Unconventional Splicing of HAC1/ERN4 mRNA Required for the Unfolded Protein Response

SEQUENCE-SPECIFIC AND NON-SEQUENTIAL CLEAVAGE OF THE SPLICE SITES*

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Accumulation of unfolded proteins in the endoplasmic reticulum (ER) activates an intracellular signaling pathway from the ER to the nucleus, termed the unfolded protein response. We and others recently identified transcription factor Hac1p/Ern4p responsible for the response in Saccharomyces cerevisiae and found that Hac1p expression is controlled by the regulated splicing of HAC1 mRNA. Walter and co-workers (Sidrauski, C.; Cox, J. S., and Walter, P. (1996) Cell, 87, 405–413) further showed that the splicing requires tRNA ligase but not spliceosome. In this report, we carried out mutational analysis of HAC1 mRNA and revealed several unique features of the splicing. First, a mutation or deletion of the branchpoint-like sequence present in HAC1 intron did not affect the splicing. Second, cleavage of the splice sites was sequence-specific and thus completely blocked by some point mutations introduced at the 5’ or 3’ splice site. Third, cleavage of the 5’ and 3’ splice sites could occur independently as judged by the nature of splicing intermediates accumulated. Fourth, swapping the nucleotide sequences of the 5’ and 3’ splice sites inhibited the ligation but not the cleavage step. We conclude that signaling from the ER activates putative endonucleases that can carry out sequence-specific cleavage of the splice sites in a random order.

Accumulation of unfolded proteins in the endoplasmic reticulum (ER) activates an intracellular signaling pathway from the ER to the nucleus, resulting in transcriptional induction of molecular chaperones and folding enzymes localized in the ER (1–4). This induction system, termed the unfolded protein response (UPR), is observed in all eukaryotes examined and is required for survival under the conditions that continuously accumulate unfolded proteins in the ER ("ER stress") in budding yeast Saccharomyces cerevisiae (5–8) as well as in mammalian cells (9–12), suggesting that the UPR has been quite conserved during evolution of eukaryotic cells.

We and others recently demonstrated that a basic leucine zipper protein Hac1p/Ern4p functions as the transcription factor responsible for the UPR in S. cerevisiae; haploid cells lacking Hac1p (hac1Δ) are unable to induce transcription of any of the target genes of the UPR and exhibit sensitivity to ER stress (7, 8, 13). Furthermore, Hac1p expression was found to be regulated posttranscriptionally in a completely unexpected manner; HAC1 mRNA is constitutively expressed but becomes spliced in response to ER stress (13, 14). Thus, an intron of 252 nt is removed from 1.4-kb precursor mRNA (pre-mRNA) to produce 1.2-kb mature mRNA (see Fig. 1A). The splicing event entirely depends on the signaling from the ER, and expression of mature mRNA activates the UPR. Since the 5’ splice site is located within the coding region, this splicing replaces the C-terminal portion of Hac1p. Pre- and mature mRNAs encode a protein of 230 and 238 aa, respectively, although these two types of Hac1p are supposed to share identical N-terminal 220 aa. Interestingly, only ER-stressed cells produce detectable amounts of Hac1p of 238 aa, which is thus translated from mature mRNA. Although Cox and Walter (13) ascribed the absence of 230-aa-Hac1p potentially synthesized from pre-mRNA to its extreme instability, we showed that there is essentially no difference in stability between 230-aa- and 238-aa-Hac1p and that the absence of 230-aa-Hac1p is due to the lack of translation of pre-mRNA. Namely, Hac1p is synthesized only after the mRNA splicing takes place, leading to activation of the UPR (14).

This splicing is also quite unique in that sequences around the 5’ and 3’ splice sites do not match the consensus found in S. cerevisiae and higher eukaryotes (GT-AG or AT-AC; Refs. 15 and 16). Walter and co-workers (17) further showed that splicing of HAC1 pre-mRNA is not mediated by the conventional pre-mRNA processing system. The splicing was not affected by conditional mutation of two components of the spliceosome (prp2Δ and prp8Δ), and tRNA ligase was found to be directly involved in the final step of the splicing, joining the two exons after ER stress-induced cleavage of HAC1 pre-mRNA. In this report, we took a different approach to gain insight into the mechanism of this unconventional type of mRNA splicing. The results obtained by mutational analysis of HAC1 mRNA will be discussed in relation to the features known for conventional pre-mRNA splicing as well as tRNA splicing.

EXPERIMENTAL PROCEDURES

Strains and Microbiological Techniques—The yeast strain used in this study was KY1145 (MATa leu2-3, 112 ura3–52 his3–Δ200 trp1–Δ901 lys2–801 hac1Δ::TRP1 ura3–52::URA3-UPRE-CYC1-lacZ) (14). The composition of synthetic complete medium used for selection of transformants such as SC(-Ura, Leu) has been described (18). Tunica-mycin was obtained from Sigma (T-7765) and used at a concentration of 5 μg/ml throughout the experiments. Transformation of yeast cells was performed by the lithium acetate method (19). Construction of Plasmids—Recombinant DNA techniques were carried out as described (20). The parental single-copy plasmids carrying the HAC1 gene, YCP-HAC1WT, and YCP-HAC1WTXbaI, were described previously (14). Plasmids with some mutated HAC1, YCP-HAC1-mBp and YCP-HAC1-ΔS13 (Fig. 1), were constructed by site-directed mutagenesis (21). Other mutations were introduced into YCP-HAC1WTXbaI by replacing the 0.18-kb XbaI-HindIII fragment or
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0.11-kb HindIII18EcoRI930 fragment with the corresponding fragment of the product obtained by polymerase chain reaction (PCR)-mediated mutagenesis after its sequence had been confirmed.

**Northern Blot Hybridization Analysis**—Northern blot hybridization analysis was carried out as described previously (6, 7, 14). The positions of probes specific for the first or second exon of HAC1 are illustrated in Fig. 1A.

**β-Galactosidase Assays**—Cellular UPR activity was monitored by measuring the level of β-galactosidase expressed from the UPRE-CYCI-lacZ reporter gene that had been integrated into the chromosome of KMY1145. Induction of β-galactosidase by tunicamycin entirely depends on both the splicing of HAC1 pre-mRNA induced by the signaling from the ER and the direct interaction between Hac1p thus produced and cis-acting unfolded protein-response element (UPRE) (7, 14). Assays for β-galactosidase activity in yeast were carried out as described previously (6).

**RESULTS**

**The Branchpoint-like Sequence Present in HAC1 Intron Is Not Required for the Splicing**—We utilized the mfold server by Zuker and Turner on the internet to obtain a possible secondary structure of HAC1 pre-mRNA at 30 °C. All of the nine structures obtained with minimum energy lower than −421.6 kcal/mol gave rise to an identical secondary structure for the intron-containing region which, as shown in Fig. 1B, contained four stem-loop structures (designated SL1 to SL4 from the 5′ side). Interestingly, the 5′ or 3′ splice site was predicted to be localized in the loop of SL1 or SL4, respectively, each loop consisting of seven nucleotides (Fig. 2). A branchpoint (Bp)-like sequence (UACUAAG) present in HAC1 intron (17) was found around the loop of SL3. The Bp sequence known to be almost invariant in S. cerevisiae (UACUAAC) is utilized to form the lariat intermediate during the first step in conventional pre-mRNA splicing and is also important for formation of splicing complex as well as commitment complex (Ref. 22 and references therein). The Bp-like sequence in HAC1 intron is found 28 nucleotides upstream of the 3′ splice sites, which matches well with Bp sequences usually located 20–40 nucleotides upstream of the 3′ splice sites (15).

We thus mutated the Bp-like sequence and examined its effect on splicing of HAC1 mRNA induced by tunicamycin, which is known to elicit ER stress by inhibiting N-glycosylation of newly synthesized proteins, leading to activation of the UPR (2, 23). In the hac1Δ strain carrying the wild-type HAC1 gene on a single-copy expression plasmid (referred to as YCp-HAC1WT in Fig. 1C) as well as wild-type strain, splicing of constitutively expressed 1.4-kb HAC1 pre-mRNA was induced within 1 h after addition of tunicamycin to produce 1.2-kb mature mRNA as reported previously (13, 14). Under these conditions, β-galactosidase expressed from the UPRE-CYC1-lacZ reporter gene was induced approximately 10-fold (Fig. 1C). When the Bp-like sequence in HAC1 intron was mutated from UACUAAG to AUGAUUG without altering the GC content and the resulting mutant HAC1 gene designated mBp was introduced into the hac1Δ strain, the splicing occurred nor-

**Fig. 1. Effects of a mutation or deletion of the branchpoint-like sequence present in HAC1 intron on the UPR.** A, a schematic structure of HAC1 pre-mRNA. The two Hac1p-coding regions are shown by the boxes, and the location of the intron of 252 nt is indicated by the double-headed arrow. bZIP and An denote the basic leucine-zipper region and the poly(A), respectively. Because the 5′ splice site is located within the open reading frame of 230 aa, ER stress-induced mRNA splicing causes removal of the C-terminal 10 aa and fusion of the remaining 220 aa with a stretch of 18 aa encoded by the second exon (cf. reference 14 for precise locations of the 5′ and 3′ splice sites). The positions of probes specific for the first or second exon of HAC1 are also indicated. B, a possible secondary structure of HAC1 intron-containing region. Four predicted stem-loop structures are designated as SL1 to SL4 from the 5′ side. The locations of the 5′ and 3′ splice sites and the branchpoint (Bp)-like sequence are also shown. The nucleotide sequences of the consensus Bp, Bp-like sequence present in HAC1 intron, and mutant Bp analyzed are shown below. C, the hac1Δ strain (KMY1145) was transformed with a single-copy expression vector alone (YCp-V), the wild-type (WT) HAC1 gene carried on the same vector (YCp-HAC1WT), or a mutant version of HAC1 containing either mutant Bp (mBp) or deletion of SL3 (ΔSL3). Transformants were grown at 30 °C in SC(-Ura, Leu) medium to a mid-log phase, and aliquots were incubated in the presence (+) or absence (−) of tunicamycin (TM). Samples taken after 3 h were used for β-galactosidase assays, and the activities are presented as means ± S.D. (bars), based on duplicate determinations with three independent transformants. Separate samples taken after 1 h were used for extracting total RNAs that were analyzed by Northern blot hybridization using DNA probe specific for the first exon of HAC1 or yeast actin ACT1. The positions of precursor and mature HAC1 mRNAs are indicated. *, denotes a band of 0.7 kb consisting of only the first exon of HAC1.
mally, and β-galactosidase was well induced. We further deleted the entire SL3 from HAC1 (designated ΔSL3); however, the deletion did not affect either the splicing of HAC1 pre-mRNA or induction of β-galactosidase significantly. These results clearly indicated that the Bp-like sequence in HAC1 intron is not required for the regulated splicing of HAC1 pre-mRNA.

Sequence-specific Cleavage of the Splice Sites—Next we examined whether nucleotide sequences around the splice sites were specifically recognized by putative endonucleases during the splicing reaction. To introduce various point mutations at the 5′ or 3′ splice site, we carried out PCR-mediated mutagenesis using a mutant version of HAC1 designated YCp-HAC1WT(XbaI) as described under “Experimental Procedures.” Although YCp-HAC1WT(XbaI) contained an XbaI site at nucleotide 640 that changed Leu211-Asp215 to Ser-Arg, the two amino acid changes did not affect the level of either HAC1 mRNA or Hac1p but only slightly reduced the transcriptional activator activity of Hac1p (14). A point mutation was introduced into each of the seven nucleotides predicted to form a loop structure of SL1 (5′ splice site) or SL4 (3′ splice site) and one flanking nucleotide each at both sides, where G was changed to C, A was changed to T, and vice versa so that the GC content was not altered (Fig. 2). For convenience, nucleotides at the 5′ side or 3′ side of the cleavage site were minus- or plus-numbered. After introduction of each point mutant into the hac1Δ strain and after 3 h of incubation in the presence or absence of tunicamycin, β-galactosidase activity expressed from the reporter gene was determined, and the extent of induction was compared with that of the wild-type (Fig. 2, A and B). Some of the point mutations were found to strongly inhibit the induction, whereas others were not. Interestingly, four out of the nine nucleotides (−3, −1, +3, and +5, boxed in Fig. 2) conserved between the 5′ and 3′ splice sites were most critical for the UPR; a point mutation of any of these nucleotides abolished induction of β-galactosidase almost completely, with one exception that affected the +5 nucleotide of the 3′ splice site. On the other hand, the nucleotide at +2 of either splice site seemed not to be important, and the effects of point mutations at −4, −2, +1, and +4 varied considerably between the 5′ and 3′ splice sites. These results indicated that sequence integrity of the splice sites is highly important for the UPR and suggested that putative endonucleases specifically recognize nucleotide sequences at both of the splice sites.

Cleavage of the 5′ and 3′ Splice Sites Can Occur Independently—Northern blot hybridization analysis was carried out to determine which step of the splicing was blocked by point mutations introduced at the 5′ or 3′ splice site (Fig. 3). Total RNAs isolated from transformants cultured for 1 h in the presence or absence of tunicamycin were analyzed using a probe specific for either the first exon or the second exon of HAC1. In hac1Δ cells carrying YCp-HAC1WT(XbaI), constitutively expressed 1.4-kb pre-mRNA (lanes 1 and 13) was spliced by tunicamycin treatment to produce 1.2-kb mature mRNA (lanes 2 and 14). In this experiment, a significantly increased amount of 0.7-kb band was detected with probe specific for the first exon (lane 2) as compared with the previous experiment using YCp-HAC1WT (a band marked by an asterisk in Fig. 1C). Because this band was not detected using probe specific for the second exon (Fig. 3, lane 14) or intron (data not shown), it must consist only of the first exon. Similarly, a band of 0.4 kb detected with probe specific for the second exon (lane 14) must consist only of the second exon, whose amount was significantly higher than that obtained with YCp-HAC1WT (data not shown). Therefore, the increased amounts of these intermediates are likely to result from slightly inefficient ligation of the

![Figure 2](image.png)

**Fig. 2. Effects of point mutations introduced at the 5′ and 3′ splice sites on the UPR.** Sequences of the nine nucleotides around the 5′ (panel A) or 3′ (panel B) splice site of the wild-type (WT) HAC1 gene and point mutants tested are shown at the bottom of each panel. Nucleotides conserved between the 5′ and 3′ splice sites are boxed. Each central seven nucleotides are predicted to be localized in the loop structure of SL1 (5′ splice site) or SL4 (3′ splice site) as illustrated at the top of each panel. The actual cleavage sites are indicated by the arrows. Nucleotides at the 5′ or 3′ side of the cleavage site are minus- or plus-numbered, respectively. The hac1Δ strain (KMY1145) was transformed with HAC1 containing an XbaI site at nucleotide 640 (YCp-HAC1WT(XbaI), see “Results”) or a point mutant introduced at either 5′ (panel A) or 3′ (panel B) splice site in YCp-HAC1WT(XbaI). β-Galactosidase assays were carried out as described in Fig. 1C, and the activities are expressed as a percentage of WT after subtracting β-galactosidase activities in unstressed cells from those in cells treated with tunicamycin for 3 h.
two exons due to the nucleotide alteration introduced to create an XbaI site at nucleotide 640, 16 nucleotides upstream of the 5′ splice site. Alternatively, the alteration introduced might have affected RNA stability.

The amounts of mature mRNA detected in tunicamycin-treated cells carrying various mutant versions of HAC1 (Fig. 3) were generally well correlated with cellular UPR activities determined by β-galactosidase assays (Fig. 2). Especially, virtually no mature mRNA was detected when a point mutation was introduced at −1 of the 5′ splice site (Fig. 3, lanes 6 and 18) or at −1 of the 3′ splice site (lanes 10 and 22). Instead, certain novel bands were detected under these conditions. In tunicamycin-treated cells carrying a point mutation at −1 of the 5′ splice site, probe specific for the first exon detected a band of 1.0 kb (lane 6) that was not detected with probe for the second exon (lane 18) but was detected with probe specific for intron (data not shown). Thus, the 1.0-kb band must represent a splicing intermediate consisting of the first exon and intron. In addition, the 0.7-kb band consisting of only the first exon was missing (lane 6), but the 0.4-kb band consisting of only the second exon was still detected (lane 18). These results strongly indicated that the point mutation at −1 of the 5′ splice site completely blocked the cleavage at the 5′ splice site without affecting the cleavage at the 3′ splice site. An almost identical pattern was obtained when a point mutation was introduced at +5 of the 5′ splice site (data not shown).

On the other hand, a point mutation at +1 of the 5′ splice site showed milder effects on the splicing than the −1 mutation. β-Galactosidase was slightly induced (Fig. 2A) and a correspondingly small amount of mature mRNA was detected (Fig. 3, lanes 8 and 20). In this case, two intermediates were detected simultaneously (lane 8), which were assigned to consist of the first exon and intron or only the first exon by differential hybridization to the probes used, indicating that the cleavage at the 5′ splice site was hampered but not blocked completely by the +1 mutation. Point mutations at −4, −3, and +3 of the 5′ splice site produced similar patterns (data not shown).

In contrast, in tunicamycin-treated cells carrying a point mutation at −1 of the 3′ splice site, two bands migrating at around the 0.7-kb fragment were detected with probe specific for the second exon (lane 22) or with probe specific for intron (data not shown), but not with probe for the first exon (lane 10). Hybridization with a pair of probes specific to either 5′ side or 3′ side of the intron indicated that the faster migrating band lacked the 5′ side of intron (data not shown). Thus, these bands must represent splicing intermediates consisting of the second exon and intron of different lengths. Furthermore, the 0.4-kb band consisting of only the second exon was absent (lane 22), and normal level of the 0.7-kb band consisting of only the first exon was detected (lane 10). These results strongly indicated that the point mutation at −1 of the 3′ splice site completely blocked the cleavage at the 3′ splice site without affecting the cleavage at the 5′ splice site. Point mutations at −3, +3, +4 of the 3′ splice site showed almost identical patterns (data not shown). From these results, we concluded that cleavages at the 5′ or 3′ splice site can occur not only independently from each other but in a random order.

Swapping the Nucleotides at the 5′ and 3′ Splice Sites Inhibits Ligation but Not Cleavage—Finally, we made three additional mutant versions of HAC1 by swapping the nucleotides around the 5′ splice site and those around the 3′ splice site, and examined their effects on the UPR (Fig. 4). As already shown in Fig. 2, not only seven nucleotides predicted to be localized in the respective loop structure, but also two flanking nucleotides at the position of −4 and +5 seemed to be important for the cleavage. Especially, the G to C mutation at +5 of the 5′ splice site completely blocked the cleavage at the 5′ splice site, whereas the same mutation at +5 of the 3′ splice site affected the splicing only weakly. Therefore, we swapped a total of nine nucleotides to see whether these differential effects are indicative of the involvement of two different endonucleases in cleaving two splice sites or not.

M1(3′-5′) contained 3′-type nine nucleotides at the 5′ splice site with those at the 3′ splice site unchanged. M2(5′-5′) contained 5′-type nine nucleotides at the 3′ splice site with those at the 5′ splice site unchanged. In M3(3′-5′), the nine nucleotides at the splice sites were entirely swapped. When induction of β-galactosidase from the reporter gene was compared (Fig. 4, bottom), M1, M2, and M3 retained 81, 18, and 6% activity of WT(5′-5′), respectively, indicating that the replacement of the nucleotides at the 3′ splice site by those at the 5′ splice site showed more profound effects on the splicing. Correspondingly decreased amounts of mature mRNA were detected by Northern blot hybridization in tunicamycin-treated cells carrying each of the mutant versions as compared with those carrying the wild-type HAC1 (Fig. 4). Sequencing of reverse transcriptase-coupled PCR products showed that each mature mRNA produced contained an exact exon-exon junction predicted from the altered sequences of the splice sites (data not shown). Contrary to the results shown in Fig. 3, however, the splicing intermediate consisting of the first exon and intron (1.0 kb) or the second exon and intron (0.7 kb) was not detected with

![Fig. 3](image-url)
In conclusion, our results reported here unraveled a novel mechanism for protein-mediated RNA splicing: sequence-specific and non-sequential cleavage of the splice sites.

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FIG. 4. Effects of swapping the nine nucleotides of the splice sites on the UPR. The hac1Δ strain (KMY1145) was transformed with the wild-type (WT) or a mutant version (M1–M3) of HAC1 containing an XbaI site at nucleotide 640. M1(3'-5') contains 3'-type nine nucleotides at the 5' splice site with those at the 3' splice site unchanged, whereas M2(5'-3') contains 5'-type nine nucleotides at the 3' splice site with those at the 5' splice site unchanged. In M3(3'-5'), the nine nucleotides at the splice sites are completely swapped. β-Galactosidase assays were carried out as in Fig. 1C, and the activities are expressed as a percentage of WT as in Fig. 2. RNAs were analyzed by Northern blot hybridization as in Fig. 3. Schematic structures and lengths (kb) of detected bands are indicated.

probe specific for the first or the second exon, respectively, in tunicamycin-treated cells carrying M2 (lanes 6 and 14) or M3 (lanes 8 and 16) although the extent of induction was less than 20% of WT in these cells. Instead, evidently increased amounts of the splicing intermediate consisting of only the first exon (0.7 kb, lanes 6 and 8) or only the second exon (0.4 kb, lanes 14 and 16) were detected. It seemed very likely that swapping the nine nucleotides inhibited the ligation but not the cleavage step, raising the possibility that the cleavage at the 5' and 3' splice sites is achieved by the same endonuclease.

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phosphodiester bond between the 5'-end of the intron and the adenosine in the Bp sequence located upstream of the 3' splice site, thus producing a lariat structure. Second, cleavage of the 3' splice site occurs, leading to release of the lariat intron and simultaneous ligation of the two exons (15, 16, 24 and references therein). On the other hand, tRNA splicing is catalyzed by the sequential action of three protein enzymes: a site-specific endonuclease, a tRNA ligase, and a phosphotransferase. In contrast to conventional pre-mRNA splicing, nucleotide sequences at the splice sites in tRNAs are not conserved, and the precise cleavage at the two splice sites is explained by a "ruler mechanism" in which a fixed distance to the splice sites is measured from a certain position in the mature domains of the tRNAs. Furthermore, the two splice sites are cleaved independently (25, 26 and references therein).

Recent work conducted in two laboratories established that the regulated splicing of mRNA encoding transcription factor Hac1p/Ern4p is required for the UPR (13, 14). Walter and coworkers (17) demonstrated that the splicing of HAC1 pre-mRNA is not mediated by spliceosome-dependent pre-mRNA processing as mentioned earlier. The results reported in this paper further substantiate this notion by demonstrating that the Bp-like sequence present in HAC1 intron is not required for the splicing (Fig. 1) and that cleavage of the two splice sites appeared to occur in a random order (Fig. 3).

Direct involvement of tRNA ligase in the splicing of HAC1 pre-mRNA (17) has evoked the possibility that cleavage of HAC1 pre-mRNA is also catalyzed by tRNA endonuclease. This hypothesis can now be pursued as a result of recent success in cloning genes that encode subunits of tRNA endonuclease (26).

However, our results revealed some important differences in the mode of recognition of the splice sites between the tRNA endonuclease and putative endonucleases responsible for the UPR. In contrast to tRNA splicing, sequence integrity at the splice sites of HAC1 pre-mRNA appeared to be very important for cleavage (Fig. 2). In addition, we previously showed that insertion of two nucleotides into a position between −2 and −1 of the 5' splice site completely blocked the splicing (14), whereas a similar insertion should result in a predictable shift of the cleavage site in the case of tRNA splicing (27).

Accumulation of unfolded proteins in the ER is considered to be sensed by Ire1p/Ern1p, a transmembrane protein kinase localized in the ER, and the signal is transmitted across the lipid bilayer through oligomerization and autophosphorylation of Ire1p (5, 6, 28). While this paper was in review, Sidrauski and Walter (29) reported that the purified C-terminal portion of Ire1p containing the kinase domain and the tail domain similar in sequence to mammalian RNase L has endonuclease activity that successfully cleaves HAC1 pre-mRNA in vitro. The effects on the splicing of a point mutation they introduced at −1 of the 5' or 3' splice site are consistent with the results shown in Fig. 3. In addition, our results of the swapping experiment (Fig. 4) may support the involvement of the Ire1p homodimer in the cleavage of the two splice sites. However, their primer extension analysis in vitro shifted the actual cleavage sites toward the 5' side by one nucleotide from those we determined by mutational analysis in vivo (14) for both 5' and 3' splice sites; namely, they claim that the cleavage occurs between −2 and −1 (see Fig. 2). The reason for this apparent discrepancy remains to be clarified.

In conclusion, our results reported here unraveled a novel mechanism for protein-mediated RNA splicing: sequence-specific and non-sequential cleavage of the splice sites.

In addition to autocatalytic self-splicing of group I and group II introns, two types of protein-mediated RNA splicing, namely spliceosome-dependent pre-mRNA splicing and tRNA splicing, have gained much attention and which have revealed completely different mechanisms. In conventional pre-mRNA splicing, sequences at the 5' and 3' splice sites are strictly conserved (GT-AG or AT-AC) and sequence-specific cleavage at the splice sites is achieved by involvement of various small nuclear RNAs and numerous proteins complexed in a spliceosome. In addition, cleavage occurs in two sequential steps. First, cleavage of the 5' splice site occurs in concert with the formation of 2'-5'
REFERENCES

1. Lee, A. S. (1987) Trends Biochem. Sci. 12, 20–23
2. Kozutsumi, Y., Segal, M., Normington, K., Gething, M. J., and Sambrook, J. (1988) Nature 332, 462–464
3. McMillan, D. R., Gething, M. J., and Sambrook, J. (1994) Curr. Opin. Biotech. 5, 540–545
4. Shamu, C. E., Cox, J. S., and Walter, P. (1994) Trends Cell Biol. 4, 56–60
5. Cox, J. S., Shamu, C. E., and Walter, P. (1993) Cell 73, 1197–1206
6. Mori, K., Ma, W., Gething, M. J., and Sambrook, J. (1993) Cell 74, 743–756
7. Mori, K., Kawahara, T., Yoshida, H., Yanagi, H., and Yura, T. (1996) Genes Cells 1, 803–817
8. Nikawa, J., Akiyoshi, M., Hirata, S., and Fukuda, T. (1996) Nucleic Acids Res. 24, 4222–4226
9. Li, X., and Lee, A. S. (1991) Mol. Cell. Biol. 11, 3446–3453
10. Li, L. J., Li, X., Ferrario, A., Buckner, N., Liu, E. S., Wong, S., Gomer, C. J., and Lee, A. S. (1992) J. Cell. Physiol. 153, 575–582
11. Little, E., and Lee, A. S. (1995) J. Biol. Chem. 270, 9526–9534
12. Morris, J. A., Donner, A. J., Edwards, C. A., Hendershot, L. M., and Kaufman, R. J. (1997) J. Biol. Chem. 272, 4327–4334
13. Cox, J. S., and Walter, P. (1996) Cell 87, 391–404
14. Kawahara, T., Yanagi, H., Yura, T., and Mori, K. (1997) Mol. Biol. Cell 8, 1845–1862
15. Kreivi, J. P., and Lamond, A. I. (1996) Curr. Biol. 6, 892–895
16. Tarn, W.-Y., and Steitz, J. A. (1997) Trends Biochem. Sci. 22, 132–137
17. Sidrauski, C., Cox, J. S., and Walter, P. (1996) Cell 87, 405–413
18. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
19. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 165–168
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Second Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Konkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
22. Berglund, J. A., Chua, K., Abovich, N., Reed, R., and Roshbash, M. (1997) Cell 89, 849–858
23. Reyes, V. M., and Abelson, J. (1988) Cell 55, 719–730
24. Shamu, C. E., and Walter, P. (1996) EMBO J. 15, 3028–3039
25. Sidrauski, C., and Walter, P. (1997) Cell 90, 1031–1039

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