carrying these deletions were transformed into the strains NNY and FC8-24D, and biological and biochemical phenotypes were assayed. The results of β-galactosidase assays in strain NNY are shown in Table 1. (Strain NNY was used for all β-galactosidase measurements since it routinely provided two- to threefold-higher levels of activity than FC8-24D, although the relative values obtained for wild-type versus mutant constructs were the same.) Surprisingly, the deletion which removed only the majority of the correct TACTAAC box, Δ8, still allowed the expression of approximately 10% of wild-type β-galactosidase activity. In contrast, a slightly larger deletion, Δ20, which removed 12 more nucleotides 5′ to the TACTAAC box, including the last four bases of the TACTAAC-like sequence TACTAAG, virtually abolished β-galactosidase activity. Similarly, the removal of the correct TACTAAC box and both TACTAAC-like sequences, Δ203, had the same consequence.

The observation that deletion of six of the seven base pairs from the correct TACTAAC box did not abolish β-galactosidase activity suggested that a low level of splicing could occur which was dependent on the sequences just 5′ to the TACTAAC box. However, an alternative possibility was that some of the β-galactosidase activity in these constructs was not dependent on splicing. We reasoned that if the enzymatic activity, both in wild-type and in Δ8, was dependent on the function of the splicing machinery, then other mutations which block splicing should also prevent expression of this β-galactosidase activity.

RNA2 is a locus at which temperature-sensitive alleles have been identified which lead to the accumulation of precursors to spliced nuclear genes at 36°C (29). To test whether the residual β-galactosidase activity is affected by the *rna2* lesion, we transformed an *rna2* strain, NNY1-*rna2*, with both pYAHB-1 and pYAHB-Δ8 and followed the β-galactosidase activity after a shift to 36°C. As a critical control, we also transformed the same strain with a plasmid, pYAB-ΔIVS, which contains an actin-*lacZ* fusion with the IVS precisely removed (13).

While the activity of the fusion without the IVS was relatively unaffected, the β-galactosidase activity from strains transformed with either pYAHB-1 or pYAHB-Δ8 declined with time (Fig. 3). The disappearance of the resid-

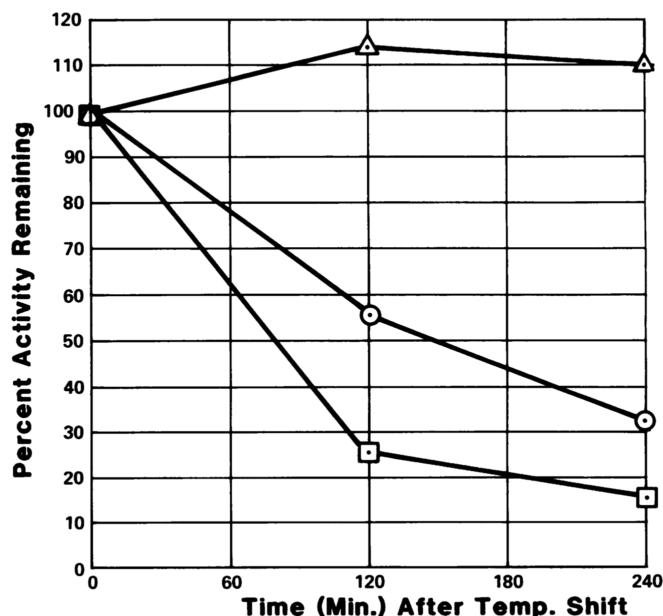

FIG. 3. Strain NNY-*rna2* was transformed with pYAHB-1, pYAHB-Δ8, and pYAB-ΔIVS (see the text). Transformants were maintained at 23°C and then shifted to 37°C and assayed for β-galactosidase activity at various times. Symbols: △, pYAB-ΔIVS; □, pYAHB-1; ○, pYAHB-Δ8.

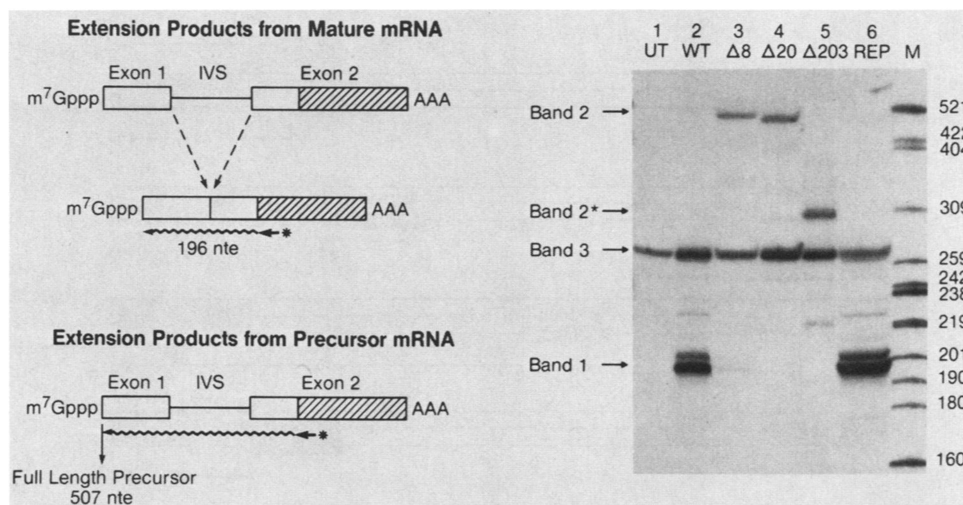

FIG. 4. Total RNA from strain FC8-24D transformed with the following plasmids was subjected to primer extension analysis as described in the text. Sizes of the cDNA products are shown schematically for unspliced precursor and for mature mRNA. Lanes: 1, untransformed parent; 2, pYAHB-1; 3, pYAHB-Δ8; 4, pYAHB-Δ20; 5, pYAHB-Δ203; 6, pYAHB-Rep; M, marker DNA (numbers on right are in base pairs). The difference in the size of the full-length precursor (band 2) results from the different sizes of the deletions in each case. In the case of Δ203, the size of the full-length precursor is noted as band 2*. The primer for this experiment is the 21-mer 5'-TAACGCCACGACCCAAATCGGC-3', which hybridizes specifically to the actin-*HIS4* junction. Band 3 is a background band, not related to the fusion, and is also found in the untransformed parent (lane 1). As described previously (25), we detected two predominant transcriptional start sites resulting in a doublet for band 1. In pYAHB there appear to be additional minor initiation sites, as indicated by the faint bands between band 1 and band 3. nte, Nucleotides; m^7G, 7-methylguanosine (cap structure).

ual activity produced in the Δ8 mutant with time demonstrates that this activity is sensitive to the loss of the *rna2* gene product and is thus dependent on splicing.

As a confirmation that the β-galactosidase activities were

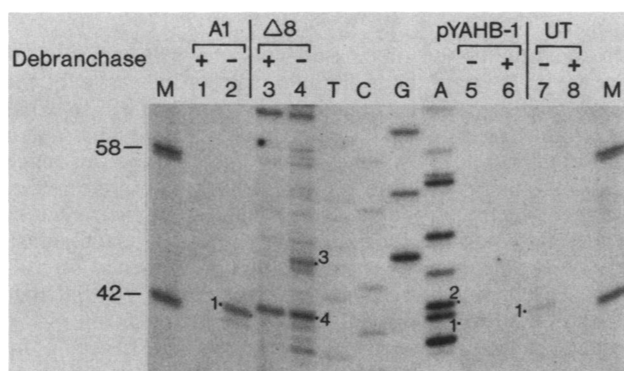

FIG. 5. Poly(A)$^+$ RNA from strains transformed with derivatives of pYAHB-1 was subjected to primer extension analysis from a primer which hybridizes to the 3' end of the actin intron with (+) or without (−) prior treatment with debranchase. Primer extension stops are positioned relative to the sequence of the wild-type actin intron generated by using the same primer on an M13 template. Samples and amounts of RNA per lane are: lanes 1 and 2, FC8-24D transformed with pYAH-I2.A (GTATAT → ATATAT; Vijayraghavan et al., submitted), 1 μg of RNA; lanes 3 and 4, FC8-24D transformed with pYAHB-Δ8, 15 μg of RNA (used to increase weak branch-point signal); lanes 5 and 6, NNY1 transformed with pYAHB-1, 5 μg of RNA; lanes 7 and 8, NNY1, 5 μg of RNA. For mapping the mutant branch points we used strain FC8-24D which is deleted for the actin chromosomal IVS. It is worthwhile noting that bands that are resistant to debranchase could still be branch points, as some branches are resistant to this debranchase treatment (30). The primer for this experiment is 5'-CTAAACATATAATATAGACACAAA-3'. Lane M, Marker DNA (numbers on left are in base pairs). Band 1 corresponds to a reverse transcriptase stop at the authentic branch point in the TACTAAC signal. Band 2 corresponds to the reverse transcriptase stop at the branch point in the TACTAAC signal of the pYAHB-1 construction, which harbors a two-base insertion between the primer site and the TACTAAC sequence. Band 3 is the reverse transcriptase stop at the branch point in the cryptic TACTAAG signal. Band 4 is a reverse transcriptase stop possibly caused by a strong secondary structure.

an accurate reflection of the amount of correctly spliced mRNA being produced, we analyzed the fusion transcripts by primer extension from a primer located in the 3' exon. This primer had the advantage of being specific for the fusion. In addition, because the 3' exon is found in precursor and mature mRNA, this primer produced cDNA products from both spliced and unspliced transcripts (see schematic to Fig. 4).

Total RNA (25 μg) from the appropriate strains was annealed to a 5'-end-labeled 21-nucleotide primer. The primer was then extended with reverse transcriptase, and the cDNA products were separated on a 6% sequencing gel. In strains carrying the wild-type plasmid, pYAHB-1, the major cDNA product was the correct length, 196 nucleotides, to correspond to correctly spliced mature mRNA (Fig. 4, lane 2, band 1). In the smallest deletion, Δ8, which leaves intact the sequence TACTAAG, a low level of spliced product was still detected (lane 3, band 1), but the predominant extension product corresponded to full-length precursor (band 2). However, the larger deletions, Δ20 and Δ203, only had cDNA products corresponding to full-length precursor (lane 4, band 2, and lane 5, band 2*).

We conclude from these experiments that removal of the TACTAAC box reduces splicing to approximately 10% of wild-type levels. This residual splicing is dependent on the sequences located just upstream of the TACTAAC box. The most likely explanation for this residual splicing is that the TACTAAC-like sequence, TACTAAG, in this region can

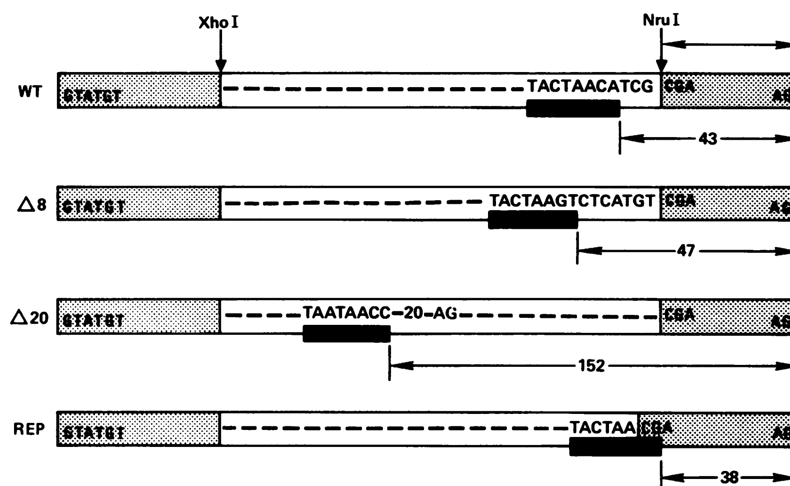

FIG. 6. The intron is illustrated showing the positioning of the TACTAAC signal in each case within the intron. Shaded regions represent the regions outside the *Xho*I and *Nru*I sites which are not altered in any of the constructs. The TACTAAC signal in each case is underlined for clarity. WT indicates the parental construction pYAHB-1.

substitute for the TACTAAC box in the splicing process, although at a reduced efficiency.

If the TACTAAG sequence is substituting for the TACTAAC box in the Δ8 deletion, we would expect the site of branch formation to be at the last A of the TACTAAG sequence. To verify this, we mapped the branch point in pYAHB-1 and pYAHB-Δ8 by primer extension from a primer which hybridized to the 3′ end of the intron. A branch structure serves as a strong stop for reverse transcriptase (26, 32). Evidence that primer extension stops are due to a branch structure is provided by treating the RNA with a debranchase activity (30) before reverse transcription. This activity has been shown to cleave a 2′-5′ linkage and remove the block to reverse transcriptase.

We mapped the branch point in pYAHB-1, and, as expected, the branch site was located at the last A of the TACTAAC box (Fig. 5, lanes 5 and 6, band 2). The reliability of this technique was demonstrated by mapping the branch point either in the chromosomal actin gene transcripts (Fig. 5, lanes 7 and 8, band 1) or in a fusion with a point mutation at the 5′ splice junction (G to A [U. Vijayrahgavan et al., submitted]) which accumulates branched intermediates (Fig. 5, lanes 1 and 2, band 1). As expected, in both cases the branch points mapped to the last A of the TACTAAC box. Primer extension of the Δ8 transcripts generated a small amount of a cDNA product the right length to correspond to a branch at the cryptic TACTAAG sequence (Fig. 5, lane 4, band 3). The cDNA product located at the TACTAAG sequence was eliminated by the debranchase activity (Fig. 5, lane 3). This indicates that the TACTAAG sequence is functioning as the TACTAAC box in the Δ8 construct. This result is consistent with fingerprints of the lariat intermediate and excised intron of Δ8 produced in an in vitro splicing reaction (data not shown).

Efficiency of TACTAAG can be improved. Having demonstrated that the residual splicing is a result of the use of the cryptic TACTAAC box, TACTAAG, we then asked whether we could restore splicing to an efficient rate by altering this sequence to more closely resemble the consensus. For this purpose, we inserted the *Dde*I (filled in)-*Xho*I restriction fragment from within the actin intron into the *Xho*I-*Nru*I sites of pYAHB-1. The result of this construction, pYAHB-Rep, was the conversion of the cryptic TACTAAG sequence to TACTAAC (Fig. 6). In addition to this alteration in primary sequence, the new TACTAAC box was also positioned nine nucleotides closer to the 3′ end of the intron than was the cryptic TACTAAG box (Fig. 6).

Transformation of this construct into the NNY strain allowed full expression of β-galactosidase activity (Table 1). In addition, analysis of the fusion transcripts revealed the same spectrum of cDNA products corresponding to mature mRNA as in wild type, demonstrating that splicing had been restored to high efficiency (Fig. 4, lane 6).

DISCUSSION

In constructing the splicing-dependent β-galactosidase fusion, we hoped to obtain a simple, quantitative assay for the efficiency of the removal of an intron. This hope was realized in that the fusion does require splicing for expression of β-galactosidase activity, as demonstrated by the dependence on a functional *rna2* gene product, and quantitative differences between constructs are paralleled in the level of mature mRNAs produced. Thus, we can use β-galactosidase activity as an initial measure of splicing efficiency in constructs designed to test the role of intron features in splicing.

One of the intriguing features of the actin intron is the presence of the TACTAAC-like sequence TACTAAG in the vicinity of the TACTAAC box. The analysis of the deletions described in this paper allowed us to conclude that, at least in the absence of the normal TACTAAC box, this sequence

TABLE 2. TACTAAC-like sequences in other yeast introns

Yeast gene (reference)	TACTAAC-like sequence	Location (relative to TACTAAC)
Actin (5, 23)	TACTAAGT	14 bases 5′
RP51A (35)	TACAACT	22 bases 3′
S10-1 (15)	TTATAACA	19 bases 3′
L17A (14)	TGCTACT	23 bases 5′
L25 (14)	TACCAACA	135 bases 5′
RP28A (19)	TTCTAAT	62 bases 5′
RP28B (19)	TACCAAT	23 bases 5′
RP29 (18)	TGTTGAC	36 bases 5′
L29 (8)	TACCACG	41 bases 5′
TACTAAC consensus	TACTAACPu	

can function inefficiently as a substitute. Although the activation of alternative 5′ junctions and branch points is a well-documented phenomenon in higher eucaryotes (20, 27, 31), previous analyses of mutant yeast mRNA introns have not indicated the use of either cryptic 5′ splicing sites or alternative TACTAAC boxes. The use of the TACTAAG sequence as a functional branch site in lariat formation demonstrates that the yeast splicing machinery is capable of recognizing such signals, albeit at low efficiency.

This finding prompts the speculation that TACTAAC-like sequences in the vicinity of the TACTAAC box could play some physiologial role. Interestingly, some yeast introns have TACTAAC-like sequences in the vicinity of the TACTAAC box (Table 2). In principle, these sequences could facilitate splicing in two ways. They might serve as additional primary binding sites for factors which could then diffuse along the RNA to the correct TACTAAC box as proposed by Langford and Gallwitz (11). Alternatively, these TACTAAC-like sequences could function as branch points at low levels, thus increasing the overall rate of splicing.

If the role of the cryptic TACTAAG box in the actin intron is to provide a second branch point used at a low level in the wild-type intron, then we should be able to detect branches formed at this position in the wild-type intron. Our analyses of the branch points in the actin intron did not detect any branches formed at the cryptic site. The most sensitive assay is provided by the examination of the branch points in mutants which accumulate a large amount of the lariat intermediate. These include point mutations in the 5′ consensus sequence and insertions between the TACTAAC box and the 3′ splice site. Analyses of the branch points in these mutants reveal only branches formed at the correct TACTAAC box (Vijayraghavan et al., submitted; A. Cellini, E. Felder, and J. Rossi, EMBO J., in press). In these experiments, branching at the cryptic TACTAAG at 5% of the level at the correct TACTAAC box would be easily detectable. We conclude that the cryptic TACTAAG box is not used in the presence of the normal TACTAAC and is only activated as a branch point by deletion of the correct TACTAAC box.

Why is the TACTAAG sequence not used in the presence of the authentic TACTAAC box? A plausible explanation is that the cryptic TACTAAG element cannot compete with the correct TACTAAC box for the binding of components required for splicing. Removal of the competing TACTAAC box by deletion allows the TACTAAG box to bind splicing factors and function during the splicing process. Interestingly, point mutations within the authentic TACTAAC box which decrease the efficiency of splicing can also cause branches to be formed at the cryptic TACTAAG box (Vijayraghavan et al., submitted).

Although the sequence TACTAAG can function as a branch site, the efficiency of branch formation at this site is quite poor (Fig. 4 and 5). To account for such poor efficiency, the two most obvious variables are the difference in primary sequence and the slight alteration in position within the intron relative to the authentic TACTAAC box. In primary sequence, the cryptic element differs from the normal TACTAAC box in three ways: (i) an absolutely conserved residue, the last C, is now a G; (ii) the highly conserved A just 3′ of the TACTAAC is replaced by a T; and (iii) additional flanking nucleotides which are conserved in the vicinity of yeast TACTAAC boxes (C. Guthrie et al., ICN-UCLA Symp. Mol. Biol., in press) are absent. The most striking alteration here is the replacement of the absolutely conserved C with a G. Yet, when this alteration is introduced into the authentic TACTAAC box, splicing still occurs at greater than 70% of the wild-type level (L. Fouser and J. Friesen, Cell, in press). In that the other primary sequence differences appear to be minor, this suggests that the cryptic TACTAAG element is used inefficiently for other reasons.

One obvious possibility is the location of the TACTAAG signal in relation to the 3′ splice site. The cryptic TACTAAG box is six nucleotides further from the splice junction than is the wild-type element. Significant variability (7 to 55 nucleotides) in this spacing has been found to be the rule in yeast introns (Guthrie et al., in press). In this regard, it is of interest to note that of the 16 yeast introns for which sequences have been determined, in only 1 of these does the spacing between the TACTAAC sequence and 3′ splice site exceed that of the TACTAAG construct (55 versus 48 nucleotides). We therefore suggest that this slight perturbation in spacing, in conjunction with a nonideal primary sequence, accounts for the inefficient use of the TACTAAG sequence in the splicing reactions. This is reminiscent of the observation that in mammalian introns there appear to be significant constraints on the distance between the branch site and the 3′ junction (27, 31). In contrast to the mammalian system in which deletion of the primary branch site leads to efficient activation of other sites (27, 31), activation of cryptic sites in yeasts is rigorously dependent upon complete, or nearly complete, homology with the authentic TACTAAC signal. This point is made especially clear by our own and others' (11) observations that actin intron deletions of both the TACTAAC and TACTAAG sequences virtually abolish splicing even though the 3′ splice signals remain intact.

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