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RNA splicing and intron turnover are greatly diminished by a mutant yeast branch point

(RNA lariat intermediate/TACTAAC box)

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ABSTRACT **Two mutant genes, both of which contain an A→C transversion at the absolutely conserved branch point of the yeast "TACTAAC box" (TACTAAC→TACTACC), were constructed and introduced into yeast cells. Splicing and gene expression are almost completely eliminated by this mutation, but a low level (≈0.1%) of proper splicing is detectable. Branch point mapping indicates that the mutant branch is formed at the normal location—i.e., to cytidine rather than adenosine. The mutant branch is also a very poor substrate for the HeLa cell debranching enzyme. Although splicing of the mutant transcripts is very poor, the cells contain a high level of mutant intron because these excised introns are remarkably stable. The results imply that the normal branch point is important not only for branch formation and splicing but also for intron turnover.**

Recent studies in a number of laboratories have clarified the pathway by which pre-mRNA processing (splicing) takes place. In both yeast (*Saccharomyces cerevisiae*) (1–3) and mammalian cells (4–7), a two-step process has been described. First, 5′ splice-site cleavage and lariat (branch point) formation takes place, in which the 5′ end of the intron is attached to the branch point via a 2′-5′ phosphodiester bond. In yeast, the last adenosine of the "TACTAAC box" serves as the branch point, while in Metazoa the branch point sequence is more variable and is specified in part by distance from the 3′ splice site. Second, 3′ splice-site cleavage and exon ligation take place, during which the lariat intron is released. Both step 1 and step 2 may be coupled cleavage–ligation events, as neither 5′ cleavage nor 3′ cleavage has been detected in the absence of lariat formation or exon ligation, respectively. It appears that these processes take place predominantly or entirely in large ribonucleoprotein particles in which pre-mRNA substrate is localized after addition to a splicing extract (8–10).

The use of mutant substrates both *in vivo* and *in vitro* has contributed greatly to our understanding of the splicing process. Studies of this nature have shown that branch point selection in mammalian cells is substantially dependent on distance from the 3′ splice junction (11), whereas branch point selection in yeast is highly dependent on the invariant TACTAAC box within which the branch point is always located (2, 3). In yeast, studies of mutants have also shown that most or all of the highly conserved nucleotides at the 5′ splice junction and at the TACTAAC box are required for splicing to take place with maximal efficiency (12–17). Very important are the first guanosine of the 5′ junction and the last adenosine of the TACTAAC box, as mutations at these two positions, which undergo bond cleavage and branch formation, dramatically affect splicing (14).

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In this communication, we report the effect of an A→C transversion at the branch point adenosine of an rp51A intron [derived from a gene encoding yeast ribosomal protein 51 (18)] in which the conserved TACTAAC box has been changed to TACTACC. As expected, this mutation lowers the rate of splicing to virtually undetectable levels. However, analysis of the RNA species present in such cells provides insights into the role of the TACTAAC box on branch formation and the possible role of debranching in intron degradation.

MATERIALS AND METHODS

Strains. All plasmids were transformed, as described by Ito *et al.* (19), into DB745 (α, *ade-100, leu2-112, ura3-52*) or into PB12, a DB745 strain in which the rp51A gene has been deleted (20).

β-Galactosidase Assay. Assays were performed as described by Osley and Hereford (21).

***In Vitro* Mutagenesis and Plasmid Constructions.** All constructions were done using the HZ18 plasmid described by Teem and Rosbash (18). Mutagenesis and plasmid constructions were done as described (15).

RNA Extraction and Analysis. RNAs were purified as described (22). For primer-extension analysis, about 10 μg of total RNA was diluted in 15 μl of 66.6 mM Tris·HCl, pH 8.0/53.3 mM KCl/0.66 mM EDTA containing ≈2.5 ng of primer, end-labeled with ^{32}P. After 30 min at 42°C, 5 μl of a premixed solution containing the following was added: 1 mM of each of the deoxynucleoside triphosphates, 40 mM $MgCl_2$, 4 mM dithiothreitol, actinomycin D at 0.2 μg/ml (from a 1-mg/ml stock solution in ethanol), and 2 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). The reaction mixture was incubated 30 min at 37°C and then extracted with phenol/chloroform (1:1, vol/vol), and the nucleic acids were ethanol-precipitated, washed with 70% ethanol, and dried. The samples were then resuspended in 2.5 μl of water with RNase A at 0.3 mg/ml and incubated 15 min at 37°C. After addition of an equal volume of 98% formamide/10 mM EDTA/0.1% xylene cyanol FF/0.1% bromophenol blue, the samples were denatured 2 min at 100°C and then electrophoresed in a 6% acrylamide/8 M urea gel.

RNA blot analysis was performed as follows. After denaturation for 2 min at 100°C in 50% formamide/5 mM EDTA/0.05% xylene cyanol/0.005% bromophenol blue, 10 μg of total RNA was loaded on a 4% acrylamide/8 M urea gel. After electrophoresis, the RNA was electroblotted directly (without treatment) onto a nylon membrane (Biodyne, 0.2 μm pore size; Pall, Glen Cove, NY) for 6 hr at ≈4 V/cm at room temperature in 50 mM Tris/50 mM boric acid/1 mM EDTA, pH 8.3. Hybridization was as described (23).

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RNAs were labeled *in vivo* with ^{32}P[phosphate] essentially as described (24). In brief, the cells were grown to OD_{600} 0.5–0.6 in 10 ml of selective minimal medium without uracil and with 2% galactose. The cells were collected by centrifugation, washed in YM-1 low-phosphate medium, resuspended in 10 ml of YM-1 low-phosphate medium with 2% galactose, and incubated at 30°C for 4 hr, after which 8 mCi (1 Ci = 37 GBq) of $H_3{}^{32}PO_4$ (from ICN) was added and the incubation continued for an additional 6 hr. The RNAs were extracted (22), and the form-2 3′III–IV intron was then purified by two-dimensional acrylamide/urea gel electrophoresis as described (2), except that the first dimension was 4% acrylamide. Indeed, two-dimensional gel electrophoresis of total RNA from 3′III–IV-transformed PB12 cells gives rise to a single, major, off-diagonal spot because of the enormous quantity of 3′III–IV intron present in the strain (see *Results*). Primer-extension analysis of RNA from this spot confirmed that it contains (consists of) 3′III–IV lariat intron (data not shown).

RESULTS

Standard cloning mutagenesis procedures were used to construct the mutant plasmids shown in Table 1. All three mutants are derivatives of HZ18, our "wild-type" plasmid, which contains a wild-type yeast intron within the β-galactosidase (*Escherichia coli lacZ*) coding region. Plasmid 3′III contains an A→C transversion at the last adenosine of the TACTAAC box. 3′III–IV contains an additional G→A transition at position −3 to the TACTAAC box. 3′III–5′II is also a double mutant and combines the TACTAAC mutant of 3′III and a point mutant at the 5′ junction, 5′II (15).

All four plasmids were transformed into yeast, and the β-galactosidase levels were measured (Table 1). The point mutant 3′III has a dramatic effect on gene expression, as enzyme activity is decreased by a factor of ≈1000. The double mutant 3′III–IV gives rise to a 3-fold higher level of β-galactosidase activity. Presumably, the 3′III–IV transcript is a somewhat better splicing substrate than 3′III. The residual activity in the 3′III mutants might be due to a low level of gene expression in the absence of splicing, or, alternatively, a low level of "proper" splicing might take place. To distinguish between these possibilities, the double mutant 3′III–5′II was constructed and analyzed. As summarized in Table 1, the β-galactosidase levels observed for 3′III are reduced by a factor of 8 when the TACTAAC mutant is combined with the 5′-junction mutant, suggesting that the latter possibility is the correct one; i.e., a low level of proper splicing takes place in 3′III.

To investigate further the nature of this splicing, RNA was analyzed by primer extension (Fig. 1). The pattern of bands observed with the wild-type RNA (HZ18) template has been described (1, 18). In brief, the HZ18 lane contains a precursor RNA band (P), mRNA bands (M), a band due to the RNA branch of the lariat intermediate (ICS), and the mRNA bands derived from the chromosomal rp51A gene. Indeed this latter pair of bands is equally intense in all four lanes, indicating that an equal amount of RNA from all four strains was analyzed. RNA from the three mutant strains generates no detectable mRNA bands, as expected from the low levels of β-galactosidase activity present in these strains (Table 1). All three mutant strains contain a large amount of precursor RNA, indicating that the first step of splicing, 5′ cleavage and lariat formation, is significantly inhibited by the absence of the usual branch point adenosine (22). Surprisingly, a substantial ICS band is clearly visible in the 3′III and 3′III–IV lanes, suggesting that a substantial amount of lariat intermediate is present in these strains. A comparable level of free 5′ exon was also detected in these two strains by blot-hybridization analysis of electrophoretically fractionated cellular RNA (data not shown). The large amount of lariat intermediate and 5′ exon compared to mRNA in these two mutant strains indicates that the second step of splicing, 3′ junction cleavage and exon ligation, is also strongly inhibited by these mutations (15). By analogy with the arguments used above,

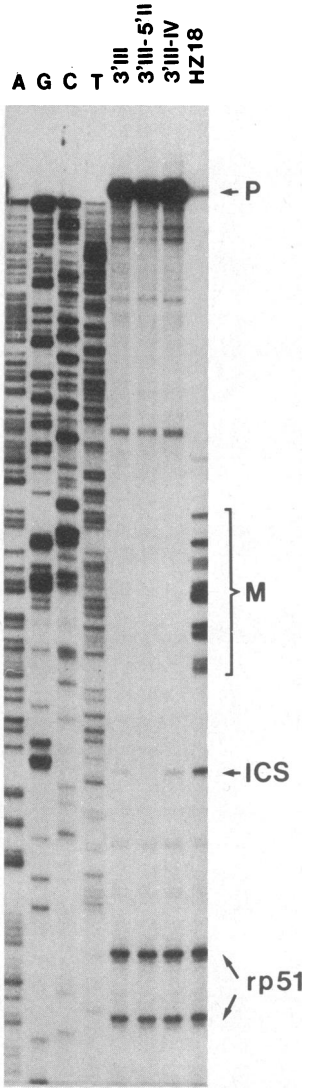

FIG. 1. Primer-extension analysis of the wild-type or mutant transcripts, using a 3′ exon primer. DB745 cells transformed with the wild-type HZ18 plasmid or with a plasmid carrying point mutations (as indicated above four lanes at right) were grown as described in *Materials and Methods*. The end-labeled oligonucleotide used as a primer is homologous to 3′ exon sequences and is the same primer used by Teem and Rosbash (18). The same end-labeled primer was used to sequence, by the chain-termination method (25), the mutated fusion gene on the 3′III plasmid after digestion with *Bam*HI and treatment with exonuclease III. The cDNAs and the sequence reactions were analyzed in parallel in a 6% acrylamide sequencing gel. cDNA molecules corresponding to unspliced fusion transcripts (P), spliced fusion transcripts (M), reverse-transcription stop at the branch point of the lariat molecule (ICS), and spliced mRNAs from rp51A are indicated. A very weak band appears, on longer autoradiographic exposure, at the ICS location in the 3′III–5′II lane. This band disappears entirely if the 3′III–5′II plasmid is used to transform DB12 A, a DB745 strain in which the endogenous rp51A gene has been deleted (data not shown). This indicates that the very low level of ICS detectable in the 3′III–5′II primer extension is due to the endogenous rp51A gene.

Table 1. Effect of the intron mutations of β-galactosidase activity

Plasmid	β-Galactosidase activity	Intron sequence (5′ to 3′)
HZ18	1	G/GTATGT–325 nt–GTATACTAAC–55 nt–AG/G
3′III	0.0008	G/GTATGT–325 nt–GTATACTA**C**C–55 nt–AG/G
3′III–IV	0.0025	G/GTATGT–325 nt–**A**TATACTA**C**C–55 nt–AG/G
3′III–5′II	0.0001	G/GTAT**A**T–325 nt–GTATACTA**C**C–55 nt–AG/G

All plasmids were transformed into DB745. β-Galactosidase activity was determined and normalized to the activity of cells transformed with the wild-type intron-containing plasmid HZ18. The consensus sequences of the intron are shown for each plasmid, with the nucleotide substitutions in the mutants underlined; slashes indicate exon/intron boundaries. nt, Nucleotides.

the absence of this ICS band from the 3'III–5'II lane (but see legend to Fig. 1) argues that it is not an artifact due to premature termination of the reverse transcriptase or due to a degradation product but, rather, is derived from bona fide lariat intermediates present in these mutant strains.

Analysis of these cDNA products at higher resolution shows that the putative ICS cDNAs comigrate precisely with the ICS cDNA from the wild-type strain (Fig. 2). As previous studies of this nature have shown that primer-extension analysis yields identical results to direct RNA mapping procedures (2, 3, 6, 7, 26) and as the same wild-type guanosine is the last incorporated nucleoside in the ICS band from 3'III and 3'III–IV RNA, the data indicate that the branch in these two RNAs is formed at the normal location, but at the mutant cytidine rather than a wild-type adenosine. A similar conclusion has been independently drawn by Fouser and Friesen (17). However, direct RNA mapping is required for definitive proof of this conclusion.

As this appeared to be the first case (of which we were aware) of a specific branch formed at a nucleotide other than adenosine, we carefully compared purified mutant and wild-type lariat introns as substrates for the HeLa cell debranching enzyme (27). To this end, mutant intron was labeled *in vivo* with ^{32}P and purified by two-dimensional gel electrophoresis of total RNA (ref. 2 and *Materials and Methods*), because the 3'III substrates are inactive for *in vitro* splicing.

Radioactive 3'III–IV lariat and radioactive HZ18 (wild-type) lariat [prepared by *in vitro* splicing of a ^{32}P-labeled SP6 substrate (28)] were incubated with limiting amounts of HeLa cell debranching activity (Fig. 3). Since the wild-type HZ18 intron was isolated as form 1 and the mutant HZ10 intron as form 2, the size differences provide a convenient means to distinguish the two substrates in a mixed incubation. The results demonstrate that 3'III–IV lariat is a much poorer (factor > 100) substrate for this activity than the HZ18 lariat. The 3' exon-containing lariat intermediate from 3'III or 3'III–IV also appears relatively resistant to debranching as assayed by primer extension (with a 3' exon primer) of total RNA subsequent to incubation with the S100 HeLa cell debranching activity (data not shown). We conclude that an unusual branch is indeed present in 3'III and 3'III–IV, consistent with the above-discussed results of primer-extension mapping.

In the course of these studies, we noticed that the level of branched RNA in these mutant strains was significantly higher when assayed with an intron primer than with an exon primer (data not shown and see below). As primer extensions

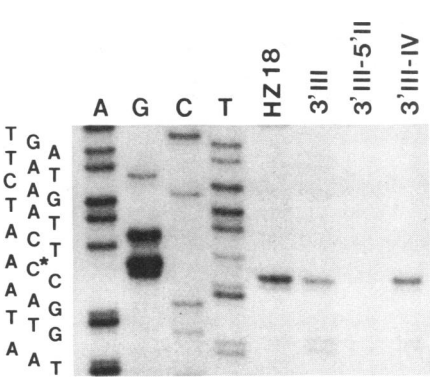

FIG. 2. Primer-extension analysis of the wild-type or mutant transcripts, using a 3' exon primer. A portion of a gel homologous to the one presented in Fig. 1 is presented here at higher resolution to show the precise mapping of the ICS reverse-transcription stop on the wild-type or the mutant transcripts. Letters at left indicate the sequence complementary to the sequence ladder and correspond to the RNA sequence transcribed from the 3'III mutant plasmid. Asterisk indicates the A→C transversion in the TACTAAC box.

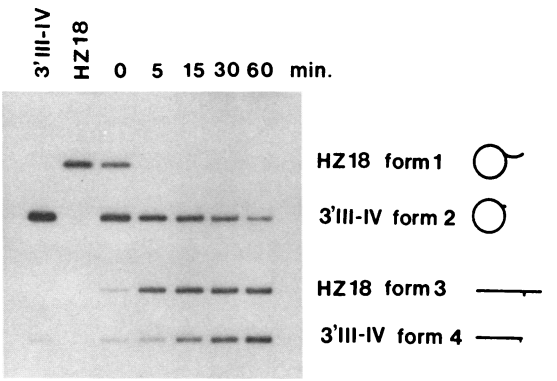

FIG. 3. Time course of debranching of 3'III–IV and HZ18 free introns. In the first lane, ≈500 cpm of *in vivo*-labeled, gel-purified form-2 3'III–IV intron was loaded. The small amount of form-4 intron present in the preparation probably corresponds to nicked form-2 intron and not to genuine debranched intron. In the second lane, ≈500 cpm of HZ18 form-1 intron, gel-purified from a preparative *in vitro* splicing reaction, was loaded. Approximately 3000 cpm of those two intron preparations were mixed together in 60 μl of 13 mM Hepes, pH 8.0/13% glycerol/16 mM EDTA containing 29 units of RNasin (Promega Biotec, Madison, WI) and 25 μl of S100 HeLa cell extract. The solution was incubated at 30°C and 10-μl aliquots were taken at the times indicated above lanes 3–7, mixed with 55 μl of 100 mM Tris·HCl, pH 7.5/12.5 mM EDTA/150 mM NaCl/1% $NaDodSO_4$ on ice. When all aliquots had been taken, 5 μl of proteinase K at 4 mg/ml was added and the reaction mixtures were incubated 15 min at 37°C. After an extraction with phenol/chloroform, ethanol precipitation, and wash with 70% ethanol, the samples were dried, resuspended in 4 μl of formamide and, after 2 min of denaturation at 100°C, loaded on a 4% acrylamide/8 M urea gel. The lariats are stable during incubation in the absence of debranching enzyme (data not shown) and the lack of "nicking" during the incubation is attested to by the stability of the linear forms during the 60-min incubation.

with an intron primer also reveal branched RNAs due to the excised intron, the data suggested that these mutant strains contained very high levels of free (excised) intron. To confirm and extend these initial observations, RNA was analyzed by gel electrophoresis followed by blotting and hybridization with an intron probe (Fig. 4). The data (note the ≈20-fold difference in exposure times) show that the intron levels are indeed much higher than what is present in the wild-type (i.e., HZ18-containing) strain. They also indicate that, of the four characterized intron forms, only the short forms, and predominantly the short branched form, are detectable in RNA from the mutant strains.

The high levels of intron in these two mutant strains presented a puzzle. How can splicing in these mutant strains be very inefficient, suggesting that the intron is excised from the primary transcript inefficiently, yet the levels of intron be much higher than that observed in the wild-type strain? The most reasonable possibility was that the excised introns from 3'III and 3'III–IV are very stable relative to the wild-type introns. To test this hypothesis, we took advantage of the fact that all of these β-galactosidase genes are under galactose control, thereby facilitating a nonradioactive pulse–chase experiment. At various times after replacement of galactose with glucose, RNA was isolated and assayed by primer extension with an intron primer. The data show that RNA molecules that terminate at the 5' junction (most of which is the form-2 intron—see Fig. 4) are remarkably stable; a substantial level of intron still remains 4–6 hr after the addition of glucose. This observation is true for both 3'III strains (Fig. 5) and has been reproduced with a blot-hybridization assay using an intron-specific probe (data not shown). In contrast, wild-type intron decays very rapidly after glucose replacement (Fig. 5 and data not shown). The

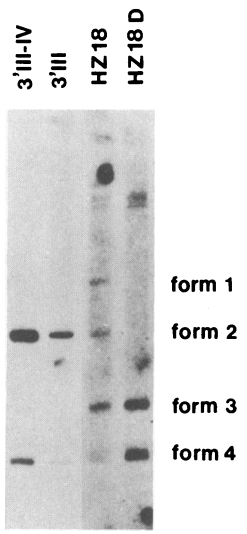

FIG. 4. Blot-hybridization analysis of the excised introns. In the first two lanes (from left to right), 10 μg of total RNA extracted from PB12 cells transformed with either 3'III–IV or 3'III were loaded. PB12 is a DB745 strain in which the rp51A gene has been deleted (20) and was used here with the mutant plasmids in order to eliminate any background due to the transcripts encoded by the endogenous chromosomal rp51A gene. The last two lanes were loaded with 10 μg of total RNA extracted from DB745 cells transformed with HZ18. RNAs were either mock-treated (lane 3: HZ18) or treated 15 min with the debranching activity from an S100 extract of the HeLa cells, as described in the legend of Fig. 2 (lane 4: HZ18D). This allows one to readily distinguish between the lariat and linear forms of the excised introns, as indicated at right. RNAs were electrophoresed in a 4% acrylamide/8 M urea gel, transferred to a nylon membrane, and hybridized with an rp51A intron-specific probe as described in *Materials and Methods*. Lanes 1 and 2 were exposed 16 hr, whereas lanes 3 and 4 were exposed 12 days.

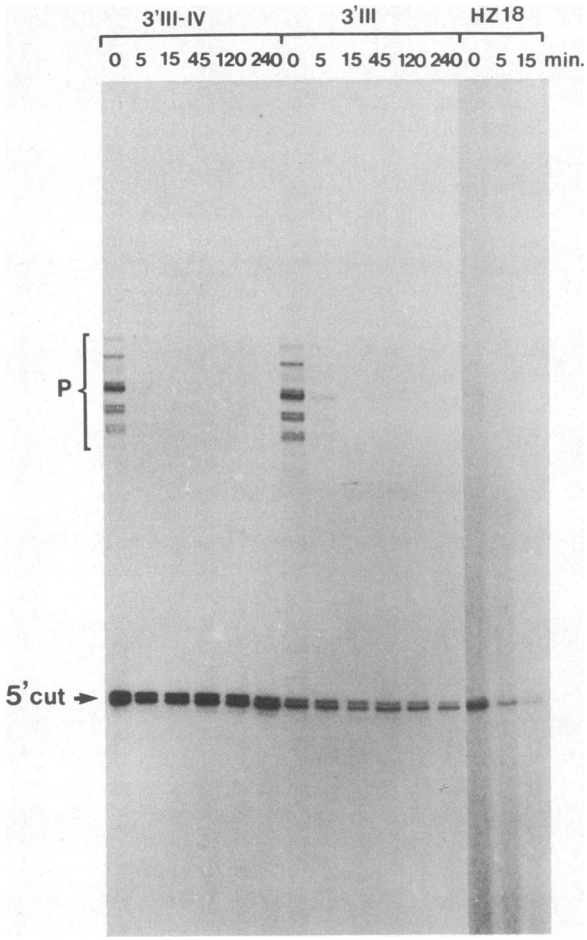

FIG. 5. Glucose-shift experiment. Cells were grown at 30°C in minimal supplemented galactose medium to an OD_{610} of 0.4. They were then harvested and resuspended in the same volume of prewarmed minimal supplemented glucose medium. Aliquots were taken and quick-cooled at the times indicated above each lane. RNAs were extracted and analyzed using an intron primer as described in *Materials and Methods*. cDNA molecules corresponding to unspliced fusion transcripts (P) and to RNA molecules cut at the 5' splice site (essentially form-2 intron in the mutants) are indicated. 5'-Cut RNA molecules generate two cDNA bands, one nucleotide apart. As described by Jacquier *et al.* (15), the upper band is most likely due to the occasional addition of a nucleotide to the end of the reverse transcript. As in Fig. 4, 3'III and 3'III–IV were transformed into PB12 cells. HZ18 was transformed into DB745 cells. The part of the gel corresponding to the mutant 3'III–IV was exposed for 12 hr. The part of the gel corresponding to the wild-type HZ18 RNAs was exposed for the same time but with an intensifying screen (DuPont Cronex Lightning Plus) at −70°C, which results in an ≈10-fold increase in sensitivity.

half-life of the wild-type HZ18 intron, as defined by such experiments, is ≈5 min (J. Rodriguez, personal communication). Precursor RNA, from both mutant and wild-type strains, also decays rapidly after glucose replacement and has a half-life much less than 5 min (Fig. 5 and data not shown). These results confirm that the increased intron levels in the two TACTACC mutants are due, at least in part, to a substantial increase in intron stability.

DISCUSSION

The data reinforce the importance of the TACTAAC box, and in particular the branch site adenosine, to the yeast splicing pathway. In agreement with others (13, 14), we have found that a single transversion at this position (TACTAAC→ TACTACC), in an otherwise wild-type background, profoundly inhibits *in vivo* splicing, as β-galactosidase activity decreases by a factor of $\approx 10^3$ and no mRNA is detectable. Also, in agreement with a previous report (14), this mutant gene is inactive as a substrate for *in vitro* splicing (data not shown).

However, there is a low level of β-galactosidase still detectable in 3'III-bearing cells. Based on the data shown in Table 1, we interpret this residual (0.1%) level of β-galactosidase as splicing-dependent gene expression. The discrepancy with a previous report of this mutation (14) may be due to the greater sensitivity afforded here by the enzyme assay or to the fact that the two studies placed the mutation in different genes. We do not know why the additional mutation in 3'III–IV (apparently) creates a better splicing substrate. This position may have a subtle effect on splicing efficiency, perhaps through an effect on the adjacent, mutant TACTACC box.

Surprisingly, the data indicate that the 3'III branch points are at the usual sixth residue of the now mutant TACTACC box, which is a cytidine rather than an adenosine. This conclusion is drawn from a high-resolution analysis in which the primer-extension stop of the lariat intermediate of 3'III was precisely mapped to the same nucleotide as that found from the wild-type gene. No other candidate branch point stops are detectable. The assignment of the branch to the cytidine in the sixth position of the TACTACC box is also consistent with the data in Fig. 3, which show that the 3'III intron branch is a very poor substrate for the HeLa cell debranching activity. As the original report of this activity (27) analyzed a number of substrates (all branched on A residues) and found them to be well cleaved by this activity, we take this result to indicate that the 3'III branch is highly unusual. Although we know of no reports of a normal,

specific branch occurring at any residue other than an adenosine, Wallace and Edmonds (26) reported a low percentage of branches occurring at cytidine.

Our finding argues persuasively for the prominent role of the TACTAAC box (and environs) in the assignment of the branch location. Faced with a cytidine at the normal location, the splicing machinery prefers to form the branch at the correct position but the "wrong" nucleotide rather than form the branch at another adenosine elsewhere in the intron. Indeed, even a pseudo-TACTAAC box (TACAAAC) located 20 nucleotides further downstream—and used as a branch point when this substrate is spliced in a HeLa cell extract (11)—is apparently ignored in preference to the correct position. The data reinforce the notion that branch site assignments in yeast and mammalian cells have significant differences, as analogous experiments in HeLa cells lead to the identification of other adenosine residues as cryptic branch points (29). This result also recalls that of Newman *et al.* (14). Faced with an AT, rather than a GT, at the 5′ junction, the splicing machinery prefers to make the 5′-junction cut at the correct position but the "wrong" nucleotide. In mammalian cells, the same mutant 5′ junction (GT→AT) is no longer recognized as an exon–intron junction and leads to the use of normally silent, cryptic 5′ junctions (30, 31).

The fact that 3′III-bearing cells contain a high precursor/mRNA ratio and a high lariat-intermediate/mRNA ratio indicates that both the first step (5′ cleavage and lariat formation) and the second step (3′ cleavage and exon ligation), respectively, are slow relative to those for a wild-type substrate (15, 22). This same argument, applied to the high level of intron present in these cells, suggests that intron turnover must also be slow in this mutant. A direct test of this hypothesis shows that this is indeed the case, as the half-life of 3′III is ≈100 times greater than that of the wild-type intron. The double mutant 3′III–IV shows an even greater stability, consistent with the higher steady-state levels of the 3′III–IV intron as compared to 3′III.

These facts notwithstanding, the marked stability of the 3′III introns might not be the only factor contributing to the very high level of intron relative to mRNA in these strains. Indeed, it is possible that at least a fraction of those "stable introns" are not produced via the normal splicing pathway but rather are derived by degradation, or endonucleolytic cleavage, of the mutant lariat intermediate prior to the second splicing step. It may be that the same putative nucleolytic activities are responsible for generating the short forms of the wild-type introns normally detected *in vivo* (2, 3, 7). We speculate that the slow rate of the second splicing step in the 3′III mutants might be responsible for the premature occurrence of these events, leading to a higher level of intron production relative to mRNA. Consistent with these possibilities is the fact that the only other point mutant reported to exhibit a slow second step is also the only other mutant we have observed with an abnormally high level of this same "small" intron form (ref. 15 and unpublished data). This derivative pathway offers an intriguing explanation for the increased stability of the mutant introns. Perhaps, as premature excision species, they are frozen in a stable "spliceosome" particle, unable to undergo the subsequent steps required for intron turnover. It should be noted, however, that the level of "free" 5′ exon in 3′III-bearing cells, as seen by RNA blot analysis (data not shown), is low and approximately equal to the level of "intact" lariat intermediate molecules detected by primer extension with a 3′ exon primer. This implies that, if the above hypothesis is correct, both the 5′ and the 3′ exon sequences are not protected in the "frozen" spliceosome particle, in contrast to the intron sequences.

It is tempting to suggest that the observations presented here reflect the stringent sequence requirements of yeast RNA metabolism. The splicing machinery is constrained to choose the sixth position of the mutant TACTACC box as a branch point, presumably because of the nature of the nucleotide sequence within and surrounding the TACTACC box. This results in very inefficient branch formation and splicing. Similarly, it may be that the 3′III intron is stable because the mutant branch point is a poor substrate for the yeast debranching activity. A further implication of this speculation is that debranching may be a normal (and perhaps required) part of the intron-degradation pathway. This thought is consistent with the data in Fig. 4, which show that the 3′III introns are not only present *in vivo* at high steady-state levels but are also predominantly of the branched forms. It is also consistent with the data in Fig. 3, which show that the 3′III intron with the abnormal cytidine branch is a very poor substrate for the HeLa cell debranching activity. As we have been able to detect the yeast analogue of the HeLa cell debranching activity in crude extracts, this should allow us to test these and other mutant branches as substrates for the homologous debranching enzyme. The study of these and other mutants should further clarify the role of the TACTAAC box in branch point assignment and the role of the branch point in intron turnover.

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