Cell, Vol. 90, 1031–1039, September 19, 1997, Copyright ©1997 by Cell Press

The Transmembrane Kinase Ire1p Is a Site-Specific Endonuclease That Initiates mRNA Splicing in the Unfolded Protein Response

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Summary

The endoplasmic reticulum (ER) communicates with the nucleus through the unfolded protein response (UPR), which senses accumulation of unfolded proteins in the ER lumen and leads to increased transcription of genes encoding ER-resident chaperones. As a key regulatory step in this signaling pathway, the mRNA encoding the UPR-specific transcription factor Hac1p becomes spliced by a unique mechanism that requires tRNA ligase but not the spliceosome. Splicing is initiated upon activation of Ire1p, a transmembrane kinase that lies in the ER and/or inner nuclear membrane. We show that Ire1p is a bifunctional enzyme: in addition to being a kinase, it is a site-specific endoribonuclease that cleaves *HAC1* mRNA specifically at both splice junctions. The addition of purified tRNA ligase completes splicing; we therefore have reconstituted *HAC1* mRNA splicing in vitro from purified components.

Introduction

Cells respond to the accumulation of unfolded proteins (UP) in the endoplasmic reticulum (ER) by increasing transcription of genes encoding ER-resident proteins. An intracellular signaling pathway common to all eukaryotic cells, called the unfolded protein response (UPR), links the ER lumen and the nucleus and, when activated, leads to increased production of ER-resident chaperones and enzymes that expedite protein folding and assembly in the ER lumen (reviewed by Sweet, 1993; McMillan et al., 1994; Shamu et al., 1994). Thus, the UPR allows eukaryotic cells to regulate the biosynthesis of ER chaperones in response to need. To date, three components involved in this signaling pathway have been identified in the yeast Saccharomyces cerevisiae: Hac1p, a DNA-binding protein with homology to the leucine zipper family of transcription factors (Cox and Walter, 1996; Mori et al., 1996; Nikawa et al., 1996); tRNA ligase, an RNA processing enzyme involved in splicing of tRNAs (Sidrauski et al., 1996); and Ire1p, a transmembrane kinase localized to the ER (or the inner nuclear membrane with which the ER is continuous) (Cox et al., 1993; Mori et al., 1993).

Upon activation of the pathway, Hac1p coordinately up-regulates transcription of the ER-resident proteins after binding to the unfolded protein response element (UPRE) shared by their promoters (Mori et al., 1992, 1996; Kohno et al., 1993; Cox and Walter, 1996; Nikawa et al., 1996). Active Hac1p results from regulated splicing of its mRNA (Cox and Walter, 1996). Induction of the UPR pathway leads to the removal of a 252 nucleotide intron near the 3′ end of *HAC1*^{u} mRNA ("u" for UPR-uninduced) to produce the spliced form of this mRNA, *HAC1*^{i} ("i" for UPR-induced). Splicing results in a change in the *HAC1* open reading frame so that it now encodes a protein, Hac1p^i, that differs in its C-terminal tail from Hac1p^u, the protein encoded by the unspliced mRNA. Hac1p^i, in contrast to Hac1p^u, can readily be detected in the nucleus of yeast cells. Because the majority of *HAC1*^{u} mRNA is found on polyribosomes, the complete absence of Hac1p^u in uninduced cells is due to a block in translation of this mRNA and/or to the rapid degradation of the Hac1p^u. Thus, the regulated processing of *HAC1* mRNA modulates the levels of Hac1p synthesis (Cox and Walter, 1996).

Splicing of *HAC1* mRNA occurs by an unprecedented pathway (Sidrauski et al., 1996). The splice junctions of *HAC1* mRNA do not resemble the consensus sequences found in pre-mRNAs processed by the spliceosome, and *HAC1* mRNA splicing is not affected by mutations that inhibit spliceosome function. Furthermore, yeast tRNA ligase (encoded by *RLG1*) is required for splicing of *HAC1* mRNA. An allele of *RLG1*, *rlg1-100*, completely blocks the UPR without affecting splicing of tRNAs (an essential function). In this mutant, *HAC1* mRNA becomes specifically degraded when the pathway is activated. Based on this observation and the known catalytic activity of tRNA ligase, we proposed that tRNA ligase joins the *HAC1* mRNA halves that are produced upon activation of an unknown nuclease. In the absence of the second step in the splicing reaction (ligation), the cleavage products were proposed to be rapidly degraded (Sidrauski et al., 1996).

Splicing of *HAC1* mRNA requires a functional, activated Ire1p transmembrane kinase. The N-terminal half of Ire1p lies in the ER lumen where it somehow senses the accumulation of unfolded proteins and transmits the unfolded protein signal across the membrane. The C-terminal half of Ire1p, containing the kinase domain, lies in the cytoplasm or the nucleus where it transmits the signal from unfolded proteins to the splicing machinery responsible for *HAC1* mRNA processing. As a Ser/Thr kinase, Ire1p belongs to a class of transmembrane kinases that includes the transforming growth factor (TGF)-β type II receptor. Like other receptor membrane kinases, Ire1p becomes activated by oligomerization and autophosphorylation by neighboring Ire1p molecules. Ire1p has a C-terminal 133 amino acid tail domain located after its kinase domain that, although dispensable for kinase activity, is required for signaling to downstream components (Shamu and Walter, 1996).

Splicing of *HAC1* mRNA is initiated by an Ire1p-mediated event that leads to nucleolytic cleavage. In principle, Ire1p could somehow regulate access of the substrate, *HAC1*^{u} mRNA, to a constitutively active endonuclease (such as tRNA endonuclease) or regulate the nucleolytic activity of the endonuclease that cleaves *HAC1* mRNA. Alternatively, Ire1p could participate directly in the splicing reaction. We show here that the cytosolic/nuclear half of Ire1p is an endoribonuclease that cleaves *HAC1*^{u} mRNA with specificity at both its 5′

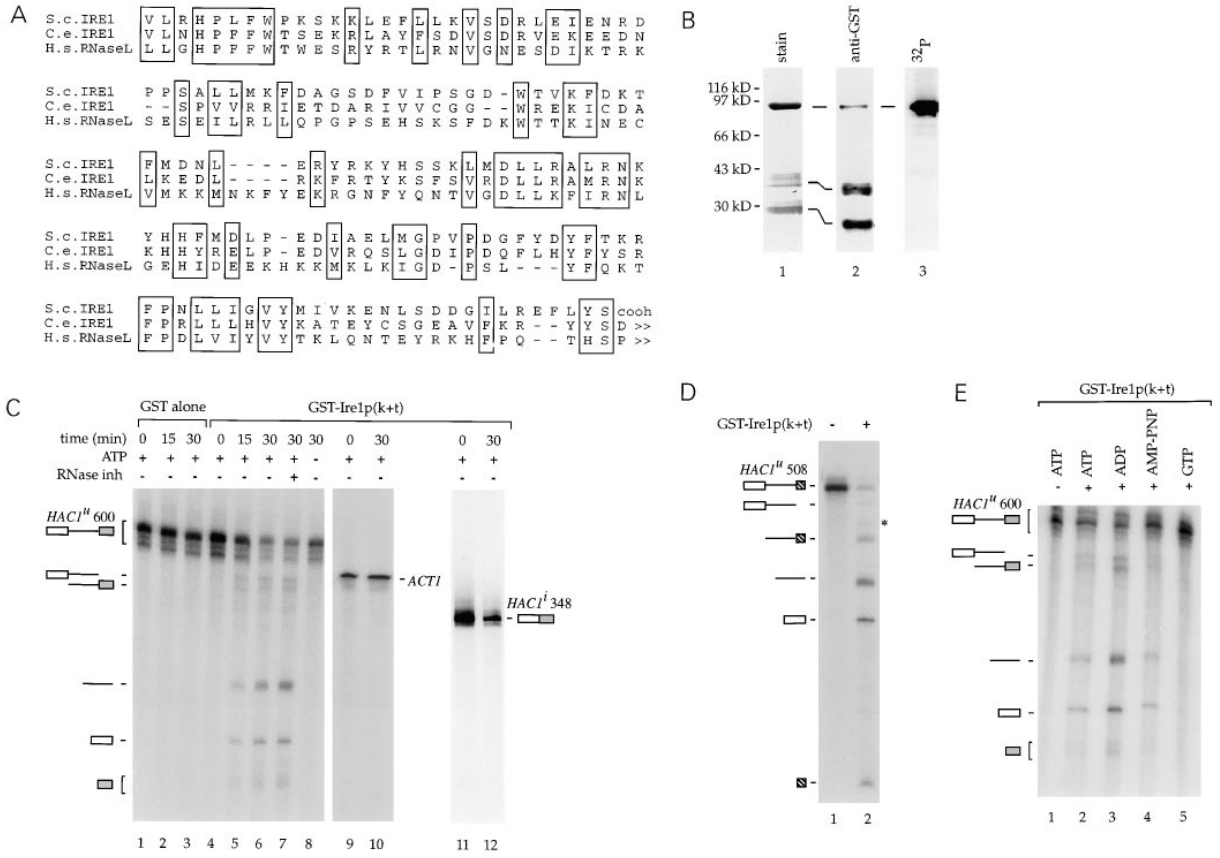

Figure 1. The Cytosolic/Nuclear Portion of Ire1p Has Both Kinase and Nuclease Activity In Vitro

(A) Amino-acid sequence alignment of the C-terminal tail domains of S. cerevisiae Ire1p, a putative C. elegans Ire1p, and human RNase-L.

(B) The C-terminal half, kinase+tail (k+t) domains of Ire1p were fused to glutathione S-transferase (GST) and purified from E. coli. The purified GST-Ire1p(k+t) fraction was electrophoresed on an SDS–polyacrylamide gel and stained with Coomassie blue (lane 1) or probed by Western blot analysis using anti-GST antibodies (lane 2). The in vitro kinase activity of the GST-Ire1p(k+t) was assayed by incubating the purified fusion protein in the presence of [γ^{32}P]ATP in kinase buffer (lane 3).

(C) Either GST alone (lanes 1–3) or GST-Ire1p (lanes 4–8) were incubated in kinase buffer with in vitro transcribed *HAC1^u* 600 RNA for the time indicated. In vitro transcribed *actin* RNA and spliced *HAC1^i* 348 RNA were used as controls for Ire1p nuclease specificity (lanes 9–10 and lanes 11–12, respectively). The products of the reaction were fractionated on denaturing 5% polyacrylamide gels. The icons indicate the different products of the cleavage reaction: 5′ exon + intron, 3′ exon + intron, intron, 5′ exon, and 3′ exon.

(D) GST-Ire1p(k+t) was incubated for 30 minutes with *HAC1^u* 508 RNA containing the same 5′ exon and intron as *HAC1^u* 600 RNA, but a smaller 3′ exon. The asterisk indicates an additional cleavage product that does not correspond to any of the expected *HAC1* mRNA fragments and that has not been identified.

(E) The GST-Ire1p(k+t) fusion protein was incubated with in vitro transcribed *HAC1^u* 600 RNA in the absence of ribonucleotide (lane 1) or in the presence of 2 mM ATP (lane 2), 2 mM ADP (lane 3), 2 mM AMP-PNP (lane 4), or 2 mM GTP (lane 5). The reaction products were resolved as described above.

and 3′ splice junctions. Indeed, when combined in vitro, Ire1p and purified tRNA ligase are sufficient to catalyze accurate splicing of *HAC1* mRNA.

Results

Ire1p Is a Site-Specific Endonuclease

Amino acid alignments reveal significant sequence similarity between the C-terminal tail domain of Ire1p (located C-terminal to the kinase domain) and mammalian RNase L, a nuclease that also contains a kinase domain and is, like Ire1p, activated by oligomerization (Zhou et al., 1993; Dong and Silverman, 1995) (Figure 1A). These striking similarities, both in sequence and mechanism of activation, led to the speculation that Ire1p might also be a nuclease (Bork and Sander, 1993; Sidrauski et al., 1996). This proposal was particularly appealing as a key step in the UPR pathway involves Ire1p-dependent but spliceosome-independent splicing of *HAC1^u* mRNA. We therefore decided to test directly whether Ire1p is the nuclease that cleaves *HAC1^u* mRNA as the first step of its splicing reaction.

To this end, we expressed the cytoplasmic/nuclear portion of Ire1p consisting of its kinase and C-terminal tail domains (k+t) as a fusion protein with glutathione S-transferase (GST) in E. coli. The fusion protein, henceforth referred to as GST-Ire1(k+t), was soluble in E. coli lysates and was purified in a single step using glutathione-Sepharose affinity chromatography. The purified protein fraction contained a major 85 kDa band, the

expected molecular weight for GST-Ire1(k+t), as well as smaller products (Figure 1B, lane 1). The 85 kDa band and the smaller products bound anti-GST antibodies (Figure 1B, lane 2); the smaller products are likely GST-derived breakdown products that copurified on the affinity column. Importantly, the kinase domain of GST-Ire1(k+t) was enzymatically active, as shown by its autophosphorylation activity upon incubation with γ-[^{32}P]ATP (Figure 1B, lane 3) (Welihinda and Kaufman, 1996).

To test for the suspected nuclease activity of GST-Ire1(k+t), we prepared a 600 nucleotide RNA substrate (*HAC1^u* 600 RNA) consisting of the *HAC1* intron (252 nucleotides) flanked on both sides by truncated exon sequences (181 nucleotides on the 5′ side and 167 nucleotides on the 3′ side). Labeled *HAC1^u* 600 RNA was prepared by in vitro transcription using T7 RNA polymerase in the presence of α-[^{32}P]UTP. Gel-purified *HAC1^u* 600 RNA always migrated as a set of multiple bands, even on denaturing gels run at elevated temperatures, presumably due to strong secondary structure (e.g., Figure 1C, lane 1). Nevertheless, as shown in Figure 1C (lanes 5–7), incubation of *HAC1^u* 600 RNA with GST-Ire1(k+t) reproducibly resulted in a series of discrete, new bands that resulted from cleavage of the substrate RNA. These bands were not obtained if purified GST was used in the reaction mixture in place of the GST-Ire1(k+t) fusion protein (Figure 1C, lanes 1–3). Moreover, under identical reaction conditions, GST-Ire1(k+t) did not cleave an RNA substrate containing the actin intron flanked by exon sequences (Figure 1C, lanes 9 and 10); a substrate commonly used for spliceosome-mediated in vitro splicing reactions (Schwer and Guthrie, 1991), neither did it cleave an RNA substrate, *HAC1^i* 348 RNA, which contained the same 5′ and 3′ exon sequences as *HAC1^u* 600 RNA but lacked the 252 nucleotide intron (Figure 1C, lanes 11 and 12). The nuclease activity of GST-Ire1(k+t) was insensitive to addition of placental RNase inhibitor (Figure 1C, lane 7), and control reactions containing limiting amounts of RNase A or RNase T1 did not yield specific cleavage products of *HAC1^u* 600 RNA (not shown). Taken together, these results show that the observed nuclease activity of GST-Ire1(k+t) exhibits specificity for *HAC1^u* 600 RNA and that the cleavage sites do not represent regions that are hypersensitive to nonspecific nucleolytic attack.

The estimated sizes of the *HAC1^u* 600 RNA fragments produced upon incubation with GST-Ire1(k+t) corresponded to those predicted of the intron, the two exons, and two putative intermediates resulting from cleavage at only one of two splice junctions. We confirmed these assignments (indicated by the icons in the margin of Figure 1 and all following figures) using RNA substrates containing exons of different lengths and point mutations that abolish cleavage at either junction (see below). As shown in Figure 1D, for example, when a different RNA substrate, *HAC1^u* 508 RNA, which contained a shorter, 75 nucleotide 3′ exon, was incubated with GST-Ire1(k+t), the band corresponding to the 3′ exon and that corresponding to the intron + 3′ exon shifted in accordance with the size of the 3′ exon (Figure 1D, lane 2), whereas the bands corresponding to the 5′ exon, the intron, and the 5′ exon + intron were unchanged (in contrast to the *HAC1^u* 600 RNA, this shorter *HAC1* transcript migrates as a single band on denaturing gels). Taken together, these results show that the transmembrane kinase Ire1p is, in fact, an endonuclease that specifically cleaves *HAC1^u* RNA at or close to both splice junctions.

Given that the kinase activity of Ire1p is required in vivo for signaling in the UPR pathway, we reasoned that the nuclease activity of Ire1p may also require the function of the kinase domain of Ire1p. Indeed, a fusion protein consisting of only the C-terminal tail of Ire1p fused to GST but lacking the kinase domain was inactive when tested for nuclease activity on *HAC1^u* mRNA (not shown). Further support for this notion came from the requirement for ATP for cleavage. The reactions discussed above were performed in kinase buffer and hence contained ATP; when ATP was omitted, no cleavage occurred (Figure 1C, lane 8), suggesting that the kinase function may be required for activation of the nucleolytic activity. Surprisingly however, we found that the ATP in this reaction could be replaced by ADP (Figure 1E, lane 3) or by the nonhydrolyzable ATP analog AMP-PNP (Figure 1E, lane 4). In contrast, GTP was not able to activate the nuclease activity (Figure 1E, lane 5). These results indicate that an adenosine nucleotide is specifically required as a cofactor for the reaction. Given that ADP and a nonhydrolyzable ATP analog can substitute for ATP, these data also suggest that the requirement for the adenosine nucleotide does not reflect a requirement for a phosphorylation event catalyzed by the kinase domain of Ire1p.

Ire1p Cleaves *HAC1^u* 600 RNA at the Correct Splice Junctions

To determine the ends of the cleavage products produced by GST-Ire1p(k+t), we used primer extension analysis. Two oligonucleotides complementary to either the intron or the 3′ exon were labeled with [^{32}P] at their 5′ ends and used to map the 5′ and 3′ splice junctions, respectively. The oligonucleotides were hybridized to either uncleaved or GST-Ire1p(k+t)-cleaved *HAC1^u* 600 RNA and primer-extended using AMV reverse transcriptase. As shown in Figure 2 (lane 2), extension of the cleaved *HAC1^u* 600 RNA product with the oligonucleotide complementary to the intron generated a product of 59 nucleotides corresponding in size to cleavage at the correct 5′ splice junction. As expected, primer extension of the uncleaved *HAC1^u* 600 RNA did not generate this fragment (Figure 2, lane 1). Similarly, primer extension of the GST-Ire1p(k+t)-cleaved *HAC1^u* 600 RNA product with the oligonucleotide complementary to the 3′ exon generated the expected 88 nucleotide product (Figure 2, lane 4). Again, this fragment was not observed when uncleaved *HAC1^u* 600 RNA was used in the reaction (Figure 2, lane 3).

From the size of the oligonucleotide fragments generated by primer extension, we conclude that GST-Ire1(k+t) cleavage of *HAC1^u* RNA in vitro occurs at the splice junctions. Previously however, the splice junctions could not be assigned without ambiguity because a G residue at the exon–exon junction in the in vivo spliced *HAC1^i* mRNA (CCAGAAG) could have been derived from either exon. The data in Figure 2 shows that the excised intron produced by GST-Ire1(k+t) contains a 5′ G residue, as

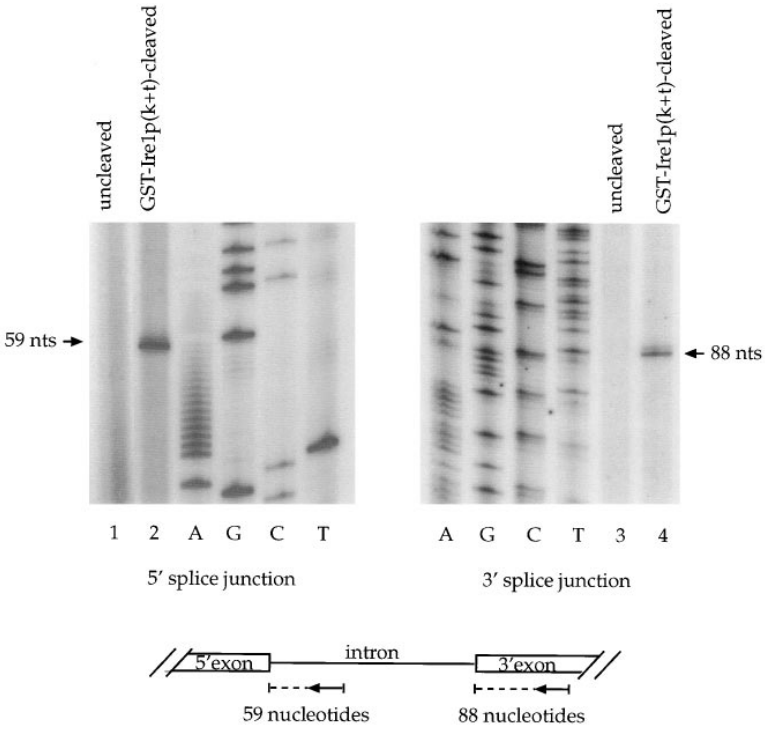

Figure 2. GST-Ire1p(k+t) Cleaves *HAC1*^{u} RNA at the Correct 5′ and 3′ Splice Junctions In Vitro

Primer extension assay on both uncleaved and cleaved *HAC1*^{u} 600 RNA was performed using oligonucleotides complementary to either the intron (lanes 1 and 2) or the 3′ exon (lanes 3 and 4). The oligonucleotides were labeled at their 5′ ends using [γ^{32}P]ATP and polynucleotide kinase and then hybridized to uncleaved (lane 1 and 3) or GST-Ire1p(k+t)-cleaved *HAC1*^{u} 600 RNA (lanes 2 and 4) and extended with AMV reverse trancriptase for 30 min. The products of the reaction were fractionated on a denaturing 8% polyacrylamide gel. Sequencing ladders were used as size markers. Note that the primers used in the sequencing ladders were different from those used to generate the primer extension products.

does the 3′ exon. Thus, the G residue found at the exon–exon junction in spliced *HAC1*^{i} mRNA is most likely derived from the 3′ splice junction.

Independent Cleavage of the 5′ and 3′ Splice Junction of *HAC1*^{u} mRNA

To assess further the fidelity of the GST-Ire1(k+t)-mediated cleavage reaction, we tested mutant *HAC1*^{u} RNAs for their ability to serve as substrates. To this end, we constructed single G→C point mutations at either the 5′ or the 3′ splice junction (*hac1[G885C]* and *hac1[G1137C]*, respectively) and tested the phenotype of these mutations in vivo. Cells that contained *hac1[G885C]* or *hac1[G1137C]* as their only copy of *HAC1* were unable to exhibit an unfolded protein response (not shown). Moreover, as shown in Figure 3A, both mutations abolished splicing of *HAC1*^{u} mRNA in vivo as assessed by Northern blot analyses of RNA isolated from strains expressing the mutant *HAC1* alleles. When the UPR was induced by tunicamycin treatment of these strains, no spliced *HAC1*^{i} mRNA was produced; instead, partially processed *HAC1*^{u} mRNA fragments accumulated. In particular, a band corresponding to the 5′ exon + intron accumulated in the strain bearing the *hac1[G885C]* mutation (Figure 3A, lane 3). This band is present, albeit less pronounced, in UPR-induced wild-type cells (Figure 3A, lane 2; Cox and Walter, 1996). Thus, the G885C mutation blocked cleavage of the 5′ splice junction but did not affect cleavage of the 3′ splice junction. Similarly, a band corresponding to the 5′ exon alone accumulated in the strain expressing *hac1[G1137C]* (Figure 3A, lane 4), suggesting that cleavage at the 3′ splice junction was blocked. From these data, we conclude that the G residues at both the 5′ and 3′ splice junctions are required for cleavage of the respective splice junction in vivo. Moreover, cleavage of the 5′ splice junction is independent of cleavage at the 3′ junction and vice versa. Thus, cleavage at both junctions occurs without an obligate order.

To assess whether the same substrate specificity is observed for cleavage of *HAC1*^{u} mRNA by GST-Ire1(k+t) in vitro, the corresponding G→C mutations were introduced into *HAC1*^{u} 600 RNA. Indeed, as shown in Figure 3B, incubation of *HAC1u[G885C]* 600 RNA with GST-Ire1(k+t) resulted in two products: a band corresponding to the 5′ exon + intron and a band corresponding to the 3′ exon; no bands corresponding to the intron or the 5′ exon were observed (Figure 3B, lane 4). Similarly, digestion of *HAC1u[G1137C]* 600 RNA resulted in bands corresponding to the intron + 3′ exon and the 5′ exon; no bands corresponding to the intron or the 3′ exon were observed (Figure 3B, lane 6). Thus, point mutations at both the 5′ and 3′ splice junctions abolished cleavage of the corresponding junction both in vivo and in vitro. Thus, by these criteria, the in vivo and in vitro cleavage reactions have indistinguishable substrate requirements. This observation strongly supports the notion that Ire1p is the endonuclease that initiates *HAC1*^{u} mRNA splicing in vivo, and moreover, that cleavage of the two splice junctions can occur independently.

In Vitro Reconstitution of *HAC1* mRNA Splicing from Purified Components

As tRNA ligase was previously identified as an essential component of the UPR pathway (Sidrauski et al., 1996), we tested next whether splicing of *HAC1*^{u} mRNA could be completed in vitro by adding tRNA ligase in addition to GST-Ire1(k+t). To this end, we obtained purified tRNA endonuclease and tRNA ligase as a kind gift from Chris Trotta and John Abelson (Caltech). To test the activity

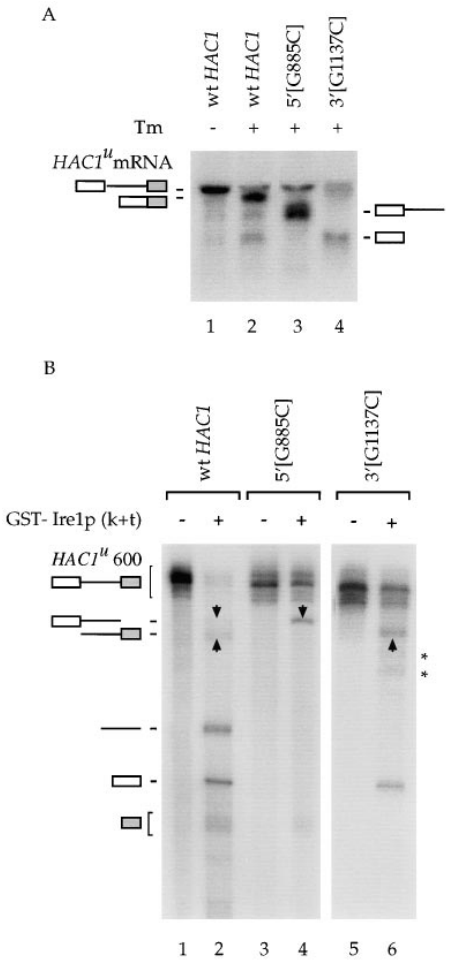

Figure 3. Independent Cleavage of *HAC1*^{u} mRNA 5′ and 3′ Splice Junctions

Point mutations in either the 5′ splice junction G*[G885C]* or the 3′ splice junction G*[G1337C]* were generated.

(A) In vivo splicing of both the 5′ and 3′ splice junction mutants was tested by transforming a *HAC1*-containing centromeric plasmid carrying the wild-type gene or either the 5′ or 3′ splice junction mutation into Δ*hac1* cells. Splicing of the wild-type *HAC1*^{u} mRNA (lane 2), *hac1*^{u} *[G885C]* mRNA (lane 3), or *hac1*^{u} *[G1337C]* mRNA (lane 4) were tested by Northern blot hybridization analysis using a probe specific for *HAC1* mRNA. Each strain was grown to mid-log phase and incubated in the absence (lane 1) or presence (lanes 2–4) of 5 μg/ml of tunicamycin. (We note that the cleavage products of the mutant mRNA are stable enough to detect in this assay, although the 5′ exon + intron fragment is more stable than the cleaved 3′ exon. This is in contrast to the mRNA fragments produced in the *rlg1-100* mutant strain in which cleaved exons and intron did not accumulate to a significant degree [Sidrauski et al., 1996]. We consider it possible that this results reflects a sustained interaction of the partially cleaved mRNA with Ire1p and/or tRNA ligase that prevents its otherwise rapid degradation.)

(B) Wild-type *HAC1*^{u} 600 RNA (lane 1–2) and *HAC1*^{u} 600 RNAs bearing the *hac1*^{u} *[G885C]* or the *hac1*^{u} *[G1337C]* mutations were incubated in buffer alone (odd-numbered lanes) or in the presence of GST-Ire1p(k+t) (even-numbered lanes), and the cleavage products were displayed on a denaturing 5% polyacrylamide gel. The asterisks represent unidentified fragments that do not correspond to expected cleavage products.

of the purified protein fractions, we first used pre-tRNA^{phe} as a substrate in a series of control reactions. As shown in Figure 4A (lane 3), tRNA endonuclease cleaved pre-tRNA^{phe} to generate fragments corresponding to the intron, 5′ exon, and 3′ exon. As expected, when tRNA ligase was also added to this reaction, a new band corresponding to the spliced tRNA^{phe} appeared (Figure 4A, lane 4). Note that GST-Ire1(k+t) did not cleave pre-tRNA^{phe} (Figure 4A, lane 2) and conversely that *HAC1*^{u} 600 RNA was not cleaved by tRNA endonuclease (Figure 4B, lane 4), further confirming the substrate specificity of Ire1p for *HAC1*^{u} RNA.

When *HAC1*^{u} 600 RNA was incubated with GST-Ire1(k+t) and tRNA ligase in the presence of ATP and GTP, a new band appeared that corresponded in size to the two joined exons (Figure 4B, lane 3, arrow). This band was not observed when tRNA ligase was omitted (Figure 4B, lane 2), and tRNA ligase had no effect on *HAC1*^{u} 600 RNA when GST-Ire1(k+t) was omitted (Figure 4B, lane 5). Similar results were obtained using the shorter *HAC1*^{u} 508 RNA; with this substrate, the band corresponding to the ligated exons was of the expected smaller size (Figure 4B, lane 8). Thus, it appears that GST-Ire1(k+t) and tRNA ligase are sufficient to catalyze splicing of *HAC1*^{u} 600 RNA in vitro.

To confirm that the new bands observed in the splicing reaction indeed corresponded to correctly spliced *HAC1* RNA, we reverse-transcribed the products of the reaction with oligonucleotides complementary to the 3′ exon followed by PCR amplification. As shown in Figure 4C, we detected a prominent smaller PCR product when tRNA ligase was added to the cleavage reaction (compare lane 3 with lanes 1 and 2, arrow). We cloned and sequenced PCR products from 5 independent reverse transcription reactions. DNA sequencing revealed that in 3 of the 5 cases, the PCR products contained the correct splice junction. Thus, in the majority of cases, the action of GST-Ire1(k+t) and tRNA ligase was sufficient to produce the accurately spliced product. In 1 of the 5 cases, the PCR product was missing 10 nucleotides from the 5′ exon; in another case, the PCR product was missing 3 nucleotides from the 3′ exon. We consider it likely that these aberrantly spliced products result from a contaminating exonucleolytic activity in the GST-Ire1(k+t) or the tRNA ligase preparation that creates some heterogenetity in the ends of the exons products prior to ligation. Such species were not detected by primer extension (Figure 2) and are therefore likely to be minor and heterogenous species in the population of the cleavage products. Taken together, these experiments demonstrate that we have successfully reconstituted Ire1p/tRNA ligase–mediated splicing of *HAC1*^{u} mRNA in vitro and further support the direct involvement of tRNA ligase in the reaction.

Discussion

We have presented evidence that the cytoplasmic/nuclear portion of the transmembrane protein Ire1p harbors two distinct enzymatic activities: in addition to its previously characterized kinase activity (Mori et al., 1993; Shamu and Walter, 1996; Welihinda and Kaufman,

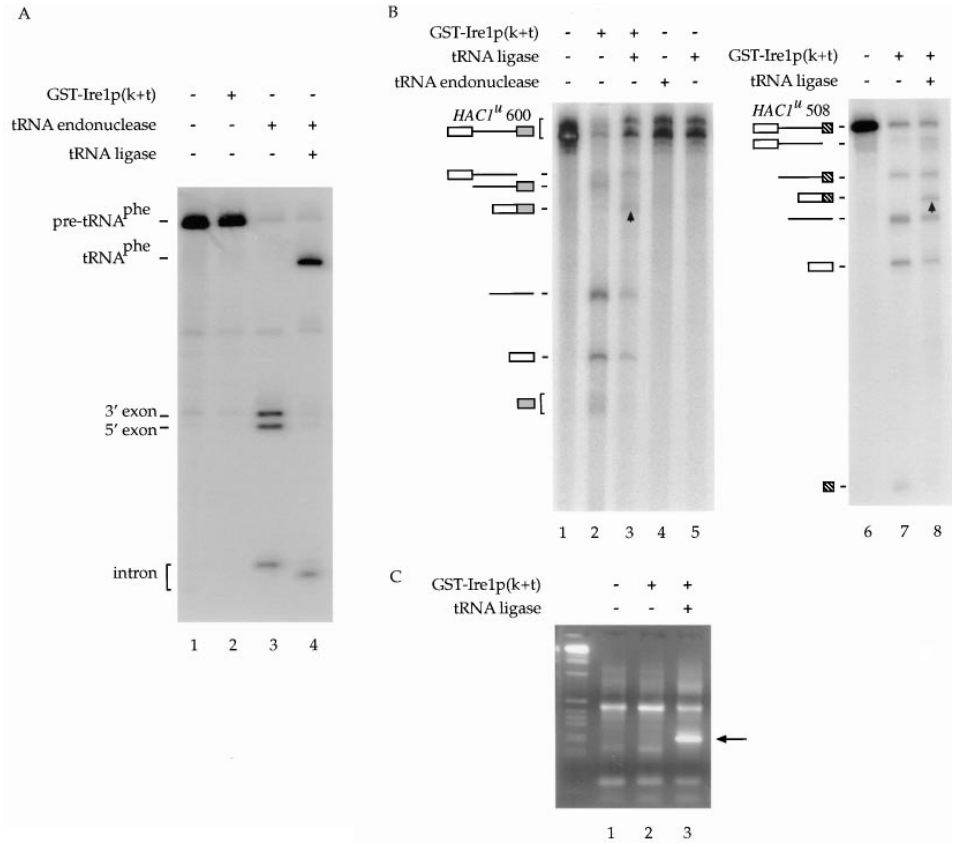

Figure 4. In Vitro Reconstitution of *HAC1*^{u} RNA Splicing

(A) In vitro transcribed pre-tRNA^{phe} was incubated in the presence of GST-Ire1p(k+t) in kinase buffer and ATP (lane 2), tRNA endonuclease alone (lane 3), or tRNA endonuclease and tRNA ligase (lane 4) in tRNA-endonuclease buffer plus ATP and GTP, and the reaction products were fractionated on a denaturing 12% polyacrylamide gel. The mobility difference of the pre-tRNA intron upon incubation with tRNA ligase is likely due to the opening of the 2′–3′ cyclic phosphodiester bond that results in an extra negative charge.

(B) In vitro transcribed *HAC1*^{u} 600 RNA was incubated in kinase buffer in the presence of ATP and GTP with GST-Ire1p(k+t) (lane 2), GST-Ire1p(k+t), and tRNA ligase (lane 3); with tRNA ligase alone (lane 5); or in tRNA endonuclease buffer with tRNA endonuclease (lane 4). In vitro transcribed *HAC1*^{u} 508 RNA containing the shorter 3′ exon was incubated in the presense of GST-Ire1p(k+t) (lane 6) or GST-Ire1p(k+t) and tRNA ligase (lane 7) as described above. (C) Reverse transcription, followed by PCR amplification, was performed on uncleaved (lane 1), cleaved (lane 2), and spliced *HAC1*^{u} 600 RNA (lane 3) using primers complementary to regions of the 5′ and 3′ exons. The PCR products were fractionated on a 1% agarose gel and stained with ethidium bromide.

1996), Ire1p displays endoribonuclease activity. In particular, we have shown that a purified GST-Ire1p fusion containing the cytoplasmic/nuclear half of Ire1p is sufficient to cleave *HAC1*^{u} mRNA, the pre-mRNA encoding the UPR-specific transcription factor Hac1p, with precision at both its 5′ and 3′ splice junctions. Thus, we propose that Ire1p is the endonuclease that initiates the spliceosome-independent *HAC1*^{u} mRNA splicing event that constitutes a key regulatory step in the UPR pathway. Moreover, we have reconstituted the entire splicing reaction of *HAC1* mRNA by adding purified tRNA ligase in addition to GST-Ire1p(k+t) to the in vitro reaction. This result supports the role of tRNA ligase in *HAC1* mRNA splicing that was previously suggested by the genetic identification of the tRNA ligase mutant allele *rlg1-100*, which blocks *HAC1*^{u} mRNA splicing in vivo (Sidrauski et al., 1996). Thus, in contrast to spliceosome-mediated mRNA splicing, which is estimated to involve more than 100 different components, the machinery that carries out regulated *HAC1*^{u} mRNA splicing is surprisingly simple: the sequential action of only two enzymes, the bifunctional kinase/endonuclease Ire1p and tRNA ligase, suffices to carry out the reaction accurately.

We embarked on this study because Ire1p resembled mammalian RNase L. Although RNase L is a soluble, rather nonspecific nuclease, it has a number of features that are intriguingly similar to Ire1p. First, considerable sequence similarity between the C-terminal tail domain of RNase L and that of Ire1p suggests a common function (Figure 1A; Bork and Sander, 1993). Indeed, for RNase L it was demonstrated that the C-terminal tail domain is required for its nuclease activity, although the domain by itself has not been shown to be active (B. Dong and R. H. Silverman, personal communication). Thus, although we consider it likely that the active sites of the nuclease activities of RNase L and Ire1p reside in the C-terminal tail domains of the respective proteins, this remains to be demonstrated directly for either enzyme.

Second, the nuclease activity of RNase L, like that of Ire1p, is thought to be activated by oligomerization (Dong and Silverman, 1995). Dimerization of RNase L

is induced by binding of 2′–5′-linked oligoadenylates (2–5A), small signaling molecules produced in cells that are treated with interferon, to the N-terminal half of RNase L, which is comprised of nine ankyrin repeats (Hassel et al., 1993; Zhou et al., 1993). Thus, as for Ire1p, the N-terminal portion of RNase L is involved in sensing a signal that leads to oligomerization, which in turn causes activation of the enzyme.

Third, both RNases contain a domain with strong sequence similarity to protein kinases. A functional kinase domain is required for Ire1p function in vivo (Mori et al., 1993; Shamu and Walter, 1996), and kinase activity as assessed by autophosphorylation has been demonstrated in vitro (Welihinda and Kaufman, 1996). In contrast, kinase activity has not yet been demonstrated for RNase L. Moreover, although the RNase activity of RNase L does not require ATP, the activity is stimulated by adenosine nucleotides, including ATP, ADP, and the nonhydrolyzable ATP analog AMP-PNP (Krause et al., 1986; Dong et al., 1994). In contrast, we have shown that, unlike RNase L, Ire1p strictly requires adenosine nucleotide as a cofactor to exhibit nuclease activity. Like for RNase L, however, ADP and AMP-PNP can substitute for ATP. Thus, the effects of adenosine nucleotide for the nuclease activity, required or stimulatory for Ire1p and RNase L, respectively, are unlikely to reflect required phosphorylation events catalyzed by the kinase domains of either protein. Instead, the adenosine nucleotide cofactor could aid directly in the chemistry of the hydrolysis reaction, or it might function indirectly, stabilizing a particular conformation after binding to either the ATP binding site of the kinase domain or elsewhere on the molecule.

The functional role of the kinase domain of Ire1p therefore remains to be determined. The fact that the GST-Ire1p(k+t) fusion construct is constitutively active in our assays implies that we have uncoupled the nuclease activity of Ire1p from its normal regulation. One possibility is that the physiological activation of Ire1p is a direct consequence of its oligomerization. Our fusion protein, then, may be constitutively oligomerized. Indeed, this is likely to be the case, as GST by itself forms homodimers (Lim et al., 1994). We have prepared recombinant Ire1p(k+t) in which the GST moiety was removed by selective proteolysis at the fusion joint, however, and have observed that, even in the absence of the GST domain, Ire1p(k+t) exhibits undiminished constitutive *HAC1*^{u} mRNA–specific nuclease activity (not shown). It remains possible that the enzyme concentration used in the in vitro assays is sufficiently high to drive self-association. Alternatively, it is possible that even at physiological concentrations Ire1p(k+t) monomers have an intrinsic tendency to dimerize (and require adenosine nucleotide binding to do so) and are normally kept apart (and hence inactive) by a yet unidentified inhibitor when the UPR is not induced. Ire1p(k+t) would then be constitutively active, because this inhibitor is absent in the in vitro reconstituted system. The kinase activity of Ire1p may function in vivo to remove the putative inhibitor through phosphorylation.

Regardless of the precise mechanism of Ire1p activation, the data presented here provide invaluable clues to the mechanism of the *HAC1*^{u} mRNA splicing reaction. In particular, through reconstitution of the splicing reaction, we have unambiguously shown that *HAC1*^{u} mRNA splicing does not occur via a spliceosome-catalyzed reaction. Spliceosome-mediated mRNA processing occurs by two sequential transesterification reactions. First, the 2′ OH group of the branch point residue attacks the 5′ splice junction to form the lariat and free 5′ exon. Second, the newly generated free 3′ OH group of the 5′ exon attacks the 3′ splice junction to form the ligated mRNA and the free intron lariat. The two cleavage reactions of a conventionally processed pre-mRNA must therefore occur in a strict sequence; cleavage of the 3′ splice site cannot occur without prior cleavage at the 5′ splice site (reviewed by Moore et al., 1993). In contrast, we observed that a point mutation at the 5′ splice site blocks cleavage at that site completely while still allowing cleavage at the 3′ splice site, both in vivo and in vitro. This result is incompatible with the chemistry of a spliceosome-catalyzed reaction.

HAC1^{u} mRNA splicing resembles much more the splicing of pre-tRNA, which is catalyzed by the sequential action of two enzymes, a tRNA endonuclease that can cleave both splice junctions in either order and a tRNA ligase (Greer et al., 1983; Peebles et al., 1983; Reyes and Abelson, 1988; O'Connor and Peebles, 1991). *HAC1*^{u} mRNA splicing and pre-tRNA splicing use the same ligase for the second step of the reaction. In contrast, the nucleases are very different: tRNA endonuclease is a constitutively active nuclease composed of four subunits, all of which are essential (Trotta et al., 1997), whereas Ire1p is composed of only one known, nonessential subunit and its nuclease activity is tightly regulated from the ER lumen. In pre-tRNA, the folded tertiary structure of the mature tRNA portion forms the structure that is recognized by tRNA endonuclease (Greer et al., 1987; Mattoccia et al., 1988; Reyes and Abelson, 1988). The enzyme then cleaves the anticodon stem at a defined distance from that structure and pays little attention to the nucleotide sequence at the junctions or within the intron. In contrast, nucleotide sequence at the intron–exon junctions of *HAC1*^{u} mRNA matters profoundly, as single G→C mutations at either junction abolish cleavage. Moreover, secondary structure predictions reveal strikingly similar stem–loop structures at both splice junctions of *HAC1* mRNA (Figure 5). In both cases, the cleavage site precedes the essential G residue, which is predicted to be located at position 3 of a seven-membered loop. The structural similarity of the two splice junctions is very appealing because our model postulates that both sites are cleaved by the same endonuclease. The predicted structural symmetry may reflect binding of Ire1p as a dimer to *HAC1*^{u} mRNA, assuming that each monomer contains an active site and that each monomer active site recognizes one stem–loop. Precedence for such a mechanism is found in Archaea: archaeal pre-tRNAs splice junctions display internal 2-fold symmetry, and the tRNA endonuclease from H. volcanni is a homodimer (Kleman-Leyer et al., 1997).

Finally, our data suggest that Ire1p, or at least the portion of the molecules that catalyze *HAC1*^{u} mRNA splicing, is localized to the inner nuclear membrane. This localization places the kinase and C-terminal tail domain in the nuclear compartment where it collaborates with the nuclear-localized tRNA ligase (Clark and

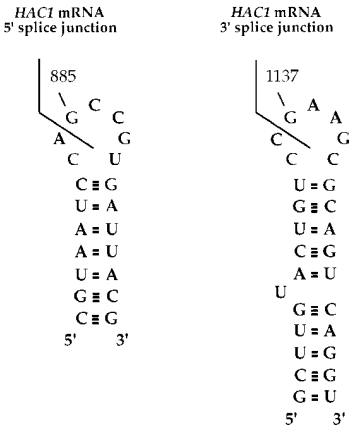

Figure 5. Secondary Structure Prediction of Both the 5′ and 3′ *HAC1* mRNA Splice Junctions

Similar stem–loop structures are predicted for the 5′ and 3′ splice junctions of *HAC1*^{u} mRNA. Ire1p cleaves before the third conserved G residue of the seven-membered loop at both junctions.

Abelson, 1987) that completes *HAC1* mRNA processing before the spliced mRNA is exported to the cytosol. Indeed, we have shown using pulse–chase experiments that cytosolic *HAC1*^{u} mRNA engaged on translating polyribosomes (Cox and Walter, 1996) is not converted to *HAC1*^{i} mRNA upon induction of the UPR (R. Chapman and P. W., unpublished data). We therefore suggest that only newly synthesized *HAC1*^{u} mRNA can become processed by Ire1p while it is still in the nucleus. Ire1p, therefore, may transmit the signal from the ER lumen directly across the inner nuclear membrane. Another possibility is that after activation in the peripheral ER membrane, activated Ire1p may become relocalized to the inner nuclear membrane, presumably by diffusing in the plane of the membrane through the nuclear pores. By analogy with tRNA processing, we suggest that Ire1p may associate with tRNA ligase and hand over the *HAC1*^{u} mRNA cleavage products to the ligase in a product-substrate channeling reaction (Greer, 1986). In the *rlg1-100* mutation, this link may be disrupted, thereby explaining the pathway-specific phenotype of this mutation that blocks *HAC1*^{u} mRNA ligation completely while having no effect on pre-tRNA processing (Sidrauski et al., 1996). With a reconstituted splicing reaction in hand, these mechanistic conjectures can now be tested directly.

Experimental Procedures

Constructs and Expression of GST-Ire1p(k+t)

The GST-Ire1p(kinase + tail) (Ire1-C-terminal domain, amino acids 556–1115) construct (pCS116) was made by PCR as described in Shamu and Walter (1996), and the PCR fragment was cloned into pGEX-2T (Pharmacia, Uppsala, Sweden). pCS116 was transformed into the DH5-α strain of E. coli. Cells were induced for 4 hr at 37°C with IPTG, and the fusion protein was purified using glutathione-Sepharose beads from Pharmacia (Uppsala, Sweden).

To make the *HAC1*^{u} 600 RNA in vitro transcription vector, a 600 bp *HAC1* fragment containing 181 nucleotides of the 5′ exon, the 252 nucleotide intron, and 167 nucleotide of the 3′ exon was amplified using Vent Polymerase (NEB, Beverly, MA) and then cloned into pBluescript IISK(−) (Stratagene, La Jolla, CA) to generate pCF150. The *HAC1*^{u} 508 RNA in vitro transcription vector (pCF187) was also constructed by PCR and contains 181 nucleotides of the 5′ exon, the 252 nucleotide intron, and 75 nucleotides of the 3′ exon. The 5′ and 3′ splice junction mutants (G→C) were generated using the Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutations were then subcloned into a yeast expression vector (pJC327) containing *HAC1* (pJC327 is a *LEU2* CEN/ARS derivative of pJC316 described in Cox and Walter, 1996). The *HAC1*^{i} 348 RNA in vitro transcription vector (pCF198) contains the same 5′ and 3′ exonic regions as pCF150 but lacks the 252 nucleotide intron. The pT7-driven actin construct contains a 543 nucleotide actin pre-mRNA fragment (nucleotides 610–1153) (gift from A. Zaug, University of Colorado, Boulder, CO).

RNA Analysis

Primer Extension

Primer extensions were performed as described in Stern et al. (1986). In brief, 10 ng of uncleaved or GST-Ire1p(k+t)-cleaved *HAC1*^{u} 600 RNA were hybridized to 1 picomol of end-labeled oligonucleotide. To map the 5′ splice junction, an oligonucleotide (nt 922–943) complementary to the intron was used. The 5′ end of the oligonucleotide was precisely 59 nucleotides downstream of the predicted 5′ splice junction. To map the 3′ splice junction, an oligonucleotide (nt 1195–1225) complementary to the 3′ exon was used. The 5′ end of the oligonucleotide was precisely 88 nucleotides downstream of the 3′ splice junction. The primers were then extended for 30 min with AMV reverse transcriptase at 40°C. The products were then precipitated and analyzed on a denaturing 8% polyacrylamide gel.

Northern Analysis

Northern blots were performed as described by Sidrauski et al. (1996).

In Vitro Transcription and Cleavage of HAC1 mRNA

In vitro transcription of *HAC1* mRNA and its mutant derivatives was carried out at 37°C for 1 hr using T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN) in 50 μl reactions containing 1 mM each of ATP, GTP, and CTP, 100 μM of UTP, 50 μCi of α^{32}P-UTP (10 mCi/ml) (Amersham Corporation, Arlington Heights, IL), and 1 μg of linearized plasmid. In vitro transcripts were purified by gel electrophoresis. The transcripts were eluted from the gel slice in 50 mM Tris–HCl (pH = 8), 1 mM EDTA, 0.3 M NaOAc/phenol/chloroform (1:1:1) overnight at 4°C and then extracted and ethanol-precipitated. The in vitro cleavage reactions were carried out at 30°C in kinase buffer (20 mM HEPES, 1 mM DTT, 10 mM $Mg(OAc)_2$, 50 mM KOAc) in the presence of 2 mM ATP and contained 2 ng (25,000 cpm) of purified labeled in vitro transcript and 0.5 μg of the GST-Ire1p(k+t) fusion protein. Samples were then extracted once with phenol-chlorophorm, ethanol-precipitated, and analyzed on denaturing 5% polyacrylamide gels.

In Vitro Splicing of HAC1 mRNA

HAC1 mRNA splicing reactions were carried out at 30°C in kinase buffer (see above) in the presence of 2 mM each of ATP and GTP. Samples were extracted once with phenol-chlorophorm, ethanol-precipitated, and analyzed on a denaturing 5% polyacrylamide gel. tRNA splicing reactions were carried out at 30°C in endonuclease buffer (Greer et al., 1987) with the addition of 2 mM each of ATP and GTP. The products of the reaction were extracted as described above and displayed on a denaturing 12% polyacrylamide gel.

Reverse Transcriptase–PCR

HAC1 mRNA was incubated in the presence of GST-Ire1p(k+t) or GST-Ire1p(k+t) and tRNA ligase as described above. Samples were extracted once with phenol-chloroform and ethanol-precipitated. First-strand cDNA synthesis was performed using oligonucleotides complementary to the 3′ exon and extending for 1 hr using AMV reverse transcriptase at 42°C. The cDNA templates were subjected to PCR amplification using oligonucleotides complementary to the 5′ and 3′ exons. The PCR products were cloned using the TA cloning kit (Invitrogen, San Diego, CA), and five independent clones were sequenced.

Acknowledgments

We thank Caroline Shamu for the GST-Ire1p(k+t) construct; Andy Libson for GST-cleaved Ire1p(k+t) protein; Chris Trotta and John

Abelson (Caltech) for purified tRNA endonuclease, tRNA ligase, and pre-tRNA^{phe} transcript; and Art Zaug (University of Colorado, Boulder) for the T7-driven actin pre-mRNA construct. We also thank John Abelson and Christine Guthrie for their valuable suggestions, and Ira Herskowitz, Regis Kelly, and members of the Walter lab for their critical comments on this manuscript. Our special thanks go to Maho Niwa and Ted Powers for their help and valuable suggestions and to Jeff Cox, Rowan Chapman, and Chris Patil for their support throughout these studies. This work was supported by a generous gift from the California Foundation for Molecular Biology and by grants from the American Cancer Society and the National Institutes of Health.

Received June 17, 1997; revised July 22, 1997.

References

Bork, P., and Sander, C. (1993). A hybrid protein kinase-RNase in an interferon-induced pathway? FEBS Lett. *334*, 149–152.

Clark, M.W., and Abelson, J. (1987). The subnuclear localization of tRNA ligase in yeast. J. Cell Biol. *105*, 1515–1526.

Cox, J.S., Shamu, C.E., and Walter, P. (1993). Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell *73*, 1197–1206.

Cox, J.S., and Walter, P. (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell *87*, 391–404.

Dong, B., and Silverman, R.H. (1995). 2–5A-dependent RNase molecules dimerize during activation by 2–5A*. J. Biol. Chem. *270*, 4133–4137.

Dong, B., Xu, L., Zhou, A., Hassel, B.A., Lee, X., Torrence, P.F., and Silverman, R.H. (1994). Intrinsic molecular activities of the interferon-induced 2–5A-dependent RNase. J. Biol. Chem. *269*, 14153–14158.

Greer, C.L. (1986). Assembly of a tRNA splicing complex: evidence for concerted excision and joining steps in splicing *in vitro*. Mol. Cell. Biol. *6*, 635–644.

Greer, C.L., Peebles, C.L., Gegenheimer, P., and Abelson, J. (1983). Mechanism of action of a yeast RNA ligase in tRNA splicing. Cell *32*, 537–546.

Greer, C.L., Soll, D., and Willis, I. (1987). Substrate recognition and identification of splice sites by the tRNA-splicing endonuclease and ligase from *Saccharomyces cerevisiae*. Mol. Cell. Biol. *7*, 76–84.

Hassel, B.A., Zhou, A., Sotomayor, C., Maran, A., and Silverman, R.H. (1993). A dominant negative mutant of 2–5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon. EMBO J. *12*, 3297–3304.

Kleman-Leyer, K., Ambruster, D.W., and Daniels, C.J. (1997). Properties of H. volcanii tRNA intron endonuclease reveal a relationship between the archaeal and eucaryal intron processing systems. Cell *89*, 839–847.

Kohno, K., Normington, K., Sambrook, J., Gething, M.J., and Mori, K. (1993). The promoter region of the yeast *KAR2* (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. Mol. Cell. Biol. *13*, 877–890.

Krause, D., Lesiak, K., Imiai, J., Sawai, H., Torrence, P.F., and Silverman, R.H. (1986). Activation of 2–5A-dependent RNase by analogs of 2–5A. J. Biol. Chem. *261*, 6836–6839.

Lim, D., Ho, J.X., Keeling, K., Gilliland, G.L., Ji, X., Ruker, F., and Carter, D.C. (1994). Three-dimensional structure of Schistosoma japonicum glutathione-S-transferase fused with a six-amino acid conserved neutralizing epitope of gp41 from HIV. Prot. Sci. *3*, 2233–2244.

Mattoccia, E., Baldi, I.M., Gandini-Attardi, D., Ciafre, S., and Tocchini-Valentini, G.P. (1988). Site-selection by the tRNA splicing endonuclease of Xenopus laevis. Cell *55*, 731–738.

McMillan, D.R., Gething, M.-J., and Sambrook, J. (1994). The cellular response to unfolded proteins: intercompartmental signaling. Curr. Opin. Biotechnol. *5*, 540–545.

Moore, M.J., Query, C.C., and Sharp, P.A. (1993). Splicing of precursors to mRNAs by the spliceosome. In the RNA World. R.F. Gesteland and J.F. Atkins, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 303–357.

Mori, K., Kawahara, T., Yoshida, H., Yanagi, H., and Yura, T. (1996). Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. Genes to Cells *1*, 803–817.

Mori, K., Ma, W., Gething, M.-J., and Sambrook, J. (1993). A transmembrane protein with a cdc2^{+}/CDC28-related kinase activity is required for signaling from the ER to the nucleus. Cell *74*, 743–756.

Mori, K., Sant, A., Kohno, K., Normington, K., Gething, M.J., and Sambrook, J.F. (1992). A 22 bp *cis*-acting element is necessary and sufficient for the induction of the yeast *KAR2* (BiP) gene by unfolded proteins. EMBO J. *11*, 2583–2593.

Nikawa, J., Akiyoshi, M., Hirata, S., and Fukuda, T. (1996). *Saccharomyces cerevisiae* IRE2/HAC1 is involved in IRE1-mediated KAR2 expression. Nucleic Acids Res. *24*, 4222–4226.

O'Connor, J.P., and Peebles, C.L. (1991). In vivo pre-tRNA processing in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *11*, 425–439.

Peebles, C.L., Gegenheimer, P., and Abelson, J. (1983). Precise excision of intervening sequences from precursor transfer RNA by a membrane associated endonuclease. Cell *32*, 525–536.

Reyes, V.M., and Abelson, J. (1988). Substrate recognition and splice site determination in yeast tRNA splicing. Cell *55*, 710–730.

Schwer, B., and Guthrie, C. (1991). PRP16 is an RNA-dependent ATPase that interacts transiently with the spliceosome. Nature *349*, 494–499.

Shamu, C.E., Cox, J.S., and Walter, P. (1994). The unfolded-protein-response pathway in yeast. Trends Cell Biol. *4*, 56–60.

Shamu, C.E., and Walter, P. (1996). Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. EMBO J. *15*, 3028–3039.

Sidrauski, C., Cox, J.S., and Walter, P. (1996). tRNA ligase is required for regulated mRNA splicing in the unfolded protein response. Cell *87*, 405–413.

Stern, S., Moazed, D., and Noller, H.F. (1986). Structural analysis of RNA using chemical and enzymatic probing monitored by primer extension. Meth. Enzymol. *164*, 481–489.

Sweet, D.J. (1993). A kinase that responds to stress. Curr. Biol. *3*, 622–624.

Trotta, C.R., Miao, F., Arn, E.A., Stevens, S.W., Ho, C.K., Rauhut, R., and Abelson, J.N. (1997). The yeast tRNA splicing endonuclease: a tetrameric enzyme with two active site subunits homologous to the archaeal tRNA endonucleases. Cell *89*, 849–858.

Welihinda, A.A., and Kaufman, R.J. (1996). The unfolded protein response pathway in *Saccharomyces cerevisiae*. Oligomerization and trans-autophosphorylation of Ire1p (Ern1p) are required for kinase activation. J. Biol. Chem. *271*, 18181–18187.

Zhou, A., Hassel, B.A., and Silverman, R.H. (1993). Expression cloning of 2–5A-dependent RNAse: a uniquely regulated mediator of interferon action. Cell *72*, 753–765.

Note Added in Proof

The observations of B. Dong and R.H. Silverman that are cited in this paper as a ''personal communication'' are now in press: Dong, B., and Silverman, R.H. (1997). A bipartite model of 2–5A-dependent RNase L. J. Biol. Chem., in press.