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Intracellular signaling from the endoplasmic reticulum to the nucleus

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Intracellular signaling from the endoplasmic reticulum to the nucleus

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Carmela Sidrauski

DISSERTATION

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in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

Date

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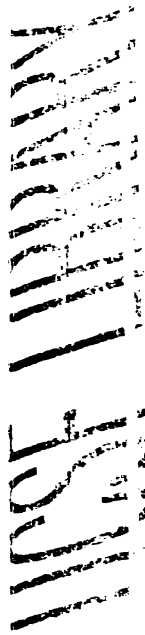

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Sidrauski, C., and Walter P. (1997). The Transmembrane Kinase Ire1p Is a Site-Specific Endonuclease That Initiates mRNA Splicing in the Unfolded Protein Response. Cell, Vol. 90, 1031-1039.

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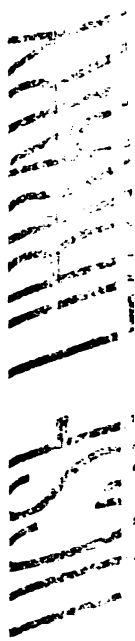
ABSTRACT

When misfolded proteins accumulate in the endoplasmic reticulum (ER), an intracellular signaling pathway, the unfolded protein response (UPR), is induced. Its activation results in the coordinate transcriptional up-regulation of ER-resident protein chaperones that expedite protein folding in this compartment. The UPR exists in all eukaryotic cells, but it is in the yeast *Saccharomyces cerevisiae* that the components involved in this pathway have been first identified. Ire1p, a serine-threonine transmembrane kinase, and Hac1p, a DNA-binding protein, are two components required for the UPR. Ire1p, which lies in the ER or inner nuclear membrane, senses the accumulation of unfolded proteins in the ER lumen and transduces the unfolded protein signal across the membrane of the organelle. Hac1p binds to the unfolded protein response element (UPRE) found in the promoters of ER-resident chaperones, which leads to up-regulation of their transcription. Hac1p is only detected in UPR-activated cells, and its appearance results from regulated splicing of its mRNA. Removal of the intron from *HAC1* mRNA is a prerequisite for its translation. This thesis describes the result of a genetic screen in *S. cerevisiae* that identified a third component required for the UPR: *RLG1*, which encodes tRNA ligase, an essential protein required for pre-tRNA splicing. A point mutation in *RLG1* abolishes activation of the pathway. In the mutant strain, *HAC1* mRNA is cleaved, but no spliced product is detected. Rather, the cleavage products are degraded. These observations suggested a new role for tRNA ligase; that is ligation of *HAC1* mRNA halves. The lack of spliceosomal consensus sequences in the splice junctions of *HAC1* mRNA and the lack of effect of mutations that block spliceosomal function on processing of this mRNA further demonstrated

that the splicing reaction occurs by an unprecedented mechanism. We have identified Ire1p as the endoribonuclease responsible for cleavage of *HAC1* mRNA. Furthermore, we were able to reconstitute the splicing reaction *in vitro* by addition of only two proteins: the bifunctional kinase/endonuclease Ire1p and tRNA ligase. Thus, characterization of the UPR has revealed unique regulatory mechanisms of signaling, including translational attenuation and a regulated mRNA processing event catalyzed by a novel splicing machinery.



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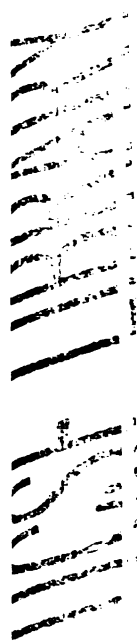

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Chapter I

Introduction

One of the hallmarks of all eukaryotic cells is that they contain membrane-bound compartments or organelles that carry out highly specialized functions. The composition and the amount of a particular organelle is not static but changes according to the needs of the cell. For example, when B cells differentiate into plasma cells, the ER proliferates significantly to accommodate the increase in the secretory flux. Thus, the nucleus not only receives information about the extracellular environment through signaling pathways originating in the plasma membrane of the cell, but it is also receives information about the status of the different organelles and modifies their activities accordingly. Intracellular signaling pathways exist that link the different organelles with the nucleus (Nunnari and Walter, 1996). These pathways usually involve transcriptional networks that regulate the composition or amount of a particular compartment in response to a cellular need for a specific function. An example of such intracellular signaling pathway is the unfolded protein response (UPR), which links the ER lumen with the nucleus. The ER is the site of entry of the secretory pathway and is the site of folding and assembly of proteins. Folding is assisted by a set of ER-resident chaperones and enzymes that prevent aggregation of folding intermediates and may also help target dead-end products to degradative pathways. In all eukaryotic cells, when misfolded proteins accumulate in the ER lumen, the transcription of ER-resident protein genes is induced in the nucleus (Lee, 1987; Kozutsumi et al., 1988. Thus, the synthesis of these ER chaperones is regulated according to demand for them inside the organelle. The unfolded protein response pathway allows transduction of the signal from the ER lumen, where unfolded proteins accumulate, to the cell nucleus, where transcription of ER-resident protein genes is activated.

In animal cells, the UPR pathway controls transcription of a set of

genes encoding ER-resident proteins that catalyze folding, assembly, and modification of proteins in the ER lumen (Table I-1) (McMillan et al., 1994; Shamu et al., 1994). These proteins were first identified as polypeptides that are highly induced upon glucose starvation (which impairs protein glycosylations and hence causes protein misfolding in the ER) and, accordingly, were called glucose-regulated proteins, or GRPs. BiP/GRP78, a member of the HSP70 family of molecular chaperones, and GRP94, a member of the HSP90 family of molecular chaperones, are thought to bind transiently to nascent proteins as they are translocated into the ER lumen, assisting in their folding and oligomeric assembly (Gething and Sambrook, 1992). They are the major molecular chaperones implicated in folding in the ER lumen. In addition, GRP 170 (a newly identified hsp70-like glycoprotein), protein disulfide isomerase (PDI) (which promotes correct disulfide-bond formation), and two PDI-related ER proteins, Erp72 and GRP58, are also up-regulated (Chen et al., 1996; Dorner et al., 1990; Mazarella et al., 1990). Lee and coworkers identified a 28 bp promoter element shared by GRP78 and GRP94, termed the GRP core, that is involved in up-regulation of these genes upon activation of the UPR (Chang et al., 1989). Thus, it has been proposed that these coordinately regulated genes are activated after binding of a common transcription factor. The identity of this UPR factor has remained elusive.

The UPR is induced by a variety of cellular insults that perturb ER function. Some of these treatments-- such as inhibition of disulfide bonding with reducing agents, inhibition of ER glycosylation with drugs such as tunicamycin, or expression of aberrant proteins that do not fold properly-- directly cause an accumulation of unfolded proteins in the ER (Shamu et al., 1994). In addition to these treatments, a variety of stresses that affect ER function but do directly increase the concentration of misfolded proteins are

inducers of the UPR. Blocking ER-to-Golgi transport, depleting the ER of Ca^{2+}, inhibiting protein degradation and overproduction of normal secretory or membrane proteins, all activate the UPR (Drummond et al., 1987; Li et al., 1993; Liu et al., 1992). It is not clear how each of these treatments results in induction of the pathway, but it is likely that by affecting the function of the organelle they indirectly lead to accumulation of unfolded polypeptides.

Although the UPR was originally identified in mammalian cells, it is in the yeast *S. cerevisiae* that the components involved in signaling have been identified. A similar set of genes is up-regulated by the response in this organism. The ER-resident proteins that are induced include BiP (encoded by *KAR2*) (Normington et al., 1989), Lhs1 (a BiP-like protein) (Craven et al., 1996), yeast PDI (LaMantia et al., 1991), Fkbp2 (a peptidyl-prolyl cis-trans isomerase) (Partadelis and Berlin, 1993), Eug1p (a PDI-like protein) (Tachibana and Stevens, 1992), and Ero1p (required for maintenance of the ER redox potential) (Frand and Kaiser, 1998; Pollard et al., 1998) (Table I-1). It is likely that additional targets of the response remain to be identified. As for the mammalian system, these genes share a common regulatory element in their promoters, the unfolded protein-response element (UPRE) (Kohno et al., 1993; Mori et al., 1992). In yeast, this single 22 bp element has been shown to be necessary and sufficient to activate transcription in response to agents that cause accumulation of unfolded proteins in the ER.

Similar to mammalian cells, the UPR can be activated in *S. cerevisiae* by addition of glycosylation inhibitors, reducing agents, and overexpression of mutant secretory proteins. In addition to these treatment, temperature-sensitive mutations in the *SEC11*, *SEC18* and *SEC53* genes cause induction of the UPR. *sec11* mutant cells lack signal peptidase activity, and when grown at the non-permissive temperature, accumulate secretory proteins that do not

fold properly due to the presence of the uncleaved signal sequence (Bohni et al., 1988). *SEC18* encodes the yeast homologue of NSF, a protein required for vesicle fusion. At restrictive temperature, transport from the ER to Golgi is block in *sec18* mutant cells. *sec53* mutant cells lack phosphomannomutase and thus are defective in protein glycosylation.

Genetic screen for yeast mutants that are unable to carry out the unfolded protein response identified a gene, *IRE1*, required for the response (Cox et al., 1993; Mori et al., 1993). *IRE1* encodes a transmembrane protein similar in structure to growth-factor receptor kinases. Yeast cells lacking *IRE1* gene function are viable under normal growth conditions but are supersensitive to treatments that induce accumulation of unfolded protein in the ER-lumen. Ire1p resides in the ER or inner nuclear membrane (with which the ER membrane is continuous) with its N-terminus inside the lumen of the ER and its C-terminal half, which contains the kinase domain, in the nucleus or cytoplasm (Figure I-1). In this orientation, the N-terminal half of Ire1p would serve as a sensor domain that detects the accumulation of unfolded proteins in the ER lumen and its kinase domain in the cytoplasm or the nucleus, where it would presumably activate downstream events in the pathway. The structural features of Ire1p make this protein perfectly suited to transduce the unfolded protein signal from the ER lumen across the membrane of the organelle. Formally, there are two possible routes of transmission of the unfolded protein signal. One possibility is that the ER-membrane localized Ire1p activates downstream events in the cytoplasm, and subsequently the signal travels into the nucleus through the nuclear pores. Alternatively, the unfolded protein signal may take a more direct route across the inner nuclear membrane with Ire1p directly activating downstream events in the nucleus (Figure I-2).

The analysis of mutations in Ire1p demonstrated that its kinase activity is required for the unfolded protein response (Mori et al., 1993). Furthermore, molecular genetic and biochemical data suggest that activation of Ire1p occurs by a mechanism similar to that found in higher eukaryotic plasma membrane receptors (Shamu and Walter, 1996). Upon induction of the pathway, Ire1p oligomerizes and is transautophosphorylated by neighboring Ire1p molecules. In addition to the kinase domain, the C-terminal domain of Ire1p contains a C-terminal extension or tail of 133 amino acids (Figure I-1). Mutant Ire1p lacking this tail has an active kinase domain but cannot induce downstream events in the pathway. Based on this observation, Shamu and Walter proposed that the C-terminal tail domain is required to bind other proteins that transmit the unfolded protein signal to the nucleus.

How dimerization and thus activation of the receptor kinase Ire1p is induced by accumulation of unfolded proteins in the ER lumen is not known. Although the ligand for the N-terminal sensor domain of Ire1p has not been identified, at least two possible models can explain how dimerization may be regulated by the presence of unfolded proteins. In the first model, Ire1p exists primarily as a monomer in an inactive form. The accumulation of unfolded proteins in the ER produces a ligand that, when bound to the N-terminal domain of Ire1p, causes dimerization and activation of the receptor. The ligand in this case may be unfolded protein themselves, a complex of unfolded proteins bound to ER-chaperones such as BiP, or a yet to be identified ligand generated by the increased activity of chaperones in the ER compartment. In this model, activation of Ire1p would resemble that of mammalian growth factor receptor kinases. An alternative possibility (a negative regulatory model) is that a chaperone such as BiP binds to Ire1p when there are few unfolded proteins in the ER, maintaining it in an inactive

monomeric state. When the concentration of unfolded proteins increases, BiP is titrated off the receptor because of its greater affinity or because the concentration of unfolded proteins is much higher than that of Ire1p, thus allowing oligomerization of the receptor (Figure I-3). Such a mechanism has been proposed for the regulation of HSF1, heat-shock factor 1, by cytosolic hsp70 in animal cells (Morimoto et al., 1994). Under non stressful conditions, HSF1 is maintained in an inactive monomeric state through transient interactions with hsp70. When the accumulation of misfolded proteins increases in the cytosol upon heat shock, hsp70 would bind to the increased pool of substrates, permitting HSF1 oligomerization into the active trimeric form. Whether similar mechanisms of activation exist in these two stress responses, in which a chaperone such as hsp70 and its ER lumen hsp70 counterpart BiP play a major regulatory role in signaling, remains to be determined.

Interestingly, *IRE1* (high insitol-requiring mutant) had been previously identified by Nikawa and Yamashita (1992) as a gene required for inositol prototrophy. In yeast, inositol-containing lipids and phospholipids are major components of membranes. The ER is the major site of cellular lipid synthesis and membrane production. The observation that *ire1* mutants are inositol auxotrophs suggested the possibility that the UPR and membrane biogenesis may be linked. Coordinating these two processes would allow the ER to grow to accommodate unfolded proteins accumulated in the organelle and newly synthesized ER lumenal resident proteins. Recent work has shown that the inositol response is coordinately regulated with the UPR (Cox et al., 1997). When unfolded proteins accumulate, chaperones are induced, and the membranes to house the extra contents are also increased. Thus, the UPR appears to be intimately involved in general aspects of ER biosynthesis.

At the time the work described in this thesis was started, *IRE1* was the only known component involved in UPR signaling. How the unfolded protein signal was sensed within the ER lumen (or the identity of upstream components of Ire1p in the pathway) and how the signal was transmitted to the transcriptional machinery (or the identity of the downstream components of Ire1p in the pathway) were not known. Chapter II describes a genetic screen designed to isolate additional mutants in the pathway. As a result of this screen, tRNA ligase was identified as a component required for signaling. Although the identification of an RNA processing enzyme known to be involved in splicing of pre-tRNAs was very surprising, the contemporaneous discovery of another component, Hac1p, by Cox and Walter provided a hint as to the possible function of this enzyme in the pathway.

Hac1p was identified in a high copy suppressor screen designed to find components that function downstream of the Ire1p transmembrane kinase Cox and Walter, 1996). Hac1p is a DNA-binding protein with homology to the leucine zipper family of transcription factors. Gel-shift experiment showed that Hac1p specifically binds to the UPRE sequence shared by target genes of the pathway. This analysis identifies Hac1p as the most distal regulator of the activity of the UPR pathway in yeast. Hac1p is detected only in UPR-induced cells. A very surprising result was that Hac1p expression is controlled by regulated splicing of its mRNA. Upon induction of the pathway, a 252 nucleotide intron is removed that is present near the 3′ end of the open reading frame (ORF) of *HAC1*^{u} mRNA (**u** for **u**ninduced). Splicing results in the production of *HAC1*^{i} mRNA (**i** for **i**nduced) and the subsequent production of Hac1p^{i} (the protein encoded by the spliced mRNA) (Figure I-4). A further unexpected result was the absence of consensus sequences in the splice junctions of *HAC1* mRNA that are common to all pre-mRNAs

processed by the spliceosome. This observation suggested that splicing of this mRNA utilizes an unconventional splicing pathway. The identification of tRNA ligase as a component of the UPR revealed one of the components involved in processing of this mRNA.

Constitutive expression of *HAC1*^{i} mRNA results in unregulated expression of Hac1p^{i} and increased transcription of all known targets of the pathway. This result demonstrates that splicing is sufficient to trigger full induction of the UPR. Processing of *HAC1*^{u} mRNA is dependent on the presence of the kinase receptor Ire1p. In *ire1* mutant cells, no splicing of *HAC1*^{u} mRNA is observed, presumably because the unfolded protein signal cannot be transduced across the membrane of the organelle. Thus, epistasis analysis positioned *IRE1* upstream of *HAC1* in the pathway.

Initial experiments suggested that the absence of Hac1p^{u} in uninduced cells is due to its rapid degradation. Excision of the intron in *HAC1*^{u} mRNA results in the replacement of the 10 amino acid long C-terminal tail present in Hac1p^{u} (which is encoded by the first part of the intron) with a slightly longer 18 amino acid long tail (encoded in the second exon), generating Hac1p^{i} (Figure I-4). It was proposed that the presence of the 10 amino acid C-termini in Hac1p^{u} conferred instability to the protein. More recently, however, Chapman and Walter (1997) have shown that Hac1p^{u} and Hac1p^{i} have the same half-life. Thus, the absence of Hac1p^{u} in uninduced cells is not a result of its instability but rather that it is never produced. *HAC1*^{u} mRNA has been localized to the cytoplasm, escaping controls that retain other unspliced pre-mRNAs in the nucleus, and associates with polyribosomes. Despite its association with the traslational machinery no protein is produced. These data strongly suggest that a translational mechanism prevents expression of the uninduced form of the protein. This block is conferred by the *HAC1*^{u}

mRNA intron. If the intron is removed, Hac1p^{u} is produced. Moreover, the intron is sufficient to confer a block in translation of an unrelated message. The details of the mechanism by which the intron of *HAC1^{u}* mRNA prevents translation are not known.

The UPR has surprised us with various novel and exciting mechanisms of regulation of gene expression. First, activation of the pathway results in regulated splicing of *HAC1^{u}* mRNA. This is the first example of a splicing event as a key regulatory step in a signaling pathway. Second, splicing results in the removal of a translational attenuation signal present in the intron of *HAC1^{u}* mRNA. This mechanism ensures that only the spliced form of the transcript is translated to produce Hac1p. Third, as described in Chapter II of this thesis, the regulated splicing of *HAC1* mRNA occurs by a non-conventional splicing mechanism that involves tRNA ligase. Processing of this mRNA bypasses the need for a functional spliceosome, which is required for splicing of all other pre-mRNAs. Instead, it requires the action of tRNA ligase for joining the *HAC1* mRNA halves to produce the spliced product. This is a novel role for tRNA ligase in splicing of a pre-mRNA. One important component that remained to be identified was the endonuclease responsible for cleavage of *HAC1* mRNA. In Chapter III, a novel function for Ire1p is described: this enzyme contains in addition to its previously characterized kinase activity, an endoribonuclease activity that is responsible for initiating splicing of *HAC1* mRNA. Furthermore, reconstitution of the complete splicing reaction *in vitro* was achieved by addition of only two proteins: Ire1p and tRNA ligase. This is in contrast to splicing of other pre-mRNAs, which is estimated to involve more than 100 different components. Chapter IV describes the initial characterization of this unique splicing machinery. Ire1p belongs to a novel class of kinase/nuclease

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Table I-1. Targets of the UPR

Mammalian	Yeast
BiP (GRP78)	KAR2
PDI (Erp59)	PDI
GRP170	
GRP94	
ERP72	
GRP58	
	FKBP12
	LHS1
	EUG1
	ERO1

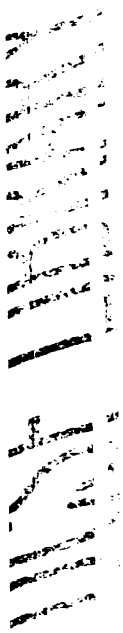


Figure I-1. Ire1p structural features

Ire1p is localized to the ER or inner nuclear membrane with its N-terminal half in the ER lumen and its C-terminal half in the cytoplasm or nucleus. Ire1p can oligomerize and trans-phosphorylates itself upon activation of the UPR. The ligand of Ire1p has not been identified. Both the kinase domain and the C-terminal tail extension are required for transmission of the unfolded protein signal.

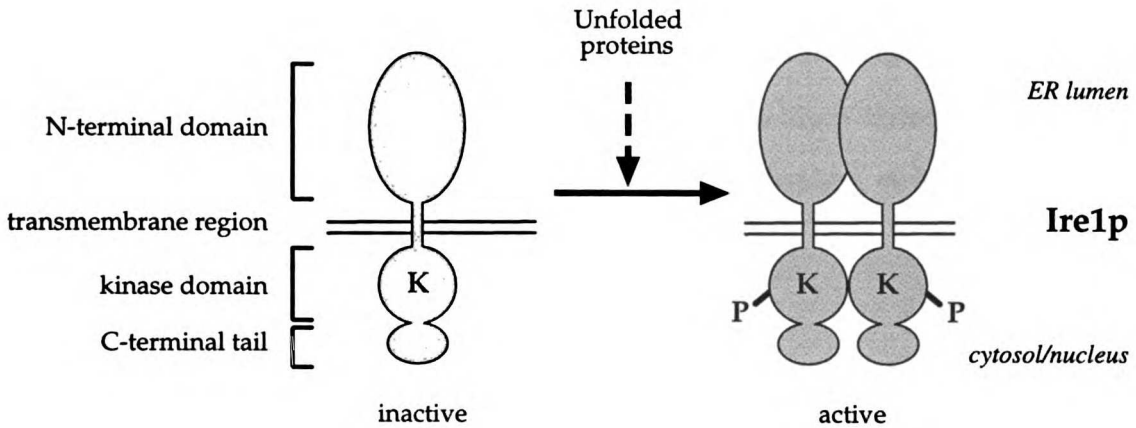
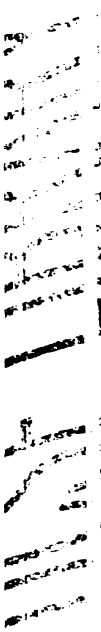

Unfolded
proteins
N-terminal domain
transmembrane region
kinase domain
C-terminal tail
K
K
K
P
P
ER lumen
Ire1p
cytosol/nucleus
inactive
active

Figure I-2. Possible routes of transmission of the UPR signal

The two possible routes of transmission of the UPR signal are distinguished by the subcellular location of Ire1p. In route a, the kinase domain of Ire1p is in the nucleus, where it transduces the signal generated by unfolded proteins (UP) across the inner nuclear membrane. In route b, the kinase domain of Ire1p is in the cytoplasm, where its activation leads to induction of downstream events in this compartment. The signal then travels to the nucleus through the nuclear pores. In both cases, signaling results in activation of transcription of UPRE-containing genes.



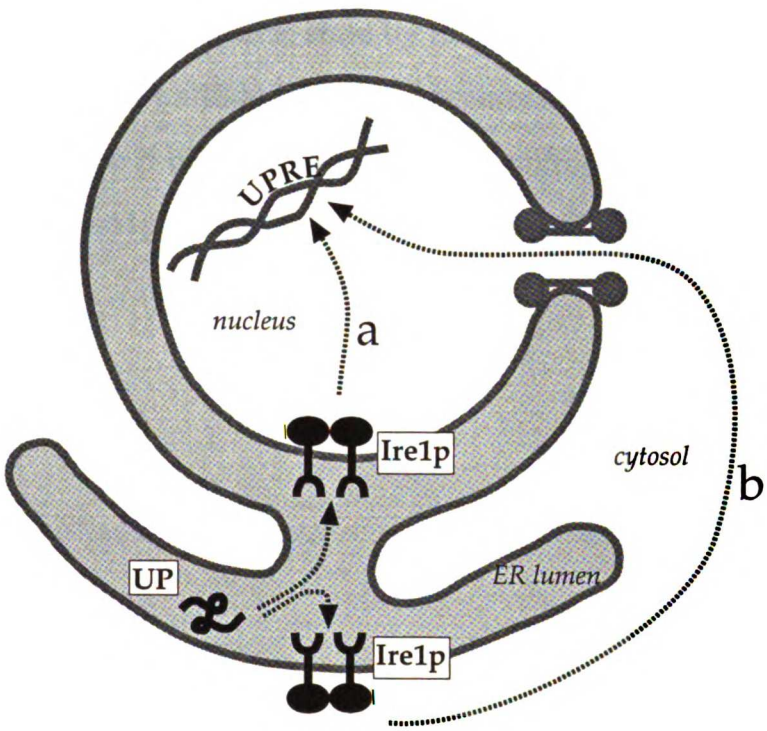
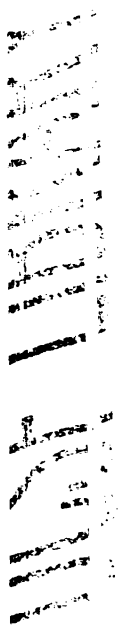
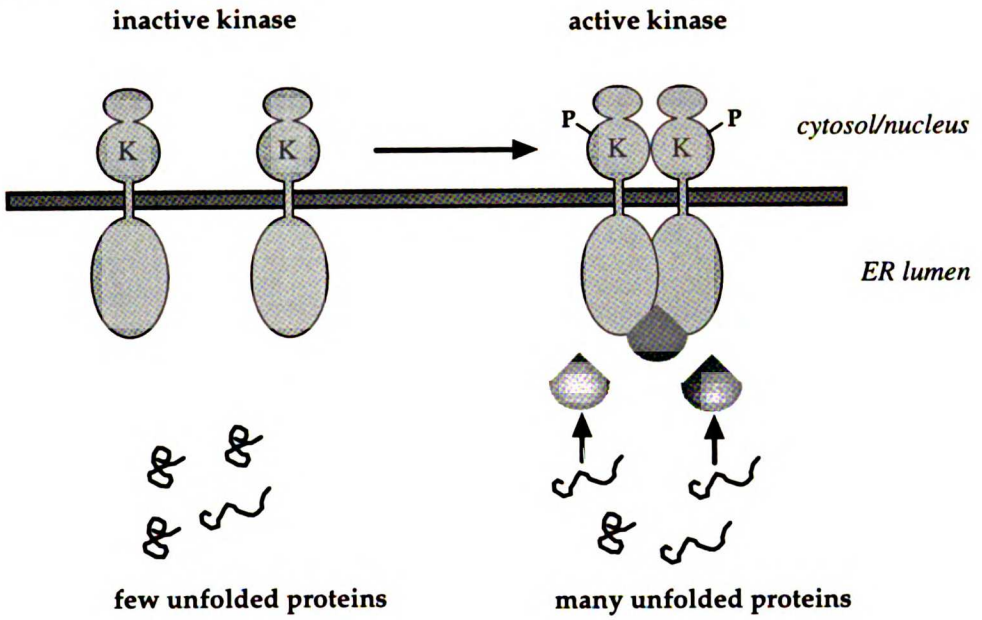

UPRE
nucleus
a
Ire1p
cytosol
b
UP
ER lumen
Ire1p

Figure I-3. Models for Ire1p activation

a) When misfolded proteins accumulate in the ER lumen, a ligand for Ire1p is generated that binds to Ire1p and induces its oligomerization and activation of its kinase. b) When the level of unfolded proteins in the ER is low, Ire1p is kept in an inactive monomeric state by binding of a ligand that prevents its dimerization. When unfolded proteins accumulate, the ligand is released from Ire1p because it binds preferentially to unfolded proteins, allowing dimerization of Ire1p (negative regulatory model).



a)


inactive kinase
active kinase
K
K
P
P
K
K
cytosol/nucleus
ER lumen
few unfolded proteins
many unfolded proteins

b)

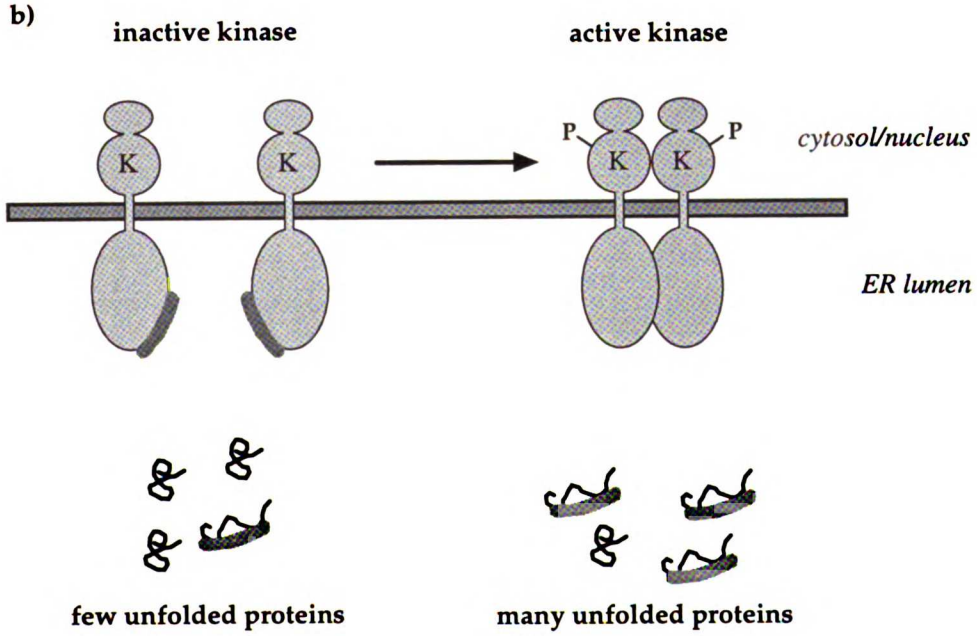
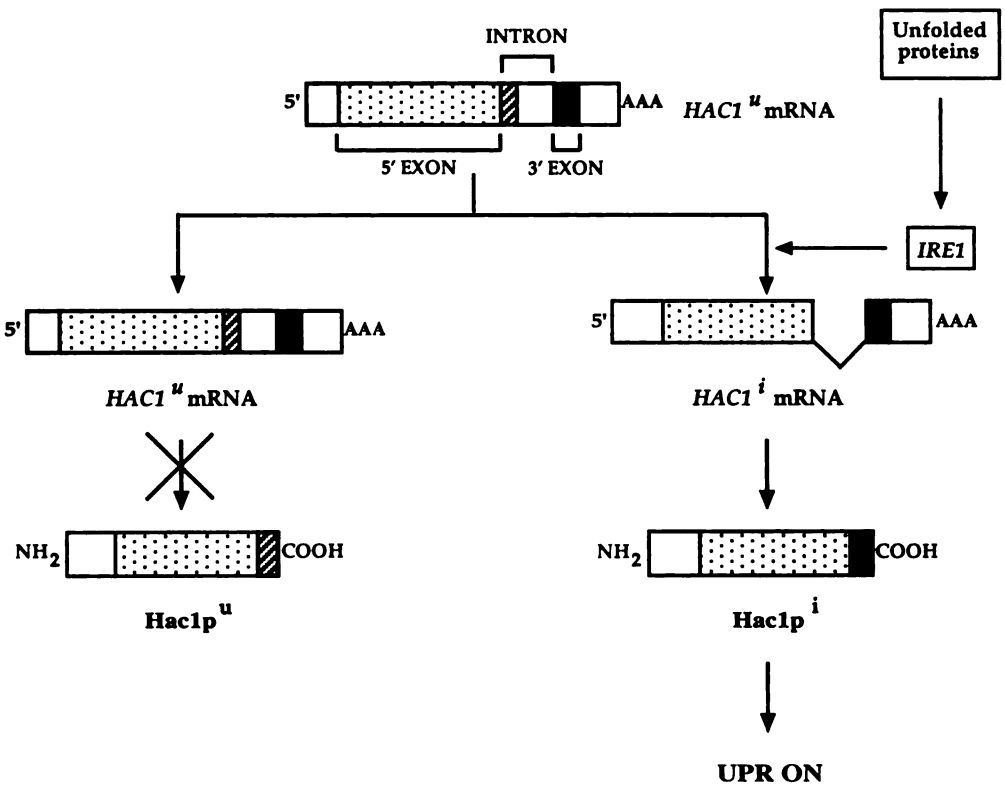

inactive kinase
active kinase
K
K
P
P
K
K
cytosol/nucleus
ER lumen
few unfolded proteins
many unfolded proteins

Figure I-4. Regulation of production of Hac1p

The right branch depicts the Ire1p-activated splicing pathway of *HAC1*^{u} mRNA. The generation of spliced *HAC1*^{i} mRNA results in production of a Hac1p^{i}. The left branch depicts the translation block exerted by the intron of *HAC1*^{u} mRNA. As a consequence, no Hac1p^{u} is produced in uninduced cells. Hac1p^{u} and Hac1p^{i} differ in their C-terminal tail.


INTRON
Unfolded proteins
5'
AAA
HAC1 u mRNA
5' EXON
3' EXON
IRE1
5'
AAA
5'
AAA
HAC1 u mRNA
HAC1 i mRNA
NH2
COOH
NH2
COOH
Hac1p u
Hac1p i
UPR ON

Chapter II

tRNA ligase is required for regulated mRNA splicing in the unfolded protein response

tRNA Ligase Is Required for Regulated mRNA Splicing in the Unfolded Protein Response

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Summary

The accumulation of unfolded proteins in the endoplasmic reticulum (ER) triggers an intracellular signaling pathway, the unfolded protein response (UPR), that leads to increased transcription of genes encoding ER-resident proteins. Transcriptional activation is mediated by a dedicated transcription factor, Hac1p, whose activity is controlled by regulated splicing of its mRNA. We have identified a mutation in tRNA ligase that disrupts the UPR in the yeast Saccharomyces cerevisiae. In this mutant, splicing of *HAC1* mRNA, but not tRNA, is blocked. In contrast, *HAC1* mRNA splicing is not impaired in cells that are blocked in spliceosome-mediated mRNA splicing. Furthermore, the splice junctions of *HAC1* mRNA do not conform to the consensus sequences of other yeast pre-mRNAs. Our results suggest that the regulated splicing of *HAC1* mRNA occurs by a novel pathway, involving tRNA ligase and bypassing the spliceosome.

Introduction

When unfolded proteins accumulate in the endoplasmic reticulum (ER), cells respond by increasing the transcription of genes encoding ER-resident proteins that assist in protein folding. This unfolded protein response (UPR) pathway monitors the concentration of unfolded proteins in the ER lumen and transduces a signal to the transcriptional apparatus in the nucleus. In Saccharomyces cerevisiae, the ER-resident proteins that are known to be induced upon activation of the UPR are Kar2p (or BiP encoded by *KAR2*), Pdi1p (endoded by *PDI1*), Eug1p (a PDI-like protein, encoded by *EUG1*), and Fkb2p (a peptidyl–prolyl *cis-trans* isomerase) (reviewed by McMillan et al., 1994; Shamu et al., 1994; Sweet, 1993).

Experimentally, the accumulation of unfolded proteins in the ER can be induced by various treatments, as follows: first, by preventing protein glycosylation with the addition of drugs such as tunicamycin (Tm); second, by preventing disulfide bond formation with reducing agents; and third, by expressing mutant secretory proteins that do not fold properly and thus accumulate in the ER. The genes encoding ER-resident proteins that are coordinately regulated by the UPR share a common upstream activating sequence, the unfolded protein response element (UPRE). This element is both necessary and sufficient to activate transcription in response to the accumulation of unfolded proteins in the ER (Kohno et al., 1993; Mori et al., 1992).

In addition to the UPRE, two other components have been identified in the yeast S. cerevisiae that are required for this signaling pathway: *IRE1* (encoding a transmembrane serine–threonine kinase) and *HAC1* (encoding a DNA binding protein with homology to the leucine zipper family of transcription factors) (Cox et al., 1993; Cox and Walter, 1996 [this issue of *Cell*]; Mori et al., 1993). Ire1p lies in the ER or inner nuclear membrane (or in both) and transmits the UPR signal across the ER membrane by a mechanism similar to those found in transmembrane kinases in the plasma membranes of higher eukaryotic cells (Shamu and Walter, 1996). Hac1p binds to the UPRE in the promoters of ER-resident chaperone genes activating their transcription. We recently found that Hac1p activity is controlled by regulated splicing of its mRNA. Upon induction of the UPR, a 252 bp nucleotide intron is removed, which leads to production of a new form of Hac1p containing a different C-terminal tail. This new tail renders Hac1p resistant to the rapid destruction that is observed in uninduced cells. The regulated processing of *HAC1* mRNA requires a functional and activated Ire1p transmembrane kinase. Thus, activation of the pathway results in a stable form of Hac1p that binds to the UPRE of target genes and increases their transcription (Cox and Walter, 1996).

Neither *IRE1* nor *HAC1* is required for viability of cells grown on rich medium; however, they are essential under conditions that induce accumulation of unfolded proteins in the ER (Cox et al., 1993; Cox and Walter, 1996; Mori et al., 1993). This can be achieved in several ways, including reducing the cellular levels of ER chaperones (Beh and Rose, 1995; Craven et al., 1996). Here, we describe the results of a genetic screen for synthetic lethality that is based on this observation. We report the identification of a new component of the UPR, namely tRNA ligase. We propose that tRNA ligase is directly involved in the regulated splicing of *HAC1* mRNA.

Results

Isolation of Mutants Defective in the UPR

ER-resident proteins are retained in the ER by virtue of their conserved C-terminal ER retention signal, HDEL. The four amino acid peptide mediates the retrieval from the early Golgi apparatus of the ER-resident proteins that have exited the ER. When the HDEL sequence is deleted, ER-resident proteins are secreted from cells (Hardwick et al., 1990; Pelham, 1989, 1990). Cells that express an HDEL-less version of Kar2p (BiP), encoded by *kar2-ΔHDEL*, as the only version of Kar2p grow at normal rates, but induce the UPR to increase the synthesis of Kar2p (Hardwick et al., 1990). This compensates for the loss of Kar2p from the ER owing to secretion. Indeed, this activation of the UPR is important, because no viable spores bearing both *Δire1* and the *kar2-ΔHDEL* alleles are produced from diploid cells heterozygous in both loci (Beh and Rose, 1995). To identify new components of the UPR pathway, we therefore designed a genetic screen to isolate mutations that, like *Δire1*, are lethal when combined with the *kar2-ΔHDEL* mutation.

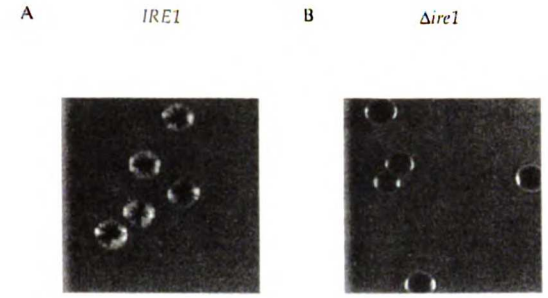

Figure 1. Mutants in the UPR Are Nonsectoring

A haploid *ade2 ade3* yeast strain carrying a *kar2–ΔHDEL* allele as the genomic copy and containing a centromeric plasmid with wild-type *KAR2* and *ADE3* genes was used in the screen (CF109). Cells were plated on low adenine–containing plates (2 μg/ml) and incubated at 30°C for 5–7 days. Strains with a functional UPR pathway can lose the plasmid as indicated by the appearance of white sectors in the colony owing to the *ade2 ade3* genotype (A). In contrast, strains that cannot induce the UPR, such as the congenic *Δire1* strain shown (CF110), cannot lose the *ADE3*-containing plasmid, and therefore nonsectoring red colonies are formed (B).

For the screen, we used a reporter strain carrying a *kar2–ΔHDEL* allele and *ade2 ade3* mutations in the genome and containing a plasmid carrying both wild-type *KAR2* and *ADE3* genes. The *ade2* mutation results in formation of a red pigment, which is no longer produced when the function of *ADE3* is also impaired. Thus, growth of this strain on plates containing low concentrations of adenine gives rise to sectoring colonies (Figure 1A), because spontaneous loss of the plasmid, and therefore the *ADE3* marker, results in cells that no longer develop red pigment (Koshland et al., 1985). As expected, when *IRE1* was disrupted in this strain, only red nonsectoring colonies were observed (Figure 1B), because cells having lost the plasmid will not grow to produce a white sector.

In contrast with previous reports that monitored spore germination, we found, however, that *Δire1 kar2–ΔHDEL* double mutant cells were viable (data not shown). Thus, formally the combination of the *Δire1* and *kar2–ΔHDEL* mutations is not synthetically lethal. For the purposes of this genetic screen, however, their growth rate was sufficiently reduced to yield a nonsectoring phenotype in our assay (Figure 1B).

Cells were mutagenized by irradiation with UV light (15% survival). From 20,000 colonies screened, we isolated 17 mutants that gave rise to nonsectoring colonies. Mutants were then tested in a secondary screen for lack of induction of the UPRE–*lacZ* reporter upon treatment with Tm. Of three mutants that were unable to induce this reporter, one belonged to a novel complementation group, as diploids heterozygous for this mutation and either *Δire1* or *Δhac1* exhibit the ability to induce the UPR. Additional complemetation tests showed that the other two isolated mutants were allelic to *ire1*. For reasons outlined below, we henceforth refer to the novel mutant as *rlg1-100*.

Characterization of *rlg1-100* Mutant Cells

rlg1-100 cells grew at normal rates and, like *ire1* and *hac1* mutants, were impaired in the induction of endogenous *KAR2* mRNA upon Tm treatment (Figure 2A; Cox and Walter, 1996) and were unable to grow on plates lacking inositol (Figure 2B). Thus, they showed phenotypes indistinguishable from cells carrying mutations in other components of the UPR.

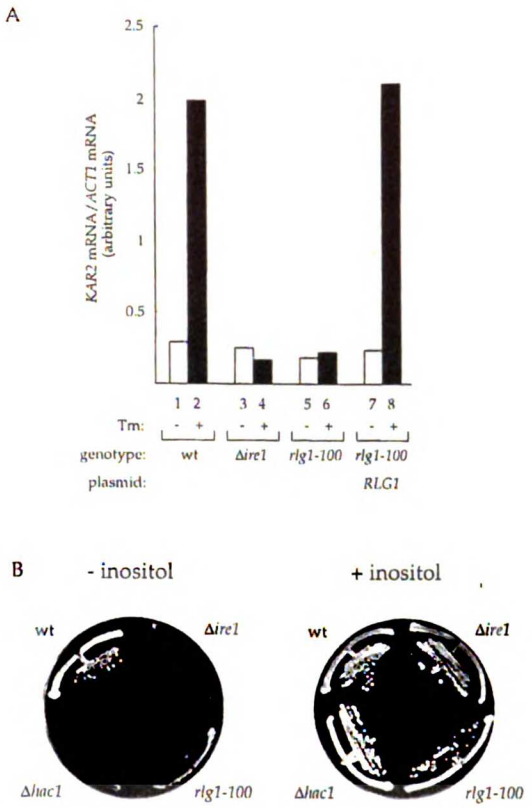

Figure 2. Characterization of the *rlg1-100* Mutant Strain

(A) Northern hybridization was performed on RNA isolated from the following strains: wt (JC102); *Δire1* cells (CS243); *rlg1-100* cells (CF181); and *rlg1-100* cells carrying a centromeric vector pCF138 containing wild-type *RLG1*. Each strain was grown to mid-log phase and incubated in the presence or absence of Tm for 2.5 hr. Total RNA was extracted and analyzed by Northern hybridization using DNA specific probes for *KAR2* and *ACT1*. The data was quantitated and *KAR2* mRNA levels were normalized to *ACT1* mRNA levels.
(B) Cells from wt (JC102), *Δire1* (CS243), *Δhac1* (JC402), and *rlg1-100* (CF181) strains were streaked for single colonies on plates containing either 100 μg/ml inositol (+inositol) or no inositol (−inositol). Plates were incubated at 30°C for 2 days and photographed.

To identify the mutant gene, we cloned genomic DNA fragments that complemented in low copy the UPR defects in *rlg1-100* cells. Subcloning revealed one gene, *RLG1*, that restored the induction of *KAR2* mRNA transcription upon Tm treatment (Figure 2A, lanes 7 and 8). The identification of this gene was surprising, because *RLG1* encodes tRNA ligase, a previously characterized essential protein that is required for pre-tRNA splicing (Phizicky et al., 1992). To ascertain whether *rlg1-100* is indeed a mutant allele of *RLG1*, we cloned the mutant gene. Sequencing revealed a single point mutation (C442T) that is predicted to change a conserved histidine at position 148 in tRNA ligase to tyrosine. To prove unambiguously that the H148Y mutation is responsible

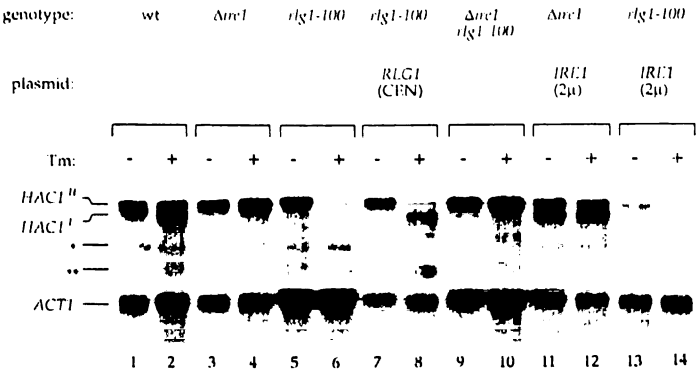

Figure 3. *HAC1* mRNA Splicing Is Blocked in *rlg1-100* Cells

Northern hybridization was performed on RNA isolated from the following strains: wt (JC102), lanes 1 and 2; *Δire1* (CS243), lanes 3 and 4; *rlg1-100* (CF181), lanes 5 and 6; *rlg1-100* (CF181) carrying a centromeric plasmic with wild-type *RLG1* (pCF138), lanes 7 and 8; *Δire1 rlg1-100* double mutant (CF203), lanes 9 and 10; *Δire1* carrying a high copy number (2μ) plasmid pCS122 with wild-type *IRE1*, lanes 11 and 12; and *rlg1-100* (CF181) carrying this same pCS122 vector, lanes 13 and 14. Each strain was grown to mid-log phase and incubated in the presence (even numbered lanes) or absence (odd numbered lanes) of Tm for 20 min. Total RNA was extracted and analyzed by Northern hybridization using DNA specific probes for *HAC1* and *ACT1*. Faint bands (denoted with asterisks) may represent the 5′ exon alone (two asterisks) and the 5′ exon plus the IVS (one asterisk) (Cox and Walter, 1996). It is not clear whether these bands correspond to splicing intermediates or dead-end products.

for the observed phenotype, we reconstructed the mutation using site-directed mutagenesis. Indeed, *Δrlg1* cells bearing *rlg1*(H148Y) on a low copy plasmid grew at normal rates but were unable to induce the UPR, whereas, as expected, *Δrlg1* cells bearing wild-type *RLG1* on the plasmid could induce the UPR (data not shown). Thus, the phenotype of *rlg1*(H148Y) cells is indistinguishable from that of *rlg1-100* cells. Based on these results, we conclude that the single identified mutation in tRNA ligase is sufficient to cause loss of the UPR and therefore named the mutant *rlg1-100*, consistent with it being a new allele of *RLG1*.

Effects of the *rlg1-100* Mutation on *HAC1* mRNA Processing

The identification of an RNA-processing enzyme as a component of the UPR was particularly intriguing in light of the discovery that a step in the pathway involves the regulated splicing of *HAC1* mRNA (Cox and Walter, 1996). Therefore, to assess directly whether tRNA ligase is involved in *HAC1* mRNA processing, we examined the fate of *HAC1* mRNA in *rlg1-100* mutant cells by Northern hybridization (Figure 3). Wild-type cells contain unspliced *HAC1*^{u} mRNA (Figure 3, lane 1), which upon induction of the UPR by addition of Tm is processed to the smaller spliced *HAC1*^{i} mRNA (Figure 3, lane 2) (Cox and Walter, 1996). This conversion is abolished in *Δire1* cells, which cannot transmit the signal induced by unfolded proteins across the ER membrane (Figure 3, lanes 3 and 4). Interestingly, in *rlg1-100* mutant cells, activation of the UPR leads to the disappearance of unspliced *HAC1*^{u} mRNA, but, in contrast with wild-type cells, no spliced *HAC1*^{i} mRNA is produced upon induction of the UPR (Figure 3, lanes 5 and 6). Importantly, the level of actin mRNA, which is a substrate of the conventional splicing machinery, was not affected in *rlg1-100* cells upon induction of the UPR (see ACT1 in Figure 3, lanes 5 and 6), indicating that the effect of the mutation is specific for *HAC1* mRNA.

Expression of an intron-less *HAC1*^{i} gene in the *rlg1-100* mutant strain results in constitutive activation of the pathway (Cox and Walter, 1996). Northern hybridization confirmed that the intron-less *HAC1*^{i} mRNA is stably expressed in *rlg1-100* mutant cells in both the absence and presence of Tm (data not shown). Thus, we can exclude the possibility that spliced *HAC1*^{i} mRNA is formed in the *rlg1-100* mutant but is then rapidly degraded. We therefore conclude that the *rlg1-100* mutation blocks production of spliced *HAC1*^{i} mRNA.

Because the *HAC1* mRNA splicing defect is only observed upon induction of the UPR, it must require transmission of the signal resulting from the accumulation of unfolded proteins in the ER to the splicing machinery. The proposed early role of Ire1p in the UPR, along with the lack of *HAC1* mRNA splicing in *Δire1* cells, predicts that Ire1p functions upstream of tRNA ligase. To establish the order of the components in the pathway directly, we determined the fate of *HAC1* mRNA in an *rlg1-100 Δire1* double mutant (Figure 3, lanes 9 and 10). As expected, the double mutant strain behaved identically to the *Δire1* mutant, i.e., only unspliced *HAC1* mRNA was observed. Furthermore, overexpression of Ire1p, which turns on the UPR constitutively in the absence of any agents that induce unfolded proteins, results in constitutive splicing of *HAC1* mRNA in wild-type cells (Figure 3, lanes 11 and 12) and leads to constitutive degradation of *HAC1* mRNA in *rlg1-100* mutant cells (Figure 3, lanes 13 and 14). Thus, the regulatory hierarchy of the components of the UPR pathway is *IRE1→RLG1→HAC1*.

tRNA Splicing Is Not Affected in *rlg1-100* Cells

Yeast cells disrupted for *RLG1* are inviable (Phizicky et al., 1992). Thus, although the *rlg1-100* mutation blocks the formation of spliced *HAC1*^{i} mRNA completely, it must not block splicing of essential pre-tRNAs to a similar degree. It is possible that the *rlg1-100* mutation is a partial loss-of-function mutation that affects the UPR more severely than tRNA splicing. Alternatively, the *rlg1-100* allele may be a pathway-specific mutation that affects only *HAC1* mRNA splicing. To distinguish between these two possibilities, we asked whether tRNA splicing is affected in *rlg1-100* cells. To this end, we labeled cells with [^{32}P]-orthophosphate and analyzed the tRNA population by two-dimensional gel electrophoresis. Control cells bearing wild-type *RLG1* driven by a regulated promoter show a discrete and previously well characterized accumulation of tRNA halves and intervening

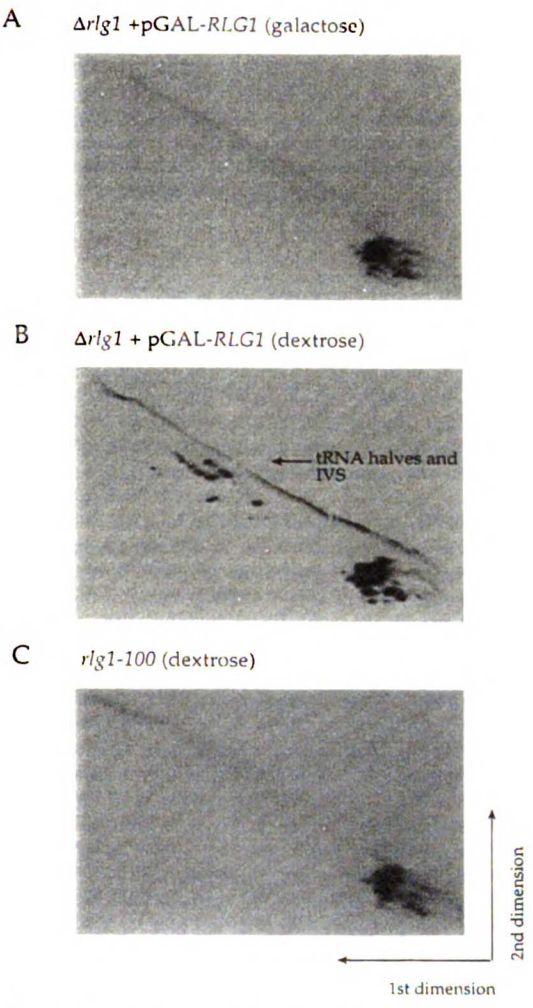

Figure 4. tRNA Splicing Is Not Affected in the *rlg1-100* Mutant Strain

The indicated strains were grown to logarithmic phase in the appropriate growth media and labeled for 30 min with [^{32}P]-orthophosphate as described in Experimental Procedures. Total RNA was extracted and displayed on two-dimensional polyacrylamide gels. First dimension is from right to left; second dimension is from bottom to top. The cluster of spots that is unique to (B) has been previously well characterized, and the identity of individual spots is known (Phizicky et al., 1992). As indicated, they correspond to tRNA halves and IVS.

sequences when expression of *RLG1* is turned off (Figure 4B). In contrast, *rlg1-100* cells do not accumulate any detectable tRNA splicing intermediates and thus behave indistinguishably from cells expressing wild-type *RLG1* (compare Figures 4C and 4A). These results suggest that the *rlg1-100* allele affects the UPR pathway specifically.

Splicing of *HAC1* mRNA Is Not Blocked in *prp2^{ts}* or *prp8^{ts}* Mutant Cells

The data presented so far suggest that tRNA ligase plays a role in *HAC1* mRNA splicing. This suggests that *HAC1* mRNA splicing may occur by a different molecular mechanism than conventional mRNA splicing. This conjecture is further supported by the lack of consensus sequences flanking the splice junctions of *HAC1* mRNA (Figure 5A). In particular, a number of bases that are highly or absolutely conserved in the splice junctions of other yeast mRNAs are not found in *HAC1* mRNA (Figure 5A, indicated by asterisks). Because splice junctions of other mRNAs are recognized by direct base-pairing with snRNAs in the spliceosome, this considerable divergence makes *HAC1* mRNA an unlikely substrate for the conventional splicing machinery. To address this conjecture experimentally, we asked whether mutations that block splicing of other mRNAs also affect splicing of *HAC1* mRNA.

prp2^{ts} cells are mutant in an essential splicing factor, an RNA-dependent ATPase that is required for the first catalytic step of mRNA splicing. In the *prp2^{ts}* mutant, splicing of pre-mRNAs is severely blocked at the nonpermissive temperature, as shown for actin mRNA in Figure 5B (compare lanes 3 and 7). In contrast, splicing of *HAC1* mRNA is not blocked, nor is the total amount of both forms of *HAC1* mRNA noticeably diminished (Figure 5B, lanes 4 and 8). Similarly, when a *prp8^{ts}* mutant strain is shifted to the nonpermissive temperature, actin mRNA splicing is blocked, whereas splicing of *HAC1* mRNA is not affected (Figure 5B, compare lanes 10 and 12). Prp8p functions during both catalytic steps of pre-mRNA splicing, where it is involved in mediating the association of two snRNPs in the spliceosome.

Unexpectedly, we observed that splicing of *HAC1* mRNA was induced even in the absence of tunicamycin at the nonpermissive temperature in both *prp2^{ts}* and in *prp8^{ts}* but not in wild-type cells (Figure 5, compare lanes 7 and 11 with lane 3). This indicates that defects in conventional mRNA splicing lead to induction of the UPR, presumably because some ER protein requires splicing for its biosynthesis. Consistent with this notion, we found that transcription of *EUG1* (encoding a PDI-like ER-resident protein) is induced at the restrictive temperature in both *prp2^{ts}* and *prp8^{ts}* but not in wild-type cells (data not shown). *EUG1* is one of the target genes of the UPR and was selected because it does not contain a heat shock element in its promoter and because its transcription is therefore insensitive to the shift to the nonpermissive temperature of the *prp* mutants.

Because spliced *HAC1^{i}* mRNA is produced at the nonpermissive temperature in both *prp2^{ts}* and *prp8^{ts}* strains even in the absence of Tm, it became important to rule out the possibility that the observed *HAC1^{i}* mRNA was produced prior to the block in Prp2p or Prp8p function. To address this concern, we determined the half-life of *HAC1^{i}* mRNA. We used a strain bearing a temperature-sensitive mutation in RNA polymerase II, in which no new mRNA transcripts are produced at the nonpermissive temperature. Thus, it is possible to measure the rate of mRNA decay by Northern hybridization. As shown in Figure 5C, *HAC1^{i}* mRNA decays with a half-life of ~20 min. The total incubation time in the previous experiment of the *prp2^{ts}* and *prp8^{ts}* strains at the nonpermissive temperature was 2.7 hr. This is longer than the time required to block mRNA splicing in both *prp2^{ts}* and *prp8^{ts}*

cells (1 hr; Jackson et al., 1988; Lee et al., 1984), and considerably longer than the half-time of *HAC1'* mRNA. We therefore conclude that the population of *HAC1'* mRNA observed in Figure 5B (lanes 7, 8, 11, and 12) must have originated after conventional mRNA splicing is blocked. This suggests that splicing of *HAC1* mRNA bypasses the block in spliceosome-mediated splicing that is induced by these mutants.

Discussion

We have identified an additional component that is required for the UPR pathway in yeast, tRNA ligase. We have shown that a single amino acid substitution in tRNA ligase is sufficient to cause a complete loss of the UPR without causing other defects in cell growth. By all criteria examined, the defects of the *rlg1-100* mutant are indistinguishable from strains carrying null mutations in the other two previously identified components of the pathway, *IRE1* and *HAC1*.

Several lines of evidence suggest that tRNA ligase participates directly in the UPR. First, we found that the *rlg1-100* mutation selectively blocks the UPR. The essential function of tRNA ligase in pre-tRNA splicing appears undiminished in *rlg1-100* cells (Figure 4), thus making it unlikely that defects in pre-tRNA processing are indirectly responsible for the observed block in the UPR. Second and most important, the *rlg1-100* mutation selectively affects *HAC1* mRNA processing (Figure 3) that we have shown in the accompanying paper to be an important regulatory step in the UPR (Cox and Walter, 1996). We have shown here that *HAC1* mRNA is specifically degraded in *rlg1-100* cells in a reaction that depends on activation of Ire1p. Third, mutations in *PRP2* and *PRP8* that block spliceosome-mediated processing of pre-mRNAs (Jackson et al., 1988; Lee et al., 1984; Teem et al., 1983) fail to impair *HAC1* mRNA splicing (Figure 5B). Fourth, the splice junctions of *HAC1* mRNA diverge considerably from the consensus sequences found in other pre-mRNAs (Figure 5A; Rymond and Rosbash, 1992). Finally, tRNA ligase is a known RNA-processing enzyme with a well characterized role in pre-tRNA splicing (Greer et al., 1983; Westaway et al., 1988). Based on these observations, we propose a model in which tRNA ligase catalyzes the obligate religation of *HAC1* mRNA halves that are produced upon activation of the UPR (Figure 6). In contrast with tRNA halves, we propose that the *HAC1* mRNA halves are rapidly degraded if they are not religated. This provides a plausible explanation as to why we do not observe the accumulation of stable intermediates. Intriguingly, our model implies that *HAC1* mRNA splicing is catalyzed, at least in part, by components for which no role in mRNA splicing has yet been described. Thus, we propose that *HAC1* mRNA splicing occurs by an unprecedented mechanism that bypasses components of the conventional mRNA splicing machinery.

The *cis* elements in *HAC1* mRNA that make it a substrate for Ire1p-regulated splicing remain to be determined. They are likely to provide invaluable clues that may help identify other mRNAs that may also use this alternative splicing pathway. Also, we note that sequences within the intervening sequence (IVS) of *HAC1* mRNA resemble, but do not match precisely, the branch point consensus sequence for conventional mRNA splicing, UACUAAC. If these sequences prove to be functionally important for *HAC1* mRNA splicing, it would suggest that both the conventional and this new pathway for mRNA splicing share some common features or components (or both).

An important question is how the H148Y mutation in tRNA ligase can exhibit a complete loss-of-function phenotype for the UPR, yet lead to no detectable defects in pre-tRNA processing. tRNA ligase is a multifunctional enzyme that contains three distinct enzymatic activities: a polynucleotide kinase, a cyclic phosphodiesterase, and adenylate synthetase. These activities are arranged in distinct functional domains on a single polypeptide chain encoded by *RLG1*, and catalyze a series of sequential reactions that together result in the joining of tRNA halves (Apostol et al., 1991; Greer et al., 1983; Phizicky et al., 1986; Xu et al., 1990). Although histidine 148 is conserved in tRNA ligase in other yeast species, it maps toward the C-terminal end of the adenylate synthetase domain, distant from residues that are important for the catalytic activity. Its location therefore sheds no immediate light onto its possible function. The absence of observable tRNA splicing defects in the *rlg1-100* mutant strain suggests that the enzymatic activities of the mutant protein required for pre-tRNA splicing are intact. Indeed, extracts of the *rlg1-100* strain have wild-type activity in pre-tRNA splicing (Apostol and Greer [UC Irvine], personal communication). Thus, *HAC1* mRNA splicing must pose structural or functional requirements on tRNA ligase that are dispensable for tRNA splicing.

The function of tRNA ligase in pre-tRNA splicing is preceded by that of tRNA endonuclease, a membrane-bound multisubunit enzyme that cleaves pre-tRNAs at both exon–intron junctions during the first splicing steps (Peebles et al., 1983). Enzymatic analyses suggest that tRNA halves produced by tRNA endonuclease are directly channeled to tRNA ligase to be rejoined. Furthermore, tRNA ligase binds pre-tRNAs with high affinity (Apostol and Greer, 1991). Thus, it has been suggested that tRNA ligase is in a physical complex with tRNA endonuclease in which it binds pre-tRNA substrates, presents them to the endonuclease, and then ligates the halves.

Considering these steps in pre-tRNA processing, several models could explain the exclusive effects of the *rlg1-100* mutation on *HAC1* mRNA splicing. First, the *rlg1-100* mutation may affect an uncharacterized enzymatic activity in tRNA ligase that is required for *HAC1* mRNA splicing but dispensable for pre-tRNA splicing. Second, the *rlg1-100* mutation may abolish a putative obligate interaction of tRNA ligase with *HAC1* mRNA. In this scenario, *HAC1* mRNA and pre-tRNAs may bind, for example, to different sites on tRNA ligase. Finally, the *rlg1-100* mutation may impair an interaction of tRNA ligase with other proteins that are required for the UPR but not for pre-tRNA splicing. Although we cannot formally distinguish between these possibilities, we favor the latter two scenarios, which could explain how *HAC1* mRNA splicing is regulated whereas pre-tRNA splicing occurs constitutively. Further support for the model that the known enzymatic activities of tRNA ligase are required for processing of *HAC1* mRNA comes from the

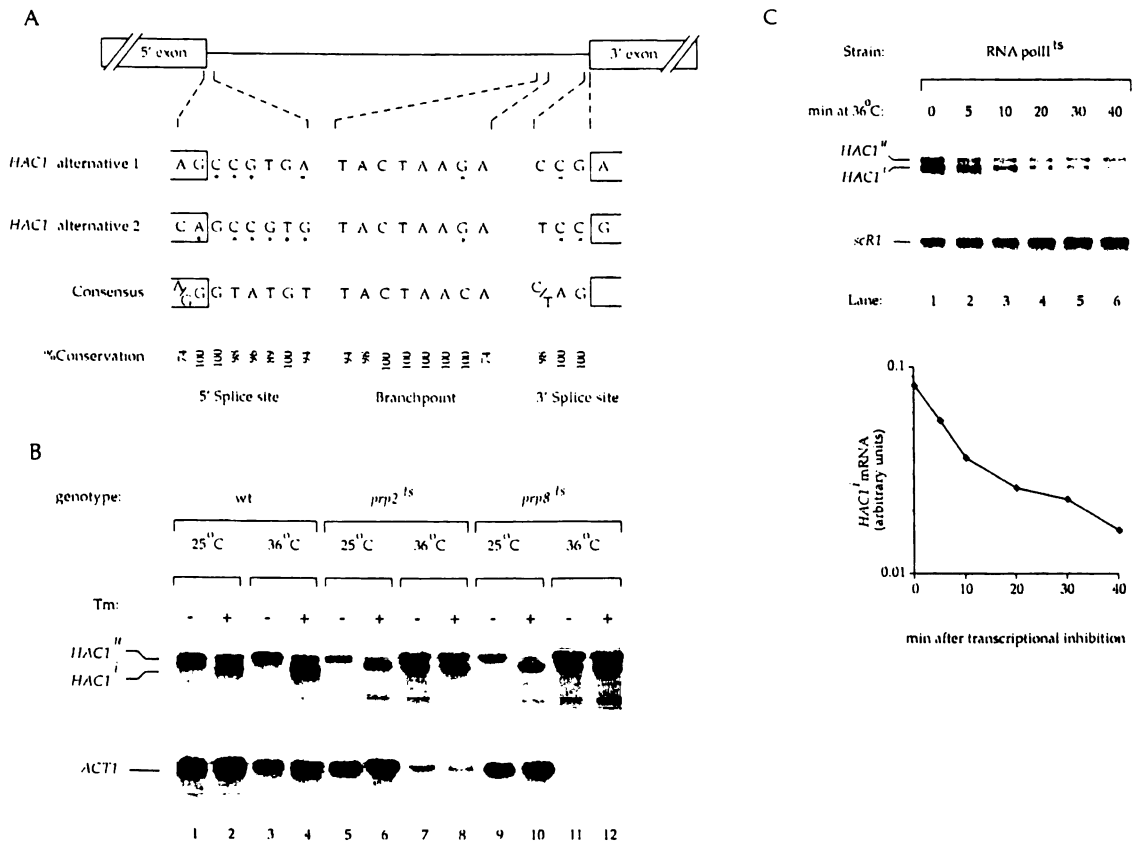

Figure 5. Splicing of *HAC1* mRNA Is Not Affected in Conditional Mutants That Block Spliceosome-Mediated Pre-mRNA Processing

(A) An alignment of the nucleotide sequences flanking the splice junctions in *HAC1* mRNA and the consensus sequences found for spliceosome-mediated mRNA splicing is shown (Rymond and Rosbash, 1992). Spliced *HAC1*^{i} mRNA contains a single G-residue at the exon–exon junction. We can presently not distinguish whether this G-residue is derived from the 5' or 3' exon. Thus, there are two possibilities for the exon/IVS junctions that are indicated by the solid and dashed boxes, respectively. The percentage of conservation of nucleotides at the splice junctions and branch point is indicated. The bases in *HAC1* mRNA that differ from well conserved consensus bases are marked by asterisks.

(B) Northern hybridization was performed on total RNA isolated from wild type (JC102), lanes 1–4; *prp2-1* (EJS42), lanes 5–8; and *prp8-1* (YEJS17), lanes 9–12; cells. Each strain was grown at the permissive temperature (25°C) or restrictive temperature (36°C) for 2 hr and then treated with (even numbered lanes) or without (odd numbered lanes) 10 μg/ml Tm for an additional 40 min. RNA was extracted and analyzed by Northern hybridization using DNA-specific probes for *HAC1* and *ACT1*.

(C) JC218 cells bearing a temperature-sensitive allele of *RBP1* encoding RNA polymerase II were treated with Tm for 20 min and then shifted to the nonpermissive temperature. At different time points after temperature shift, RNA was extracted and analyzed by Northern hybridization. Blots were probed for *HAC1* mRNA and, as a control, for the RNA polymerase III transcript *SCR1* RNA. *HAC1*^{i} mRNA was quantitated and normalized to *SCR1* RNA.

observation that a tRNA ligase mutant (K119S, changing an essential lysine residue required for adenylate synthetase activity), which is inactive for joining of tRNA halves, fails to complement the *HAC1* mRNA processing defects of the *rlg1-100* mutant strain (Apostol and Greer [UC Irvine], personal communication).

HAC1 mRNA splicing is initiated by an Ire1p-mediated event that leads to nucleolytic cleavage. In principle, tRNA endonuclease could perform one or both of the required *HAC1* mRNA cleavage steps. According to this view, Ire1p would somehow regulate access of the substrate to the constitutively active endonuclease. Alternatively, tRNA ligase may interact with another endonuclease, different from tRNA endonuclease, that cleaves *HAC1*^{u} mRNA. A particularly exciting possibility is suggested by sequence similarities between the essential C-terminal tails of Ire1p kinase and mammalian RNase L, a nuclease that also contains a kinase domain and is, like Ire1p, activated by oligomerization (Bork and Sander, 1993; Dong and Silverman, 1995; Zhou et al., 1993). Based on these structural and functional similarities, we speculate that Ire1p may itself be the endonuclease that cleaves *HAC1*^{u} mRNA and thus initiates the splicing reaction directly. Oligomerization of Ire1p kinases could, for example, activate the putative C-terminal RNase domain by phosphorylation, or inactivate putative RNase inhibitors. We are currently developing in vitro assays to test this hypothesis directly.

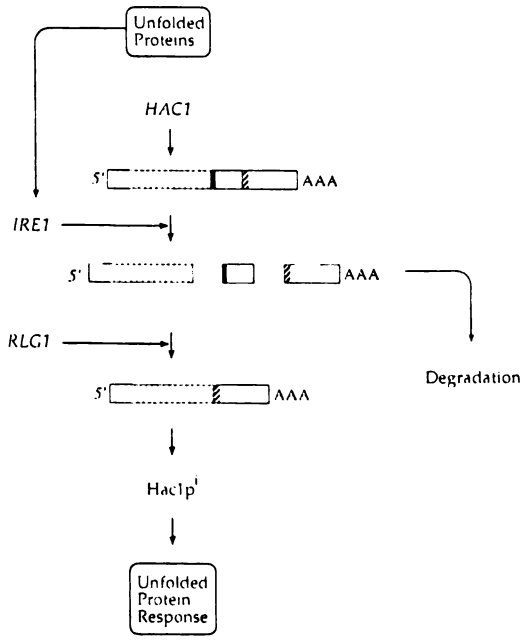

Figure 6. Model for tRNA Ligase Function in the UPR Pathway

The accumulation of unfolded proteins in the ER activates an Ire1p-dependent nucleolytic cleavage of *HAC1* mRNA. Ire1p could be directly involved in the cleavage event or, alternatively, may be required to activate a yet unknown nuclease. *HAC1* mRNA halves are then religated by tRNA ligase to generate the spliced form of this mRNA. If religation is blocked, the splicing intermediates are rapidly degraded in vivo. Spliced *HAC1* mRNA results in production of a more stable form of Hac1p that binds to the UPRE and thus activates transcription of the target genes of the UPR. Stippled, filled, and hatched boxes represent Hac1p coding information. Splicing of *HAC1* mRNA results in the removal of 10 codons from *HAC1*^{u} (black) with the concomitant addition of 18 codons (diagonally striped) to produce *HAC1*^{i}.

In wild-type cells expressing endogenous *HAC1*, we find that about 50%–60% of the *HAC1* RNA molecules are spliced when the UPR is induced by Tm treatment for 20 min. No increase in the absolute level of spliced *HAC1*^{i} mRNA was observed in cells overexpressing *HAC1* mRNA from a 2μ plasmid when Tm-treated for the same time period (data not shown). This indicates that the splicing machinery is normally saturated when the UPR is induced. In contrast, we observed that virtually all *HAC1* RNA is degraded in *rlg1-100* cells when Ire1p is activated upon Tm treatment (Figure 3, lane 6). This apparent paradox can be most easily explained if one assumes that *HAC1* mRNA ligation is the rate-limiting step of the overall splicing reaction. In *rlg1-100* mutants, the rate of nucleolytic cleavage may therefore no longer be limited by ligation, resulting in a more efficient processing, and hence degradation, of *HAC1*^{u} mRNA. Thus, our data suggest that during splicing of *HAC1*^{u} mRNA, cleavage and religation are mechanistically coupled.

HAC1 mRNA splicing, like the splicing of all other cytoplasmic RNAs, most likely occurs in the nucleus. Sequestering the splicing machinery in a different compartment confers the advantage, unique to eukaryotic cells, that access of ribosomes to the RNA is restricted until it has been properly processed. In the accompanying paper, we propose that both unspliced *HAC1*^{u} RNA and spliced *HAC1*^{i} RNA are translated. Thus, in contrast with other pre-mRNAs, *HAC1*^{u} RNA is a functional mRNA. We therefore propose that the activated *HAC1* mRNA splicing machinery catches *HAC1*^{u} mRNA during export from the nucleus to the cytosol. Indeed, enzymes involved in pre-tRNA splicing and modification are found in close proximity to nuclear pores, suggesting a link between export and processing (Clark and Abelson, 1987; Simos et al., 1996). Therefore, if our speculation is correct and Ire1p is directly involved in *HAC1*^{u} mRNA processing, we predict that Ire1p, or at least the fraction of the Ire1p molecules that could participate in splicing, lies in the inner nuclear membrane. Ire1p would then transduce the unfolded protein signal directly from the ER lumen into the nucleus.

Experimental Procedures

Media and General Methods

Media, reagents, and general procedures are as described by Cox and Walter (1996).

Plasmid Construction

To make the *kar2-ΔHDEL* integrating vector, a synthetic oligonucleotide was used to amplify the C-terminal domain of *KAR2*, converting the C-terminus of the encoded protein from FEHDEL to FGR (Hardwick et al., 1990). The PCR fragment containing the altered C-terminal domain of *KAR2* and an actin terminator were cloned into YIplac204 (Gietz and Sugino, 1988) to generate pCF104. The wild-type *KAR2*, *ADE3*, and *URA3* sectoring plasmid pCF105 is a centromeric vector made by subcloning the *ADE3* gene into the BamHI site of pMR397 (Rose et al., 1989).

pCF138 was constructed by inserting a genomic XhoI–SacI fragment containing *RLG1* into the CEN/ARS vector pRS316 (*URA3*) (Sikorski and Hieter, 1989). Similarly, pCF157 was constructed by inserting this fragment into the CEN/ARS vector pRS313 (*HIS3*) (Sikorski and Hieter, 1989).

Yeast Strains

Yeast strains used in this study are listed on Table 1. Reporter strain CF109 was constructed by homologous recombination of pCF104 (linearized with BsmI) into the chromosomal copy of *KAR2* to generate *kar2-ΔHDEL*. Correct integration was confirmed by PCR. To generate strain JC218, strain YAS880 (a gift from A. Sachs) was backcrossed twice to JC103 (Cox and Walter, 1996).

Primary Screen

Strain CF109 was mutagenized with UV light to 15% survival. Cells were plated onto synthetic minimal plates lacking tryptophan and containing 2 μg/ml adenine. Cells were plated at a density of ~300 colonies per plate. Colonies were allowed to grow 5–7 days at 30°C. Individual nonsectoring colonies were restreaked onto fresh plates, and only those mutants that gave rise to uniformly red colonies were studied.

Secondary Screen

Mutant strains were transformed with the UPRE-*lacZ* reporter construct pCF118. This vector is a centromeric derivative of pJC005 (Cox et al., 1993). Yeast patches were replica-plated onto X-Gal/Tm indicator plates and incubated overnight at 30°C. Out of 17 mutants, 3 failed to turn blue on the indicator plates.

Table 1. Yeast Strains

Strain	Genotype	Source/Reference
CF109[a]	*leu2-3,-112; his3-11,-15; trp1-1; ura3-1; ade2-1; can1-100; ade3Δ; kar2-ΔHDEL::TRP; MATα*	This study
CF110	same as CF109 except *ire1::LEU2*	This study
JC102	*trp1-1; his3-11,-15; ura3-1; ade2-1; can1-100; leu2-3,-112::LEU2 UPRE-lacZ; MATα*	Shamu and Walter, 1996
JC402	*trp1-1; ade2-1; can1-100; leu2-3,-112; his3-11,-15::HIS3 UPRE-lacZ; Δhac1::URA3; MATα*	This study
CS243	same as JC102, except *ire1::URA3*	Shamu and Walter, 1996
CF181[a]	*leu2-3,-112; trp1-1; ura3-1; ade2-1; can1-100; ade3Δ; rlg1-100; MATα* and *pUPRE-lacZ LEU2*	This study
CF203	*leu2-3,-112; his3-11,-15; trp1-1; ade2-1; can1-100; rlg1-100; ire1::URA3; MATα*	This study
EJS42	*ade1; ade2; ura1; his7; tyr1; lys2; gal1; prp2-1; MATα*	C. Guthrie
YEJS17	*ade1; ade2; ura1; his7; trp1; lys2; prp8-1; MATα*	C. Guthrie
EMPY439	*ade2-101; his3-Δ200; ura3-52; rlg1-ΔKpn1; GAL ; SUC2; MATα* and pGAL-*RLG1 URA3*	Phizicky et al, 1992
JC218	*ade2; trp1; ura3; leu2-3,-112::LEU2-UPRE-lacZ; his3-11,-15::HIS3-UPRE-lacZ; rbp1*	This study

[a] Spontaneous mutation his derivative cannot be transformed by a *HIS3* containing plasmid.

Rescue of the *rlg1-100* Mutant Phenotype

Strain CF181(*rlg1-100* first backcross) was transformed with a high copy yeast genomic library (Carlson and Botstein, 1982), and colonies were replica-plated to X-Gal/Tm plates. Colonies that turned blue were tested for their ability to grow on plates lacking inositol. Of 7,000 colonies screened, 6 turned blue on indicator plates and were also inositol prototrophs. Plasmids from these strains were rescued and sequenced. Three of them were shown to contain *HAC1*. Overexpression of *HAC1* suppresses the defects of the *rlg1-100* mutant. The remaining three plasmids had overlapping inserts, each containing the *RLG1* open reading frame. A fragment containing *RLG1* was subcloned into a low copy vector (pCF138) and shown to complement the defects of the *rlg1-100* mutant. Deletion analysis of this plasmid confirmed that the fragment carrying *RLG1* contained the complementing activity.

The *rlg1 (H148Y)* mutation also introduces a stop codon in a small open reading frame (*ORF5*) oriented in the opposite direction of *RLG1*. To confirm that the phenotype observed in the *rlg1-100* mutant strain was due to the amino acid change in the Rlg1p, we made a mutation (A612T, counted from the initiating AUG of the *RLG1* open reading frame) that introduces a stop codon in *ORF5* and that does not change the amino acid sequence of the Rlg1p. This construct restored the ability of the *rlg1-100* strain to induce the UPR, indicating that disruption of the *ORF5* is not responsible for the phenotype observed in the *rlg1-100* mutant strain. Also, a wild-type *RLG1* gene under the control of the *GAL10* inducible promoter (pBM150-RLGX, Phizicky et al., 1992) restored the ability of the *rlg1-100* strain to induce the UPR only when cells were grown in the presence of galactose. Because transcription of the small open reading frame is presumably unaffected by expression of *RLG1* by the *GAL10* promoter, we conclude that disruption of *ORF5* has no effect on the UPR.

Cloning of the Mutant *RLG1* Allele

Genomic DNA was isolated from strain CF181. The genomic copy of the *RLG1* gene was amplified with Vent Polymerase (NEB, Beverly, MA) using three overlapping pairs of oligonucleotides. The PCR fragments were subcloned using the TA cloning method (Invitrogen, San Diego, CA) and sequenced. To distinguish the original mutation from mutations introduced during the PCR procedure, two independent reactions were performed, subcloned, and sequenced for each pair of oligos. Only one mutation was found in two independent PCR reaction subclones (C442T).

Site-Directed Mutagenesis

To reconstruct the C982T mutation, an oligonucleotide ending in an Alw26I site with the desired point mutation in combination with a second oligonucleotide covering a unique HpaI site 5′ to the site of mutation was used in PCR to amplify a 400 bp fragment of *RLG1*. The amplified fragment was then digested with HpaI and Alw26I. The latter restriction enzyme allows generation of a sticky end and removal of the enzyme recognition site from the digested PCR product. Similarly, a second 600 bp PCR fragment was generated using an oligonucleotide ending in an Alw26I site in combination with a second oligonucleotide covering a downstream BglII site in *RLG1*. Digestion of both PCR products with HpaI and Alw26I and with BglII and Alw26I, respectively, left two sticky ends that when ligated restored the wild-type *RLG1* sequence at the junction. Thus, we were able to introduce the fragment with the mutation in the absence of any suitable unique restriction site in its proximity. The HpaI–BglII fragment was then subcloned into a centromeric *HIS3*-containing vector (pRS313) bearing the rest of the *RLG1* coding sequence generating pCF158 (*rlg1*[H148Y]).

Northern Analysis

Total RNA was made according to a scaled-down version of the hot phenol method (Kohrer and Domdey, 1991). RNA was analyzed by electrophoresis in a 6.7% formaldehyde, 1.5% agarose gel and transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA). Hybridization was performed at 65°C overnight in 0.5 M Na_2HPO_4, 1 mM EDTA, 7% SDS [pH 7.5]. Quantitation of Northern blots was performed on a Molecular Imager System GS-363 (BioRad, Hercules, CA).

Detection of tRNA Halves and Intervening Sequences

Labeling of Cells

Strain EMPY439 was grown on minimal media lacking uracil and containing 2% galactose/2% raffinose. The culture was then diluted back into media containing either 2% dextrose or 2% galactose/2% raffinose for an additional six generations before labeling. Strain CF181 was grown on minimal media lacking leucine and containing 2% dextrose. Cells were harvested, washed, and resuspended at a density of 2×10^7 cells/ml in low phosphate medium (Warner, 1991) with the appropriate carbon source, adapted to low phosphate medium for one generation, and labeled with 0.1 mCi/ml [^{32}P]-orthophosphate for 30 min.

Analysis of RNA

Total RNA was isolated from cells and tRNA processing intermediates were displayed on two-dimensional polyacrylamide gels as previously described (Phizicky et al., 1992). Electrophoresis in the first dimension was through 10% polyacrylamide (39:1), 4 M urea in TBE, until the bromophenol blue dye front had migrated a distance of 23 cm. Electrophoresis in the second dimension was through 20% polyacrylamide, 7 M urea, until the xylene cyanol marker was just beginning to elute.

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Chapter III

The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response

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′

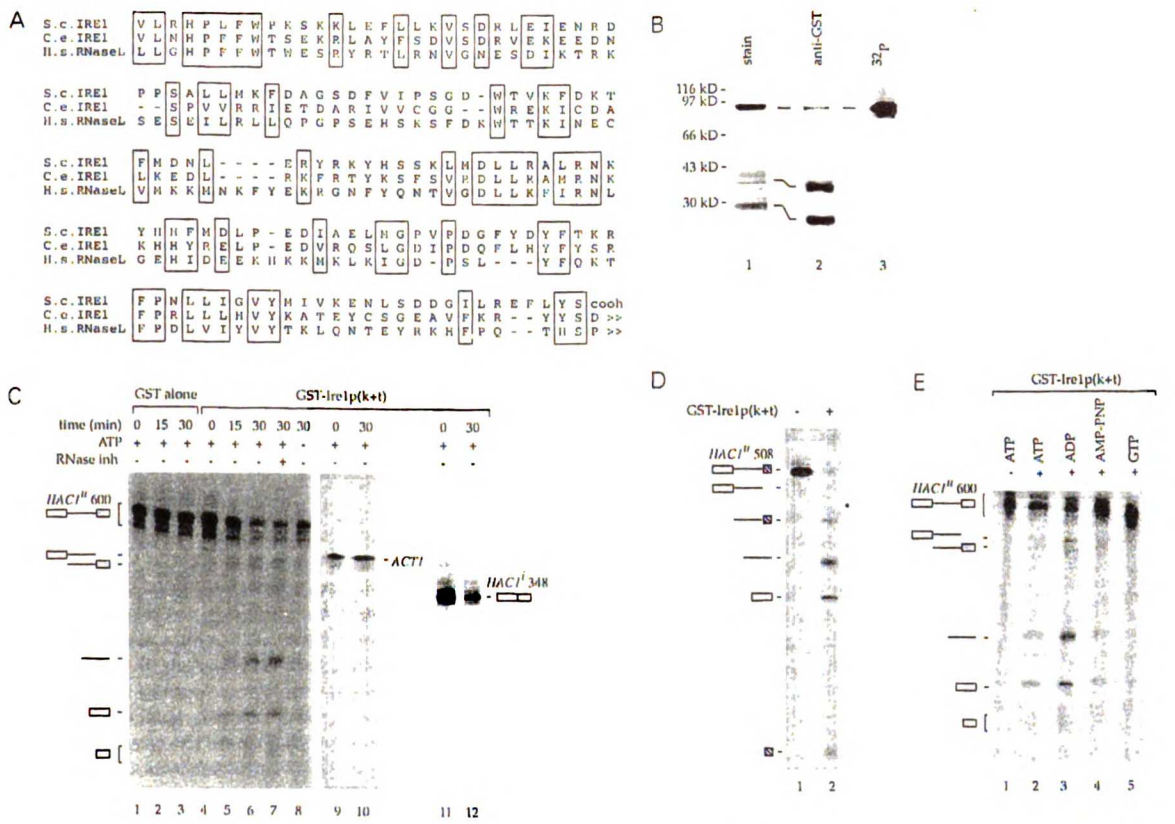

Figure 1. The Cytosolic/Nuclear Portion of Ire1p Has Both Kinase and Nuclease Activity In Vitro

(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

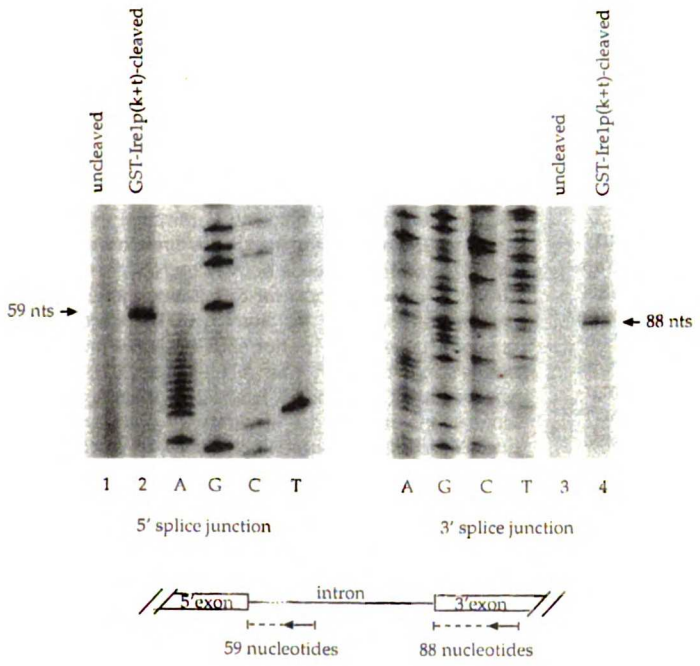

Figure 2. GST-Ire1p(k+t) Cleaves *HAC1*^{u} RNA at the Correct 5′ and 3′ Splice Junctions In Vitro

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

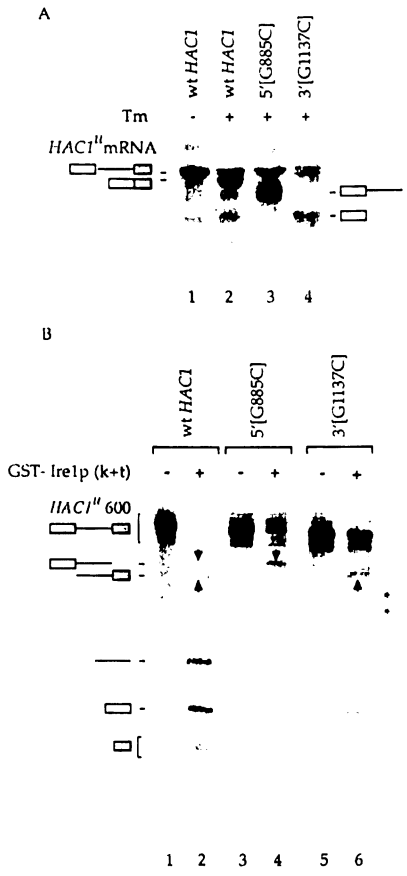

Figure 3. Independent Cleavage of *HAC1*^{u} mRNA 5′ and 3′ Splice Junctions

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,

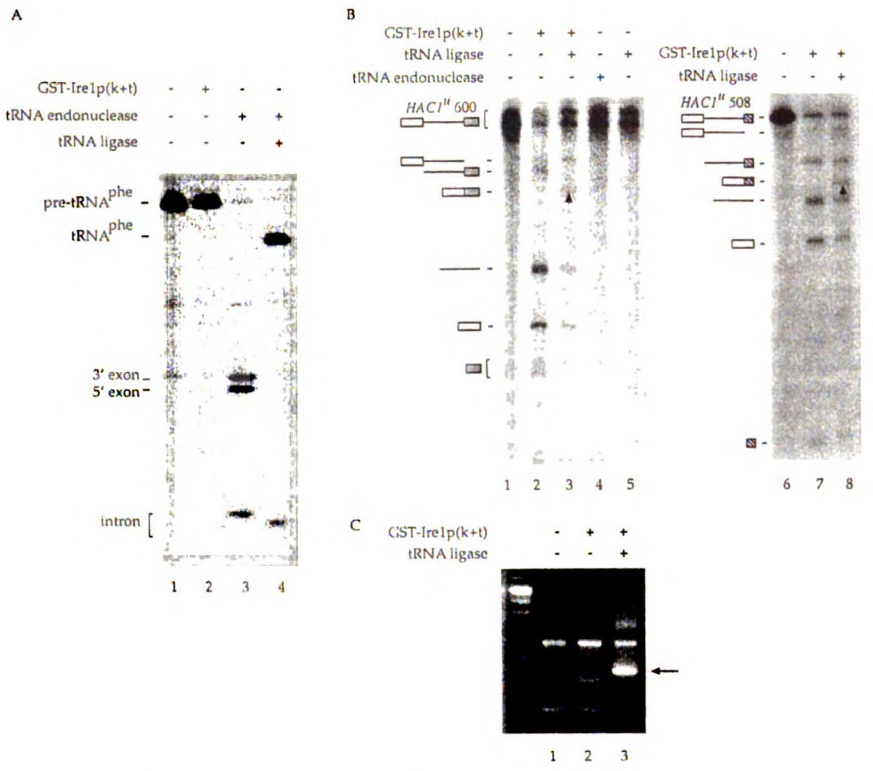

Figure 4. In Vitro Reconstitution of *HAC1*^{u} RNA Splicing

(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

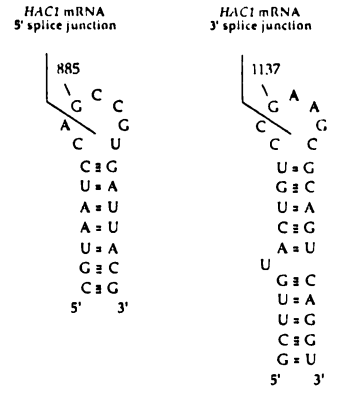

Figure 5. Secondary Structure Prediction of Both the 5' and 3' *HAC1* mRNA Splice Junctions

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.

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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.

Concluding remarks

Shortly after this work was published, Mori et al. showed that Ire1p cleaves *HAC1* mRNA *in vivo* 3′ of the conserved G in the seven-membered loop (Kawahara et al., 1998). In this paper we showed that Ire1p cleaves 5′ of this conserved G *in vitro*. In order to resolve this discrepancy, T. Gonzalez repeated the primer extension experiments designed to map the cleavage sites *in vitro* but utilized oligonucleotides complementary to sequences closer to the splice sites (unpublished results). These experiments confirmed Mori's results: Ire1p cleaves 3′ of the conserved G in the predicted splice junction stem-loops.

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Chapter IV

Preliminary characterization of the endoribonuclease activity of Ire1p

Introduction

The accumulation of unfolded proteins in the ER lumen results in splicing of the mRNA encoding the UPR-specific transcription factor Hac1p (Cox and Walter, 1996; Kawahara et al., 1997). Processing of *HAC1*^{u} mRNA (u for uninduced) is carried out by only two proteins: the transmembrane kinase/nuclease Ire1p and tRNA-ligase. Production of the spliced form, *HAC1*^{i} mRNA (i for induced), results in translation of Hac1p, as removal of the intron from *HAC1*^{u} mRNA is a prerequisite for its translation (Chapman and Walter, 1997). Ire1p is the transducer of the unfolded protein signal across the ER membrane. It somehow senses the accumulation of unfolded proteins in the ER lumen and transmits the information across the ER membrane. How such a signal transduction device functions remains an open question.

Ire1p belongs to a new class of hybrid kinase/nuclease signaling molecules. To date, the only other known member of this family is mammalian RNase L. These two proteins share a number of features (Figure IV-1). Structurally, they both contain a kinase domain followed by a conserved C-terminal tail extension that has been proposed to contain the nuclease active site. The nuclease activity of both Ire1p and RNase L is thought to be activated by oligomerization. Binding of 2'-5'-linked oligoadenylates (2-5A), small signaling molecules produced upon treatment of cells with interferon, drives dimerization and activation of RNase L *in vitro* (Dong and Silverman, 1995). In the case of Ire1p, oligomerization has been shown to occur *in vivo*, but it is not known whether the oligomerization state changes upon induction of the UPR (Shamu and Walter, 1996). The N-terminal portion of both molecules is involved in sensing the signal that leads to oligomerization and activation of the enzyme. In the case of RNase L, the N-terminal half is composed of nine ankyrin repeats,

only two of which are required for binding of 2-5A. It is thought that 2-5A binding causes a conformational change in RNase L that releases the inhibitory effect of the N-terminus on the catalytic domain (Dong and Silverman, 1997). The ligand of Ire1p has not been identified. The N-terminal half lies in the ER lumen, where it somehow detects changes in the concentration of unfolded proteins in this compartment.

RNase L and Ire1p also have several distinct features. Whereas Ire1p is a transmembrane protein that specifically cleaves *HAC1*^{u} RNA, RNase L is a soluble, rather nonspecific nuclease capable cleaving any single-stranded RNA 3' of UpUp and UpAp sequences (Dong et al., 1994; Sidrauski and Walter, 1997). The basis for this difference in substrate specificity is currently not understood. In addition, although a functional kinase domain is required for Ire1p function *in vivo*, no kinase activity has yet been demonstrated for RNase L.

How oligomerization and activation of the kinase domain of Ire1p result in induction of its nuclease activity is not known. There are many possible models to explain how Ire1p kinase activity could be instrumental in turning on its nuclease activity. Phosphorylation of Ire1p, for example, could enhance the affinity of Ire1p molecules for one another, which would result in activation if its endonuclease activity requires oligomerization. Alternatively, the kinase domain may directly phosphorylate the nuclease domain, thus activating it. A third model would assume that phosphorylation of Ire1p leads to release of a putative inhibitor that masks the nuclease function. Finally, phosphorylation may be required to relocalize Ire1p in the cell, for example, to the proximity of nuclear pores, so that it can interact with *HAC1*^{u} mRNA. In the last two scenarios, the kinase activity of Ire1p should be dispensable for its nuclease activity *in vitro*. It is also possible that the kinase activity of Ire1p may play more than one role during activation of the UPR.

The nuclease activity of RNase L is stimulated in the presence of adenosine nucleotides, including ATP, ADP, and the nonhydrolyzable ATP analog AMP-PNP (Dong et al., 1994; Krause et al., 1986). The same effect was observed for Ire1p, but in this case, a strict requirement for an adenosine cofactor for its nuclease activity was reported (Sidrauski and Walter, 1997). The effect of adenosine nucleotides on the nuclease activity of Ire1p is unlikely to reflect required phosphorylation events catalyzed by the kinase domain. Instead, these cofactors could aid directly in the chemistry of the hydrolysis reaction or might function indirectly, to stabilize a particular conformation after binding to either the ATP binding site of the kinase domain or elsewhere on the Ire1p molecule. The molecular basis of this adenyl nucleotide co-factor requirement by Ire1p and RNase L remains to be unraveled.

This chapter describes two different sets of experiments. One set was designed to identify the structural elements within *HAC1*^{u} mRNA that are required for Ire1p-mediated cleavage *in vitro*. Here, we demonstrate that the predicted stem-loop structures present in the splice junctions of *HAC1*^{u} mRNA are sufficient for cleavage by Ire1p. The second set of experiments was designed to determine the effects of phosphorylation and the oligomerization of Ire1p on its function as an endonuclease. These experiments do not provide an endpoint but are the initial steps in the characterization of this novel nuclease. An exiting new possibility revealed by this work is that the kinase domain may be required *in vivo* to couple the cleavage and ligation steps during processing of *HAC1*^{u} mRNA.

Results

The predicted stem-loop structures present at both splice junctions of *HAC1*^{u} mRNA are sufficient for Ire1p-mediated cleavage *in vitro*

Secondary structure modeling predicts similar stem-loop structures at both splice junctions of *HAC1*^{u} mRNA (Figure III-5). A fusion of Ire1p containing the kinase and tail domains (GST-Ire1p (k+t)) was shown to cleave *in vitro* at both junctions 3′ of the 3rd conserved G in the seven-membered loop. The substrate utilized in these experiments was a 508 nucleotide long RNA, *HAC1*^{u} 508 RNA, which contains both splice junctions and the intron of *HAC1*^{u} mRNA (Sidrauski and Walter, 1997). The presence of similar stem-loops at both junctions of *HAC1*^{u} mRNA led to the speculation that they are the minimal cis-elements that are recognized by the nuclease, Ire1p. To test this proposal, a 43 nucleotide long RNA which contains only the predicted 3′ splice junction stem-loop, *HAC1* 3′ splice-junction RNA, was synthesized *in vitro* in the presence of γ-[^{32}P] UTP. Incubation of this radioactively labeled substrate with GST-Ire1p (k+t) led to the appearance of two discrete cleavage products (Figure IV-2, lane 4). Recently, T. Gonzalez has shown that Ire1p cleaves at the correct junction within this smaller substrate. Similar results were obtained with a substrate containing the 5′ splice junction stem-loop (T. Gonzalez, unpublished results). Thus, the predicted stem-loops present at the splice junctions of *HAC1*^{u} mRNA are sufficient for Ire1p-mediated cleavage *in vitro*. In addition, as it was reported for cleavage of *HAC1*^{u} 508 RNA, cleavage of *HAC1* 3′ splice-junction RNA requires the presence of ATP (Figure IV-2, lane 1). Whether ATP can be replaced by ADP or a non-hydrolyzable analog in this reaction has not been tested. It is not known whether the *HAC1*^{u} mRNA splice junction stem-loops are also sufficient for cleavage by

Ire1p *in vivo*. Additional RNA secondary structure elements within $HAC1^u$ mRNA may be required to properly localize or present the substrate to Ire1p in the cell.

An Ire1p kinase impaired-mutant has only partial nuclease activity *in vitro*

Cleavage of $HAC1^u$ RNA *in vitro* requires the presence of an adenosine cofactor. ATP, ADP, or the non-hydrolyzable analog AMP-PNP but not GTP can activate the nuclease activity of Ire1p (Sidrauski and Walter, 1997). The lack of requirement for ATP hydrolysis suggested that the kinase activity of Ire1p is not required for cleavage of $HAC1^u$ RNA. However, a possible role for the kinase activity of Ire1p is to stabilize an oligomer conformation. Trans -autophosphorylation by neighboring molecules may be required for stable association. The GST-Ire1p (k+t) fusion may be active in the absence of ATP hydrolysis because it is constitutively dimerized. Indeed, this is likely to be case, as GST by itself forms homodimers (Lim et al., 1994). However, similar results were obtained with a fusion lacking GST. It is possible that the enzyme concentration used in the *in vitro* assays was sufficiently high to drive self-association. Alternatively, the kinase activity of Ire1p may be required to directly activate its nuclease domain. It could be that Ire1p as purified from *E.coli* is already, at least partially, phosphorylated at functionally important residues and thus does not require hydrolyzable ATP. In the absence of phosphatases, this phosphorylation could be stable.

In order to elucidate the role of the kinase domain of Ire1p, we generated and purified a GST-Ire1p (k+t) fusion in which the kinase activity has been impaired. GST-Ire1p K702A (k+t) bears a single amino acid substitution in the catalytic lysine residue (702) that is conserved among most kinases and is positioned in the active site. Yeast cells expressing Ire1p-K702A as their only

version of Ire1p show an *ire1* null phenotype: they are severely compromised in their ability to carry out the UPR (Mori et al., 1993). Moreover, unlike cells carrying wild-type Ire1p, no phosphorylation of Ire1p-K702A is observed upon induction of the pathway (Shamu and Walter, 1996). These data indicate that Ire1p-K702A has an inactive kinase domain and that the kinase activity is required for a functional UPR. As expected, the K702A mutation also impairs the kinase activity of Ire1p *in vitro* (data not shown) (Welihinda and Kaufman, 1996)

The ability of GST-Ire1p (k+t) K702A to cleave either *HAC1*^{u} 3' splice-junction RNA or *HAC1*^{u} 508 RNA was tested. In contrast to the wild-type GST fusion, no cleavage of the *HAC1* 3'splice-junction RNA substrate was observed upon incubation with GST- Ire1p (k+t) K702A (Figure IV-2, lane 3). A slightly different result is observed with *HAC1*^{u} 508 RNA. Partial cleavage of this substrate by GST-Ire1p (k+t) K702A can be detected (Figure IV-3, compare lanes 2 and 5). At the range of concentrations tested, the activity of the mutant was consistently lower than that of wild-type Ire1p (Figure IV-3, lanes 2-7). These results suggest that the kinase activity of Ire1p is required *in vitro* for its activation as a nuclease. A caveat for this interpretation is that the K702A mutation, which is located in the ATP binding site, may affect both the ability of the mutant protein to phosphorylate itself and its ability to bind a cofactor (ATP or ATP analogs). Thus, we cannot distinguish whether the effects of the mutation are due to its lack of phosphorylation or impaired ATP binding.

The observation that Ire1p-K702A cannot cleave the *HAC1*^{u} 3' splice-junction RNA is not surprising. As compared to *HAC1*^{u} 508, a considerably higher amount of wild-type protein has to be added to the nuclease reaction in order to detect cleavage of this RNA. This is likely to reflect Ire1p's lower affinity for this minimal substrate.

Interestingly, in the absence of cofactor, the nuclease activities of the K702A mutant and wild-type Ire1p fusion proteins are the same (Figure IV-3, lanes 8 and 9, as compared with lanes 2 and 5). Only the nuclease activity of the wild-type protein can be induced by addition of ATP or ADP (Figure IV-4). The observation that the nuclease activity of GST-Ire1p (k+t) K702A cannot be induced by addition of ADP (the stimulatory effect of this nucleotide cannot be due to protein phosphorylation) suggests that cofactor binding is affected by the K702A mutation. In addition, it indicates that these nucleotides are likely to bind to the ATP binding site of the kinase domain, where this mutation is located, and not elsewhere in the Ire1p molecule. An alternative and less exciting interpretation is that this mutation drastically affects the overall folding of the molecule. Finally, it is also possible that in the absence of phosphorylation, the nuclease activity of Ire1p cannot be stimulated by addition of a cofactor.

The discrepancy between previous experiments that showed no cleavage of *HAC1*^{u} RNA in the absence of cofactor and the partial cleavage reported here is probably due to differences in protein concentration and specific activity of the protein preparations.

In vitro inter-allelic complementation of Ire1p-K702A

As discussed in the previous section, the lack of nuclease activity of the Ire1p-K702A mutant protein may result from its lack of phosphorylation or impaired cofactor binding. We reasoned that if we could phosphorylate the Ire1p-K702A protein in trans, we could specifically assess the stimulatory role of this modification on its nuclease activity. To this end, we asked whether a kinase active, nuclease inactive mutant of Ire1p would complement GST-Ire1p (k+t) K702A. If successful, such inter-allelic complementation would indicate that phosphorylation of Ire1p stimulates its ribonucleolytic activity.

As a first step, a fusion of Ire1p containing the kinase but not the tail or putative nuclease domain, Ire1p (k), was expressed and purified. This mutant protein was expected to have kinase activity, as it contains an intact kinase domain but no ribonucleolytic activity. This was based on the observation that a tail-less mutant of Ire1p is able to phosphorylate itself *in vivo* but the UPR is abolished(Shamu and Walter, 1996). As expected, GST-Ire1p (k) is able to autophosphorylate *in vitro* (Figure IV-5, lane 6), confirming that it is an active kinase. Furthermore, GST-Ire1p (k) cannot cleave *HAC1*^{u} 508 RNA (data not shown, see Table IV-1). Thus, the tail domain of Ire1p is required *in vitro* for cleavage of *HAC1*^{u} RNA.

Incubation of wild-type Ire1p (k+t) with GST- Ire1p K702A results in phosphorylation of both the wild-type and mutant proteins (Figure IV-5, lane 1). This result confirms that under our assay conditions, wild-type Ire1p can trans-autophosphorylate the Ire1p kinase mutant fusion protein. However, when the GST-Ire1p (k+t) K702A mutant is incubated in the presence of GST-Ire1p (k), a very small amount of trans-phosphorylation is detected (Figure IV-5, lane 5). This result is not due to the presence of GST in the tail-less Ire1p fusion protein (data not shown). It is likely that due to the absence of the tail, Ire1p (k) cannot associate with the full-length mutant protein. This is in contrast with inter-allelic complementation experiment performed *in vivo*, where tail-less Ire1p is able to trans-phosphorylate a full-length mutant Ire1p protein(Shamu and Walter, 1996). In this case, however, the presence of the N-terminal domain may contribute to their association.

In an alternative approach, we used wild-type Ire1p (k+t) to phosphorylate the GST-Ire1p (k+t) K702A mutant protein that is bound to glutathione beads. We reasoned that we could first phosphorylate the K702A mutant and then wash off the wild-type fusion from the column. Unfortunately,

after phosphorylation of GST-Ire1p (k+t) K702A, we were unable to completely remove Ire1p (k+t). As seen in Figure IV-5 (lanes 2-4), high salt washes did partially remove the wild-type fusion, but a significant amount remains bound to the beads. In the later washes, GST-Ire1p (k+t) K702A also came off the beads. Thus, to date, we have been unable to establish conditions that would allow us to decipher the role of phosphorylation on Ire1p's nuclease activity.

A third ongoing approach involves the generation of point mutations in the nuclease or tail domain of Ire1p that impair its nuclease but not its kinase activity. As they contain the tail domain, these mutants should, like the wild-type full-length protein, be able to trans-autophosphorylate the Ire1p K702A mutant protein. Ire1p shows no sequence similarity to other ribonucleases besides RNase L, which has remained poorly characterized enzymatically. Thus, there is no information available, experimentally or in comparison to other hydrolases, regarding the active site. We reasoned that the residues involved in RNA catalysis should be conserved among mammalian RNase-L, *S. cerevisiae* Ire1p, *c. elegans* Ire1p and human Ire1p (which is currently being cloned and sequenced by M. Niwa in the laboratory). Mutants in these conserved residues were generated and their kinase and nuclease activities are currently being tested.

Activation of Ire1p's RNase activity may result from phosphorylation of the nuclease or tail domain of Ire1p. However, to date, there is no evidence that phosphorylation of the tail domain occurs either *in vivo* or *in vitro*. Incubation of GST-Ire1p (k+t) with a fusion of Ire1p containing this C-terminal tail, GST-Ire1p (t), does not result in its phosphorylation. This may imply that this domain is not a substrate of the kinase activity of Ire1p. A less appealing explanation is that this domain cannot fold properly in the absence of the kinase domain. Furthermore, GST-Ire1p (t) does not have any intrinsic nuclease activity (Table IV-1). Although

this portion of the molecule is critical for RNase activity, it is not sufficient. This is also the case with RNase L (Dong and Silverman, 1997).

Ire1p (k+t) oligomerizes in the presence of ATP

In vivo inter-allelic complementation and co-immunoprecipitation indicate that Ire1p oligomerizes. Similar to other transmembrane kinases, oligomerization of Ire1p has also been proposed to be instrumental in activation of the UPR (Shamu and Walter, 1996). To begin to elucidate its role in Ire1p activation, we decided to monitor the oligomerization state of Ire1p (k+t). To this end, we fractionated the purified protein by sucrose density centrifugation (5-20% gradient). Aliquots of the gradient were then analyzed by SDS-PAGE electrophoresis. In the absence of ATP, the peak of the protein profile migrated in fraction #7 (Figure IV-6 A). In contrast, when Ire1p (k+t) is first preincubated with 2 mM ATP and then loaded unto a gradient also containing ATP, the peak reproducibly shifted to fraction #8 (Figure IV-6 B). This shift suggests that the protein oligomerizes in the presence of this nucleotide. Additional studies are required to determine the number of molecules present in the complex but, by comparing the elution volumes with those of protein markers, a shift of only one fraction is suggestive of formation of dimer. In addition, the appearance of a faster migrating band on the SDS-polyacrylamide gel is observed (marked with an asterisk). This band must result from a covalent modification of Ire1p (k+t), such as phosphorylation. This result suggests that dimerization of Ire1p is stabilized by its phosphorylation. This explains the appearance of a faster migrating band on the gel and the concomitant shift to a later fraction in the gradient. An obvious next step is to monitor the behavior of Ire1p (k+t) after addition of ADP or a nonhydrolyzable ATP analog. If phosphorylation is required for dimerization, addition of these nucleotides should not induce the

conversion to a dimeric state. If, however, cofactor binding is sufficient to induce a conformational change that results in dimerization, addition of ADP or a nonhydrolyzable ATP analog should have the same effect as addition of ATP. These experiments could help elucidate the effects of phosphorylation and cofactor binding on oligomerization of Ire1p. In addition, by measuring the specific nuclease activities of the monomer and dimeric Ire1p populations, we may reveal that activation of Ire1p RNase activity requires its oligomerization.

No changes in the protein profile of GST-Ire1p (k+t) were observed upon addition of ATP (data not shown). Moreover, the protein eluted in a significantly later fraction in the gradient. This result suggests that this protein is constitutively dimerized. This is not unexpected, as GST is known to form homodimers.

The *HAC1^u* mRNA cleavage and ligation steps appear to be uncoupled in *ire1-K702A cells*

Cells expressing only Ire1p-K702A display an *ire1* null phenotype (Mori et al., 1993). For example, no *KAR2* mRNA induction, a target of the UPR, is observed in the presence of tunicamycin in a strain carrying *ire1-K702A* as the only copy of *IRE1* (Figure IV-7 B, compare lanes 3 and 4 with lanes 5 and 6). To date, splicing of *HAC1^u* mRNA had not been looked at in a strain expressing this mutant form of Ire1p. The expectation was that the *HAC1^u* mRNA splicing phenotype of an *ire1-K702A* mutant strain would be identical to that of an *ire1* strain (Figure IV-7 A, lanes 3 and 4). Surprisingly, this is not the case. Partial cleavage of *HAC1^u* mRNA is detected in the mutant (Figure IV-7 A, lanes 5 and 6). However, whereas in a wild-type strain, *HAC1^u* mRNA is processed to the smaller spliced *HAC1^i* mRNA (Figure IV-7 A, lanes 9 and 10), in the mutant, this conversion does not take place. Instead, the accumulation of splicing

intermediates is detected. This suggests that cleavage and ligation are somehow uncoupled in *ire1-K702A* cells. The unregulated cleavage of *HAC1^u* mRNA in the mutant strain is probably due to expression of Ire1p-K702A from a high-copy plasmid and not a consequence of the mutation in Ire1p.

The observation that *HAC1^u* RNA halves accumulate in *ire1-K702A* cells suggests a new role for the kinase activity of Ire1p. One possibility is that phosphorylation of Ire1p or tRNA ligase may be required for their association. The formation of a splicing complex ensures coupling of a series of RNA processing reactions. It has not been determined whether these two proteins can form a complex but we consider it likely. As was the case for previous experiments that utilize this mutant, we do not know whether the splicing phenotype observed is only a consequence of its lack of kinase activity. Thus, we studied the phenotype of other mutations in Ire1p that have also been proposed to affect the kinase function.

A second previously characterized kinase mutant is Ire1p-K702R (Shamu and Walter, 1996). Induction of the UPR target genes of the response in a strain carrying *ire1-K702R* as its only copy is 25% that of strain carrying a wild-type copy. Thus, in contrast to *ire1-K702A* cells, cells expressing Ire1p-K702R exhibit a diminished UPR. The *HAC1^u* mRNA splicing phenotype of these two mutants also differs. No accumulation of cleaved fragments is detected in *ire1-K702R* cells (Figure IV-7 A, lanes 7 and 8). Instead, the fraction of *HAC1^u* mRNA that is cleaved is converted to *HAC1^i* mRNA. Assuming that the splicing phenotype in these mutants reflects the activity of the kinase in each, these data suggest that Ire1p-K702R has residual activity that is sufficient to restore ligation of the intermediates generated.

In addition we analyzed the *HAC1^u* mRNA splicing phenotype of two other mutants. Their mutations, however, do not lie in Ire1p's kinase active site

but are located in putative activating phosphorylation sites. The CDK kinases and many transmembrane tyrosine kinases have activating phosphorylating sites on a protein loop that stretches between conserved kinase domains VII and VIII. Ire1p has four conserved serine and threonine residues in this location. Shamu and Walter tested the UPR activity of two mutants, Ire1p-S840A/S841A and Ire1p-T844A, in these conserved residues. Whereas the double mutant virtually eliminated the UPR, the single mutant only reduced the response 30% (Shamu and Walter, 1996). These results suggested that these residues correspond to Ire1p's activating phosphorylation sites. As with other mutants, it is possible that these phenotypes result from structural problems in the kinase domain that destroy its activity. As seen in Figure IV-8 (lanes 3-6), both mutants had partial nuclease activity. The double mutant had a more drastic phenotype that the single mutant but in both cases spliced *HAC1*^{i} mRNA was detected. Thus, the accumulation of splicing intermediates is a phenotype that is specific to the K702A mutation in *IRE1*.

Discussion

In this chapter we have shown that the predicted stem-loop structures present at both splice junctions of *HAC1*^{u} mRNA are sufficient for Ire1-mediated cleavage *in vitro*. This is an important first step in the identification of the cis-elements in *HAC1*^{u} mRNA that are required for recognition by Ire1p. Three nucleotides are conserved within the predicted seven-membered loops (Figure IV-10). One nucleotide corresponds to the G located 5′ to the cleavage site. This G is essential for cleavage both *in vivo* and *in vitro*. (Sidrauski and Walter, 1997) The other two conserved nucleotides have been shown to be required for cleavage *in vivo*, confirming the role of the nucleotide sequence in the junctions of *HAC1*^{u} mRNA (Kawahara et al., 1997). Thus, in contrast to tRNA endonuclease, Ire1p

appears to be a sequence-specific nuclease. More work needs to be done in order to define the important nucleotide sequences and secondary structure elements within these stem-loops that are necessary for recognition by Ire1p. The ease with which it is possible to generate substitutions and deletions in the 5′ and 3′ splice-junction RNAs should significantly facilitate this task. These small substrates are synthesized by *in vitro* transcription of oligonucleotides that also contain the T7 promoter. Thus, the generation of mutations does not involve the time-consuming site-directed mutagenesis and cloning steps. The information obtained may be useful in the search for other substrates that are cleaved by this nuclease either in yeast or higher organisms. Moreover, the mutants generated will be invaluable tools in the analysis of the mechanism of the Ire1p-mediated cleavage reaction.

It is currently not known whether these splice junction stem-loops are also sufficient for cleavage by Ire1p *in vivo*. Transplantation of the splice-junctions of *HAC1*^{u} mRNA to a "naïve" mRNA will allow us to determine if this is indeed the case. There may be other elements within *HAC1*^{u} mRNA that are required for recognition by Ire1p in the cell. The intron of *HAC1*^{u} mRNA is also predicted to contain two other stems-loops (Figure IV-9). The intron may be important for localization, transport, or presentation of the substrate to the splicing machinery.

Although the experiments addressing the role of phosphorylation and oligomerization of Ire1p on its activation as a nuclease are inconclusive, they provide a framework for further experimentation. Here, we show that the kinase-impaired mutant, Ire1p-K702A, has only partial nuclease activity *in vitro*. However, we have been unable to show that the decrease in activity is due to its lack of phosphorylation. The inter-allelic complementation experiments proposed earlier may allow us to assess the role of this modification on induction of Ire1p. Such an approach proved to be very useful in the analysis of different

mutants of Ire1p *in vivo*. An alternative explanation is that the K702A mutation affects cofactor binding to Ire1p. We can directly address this possibility by comparing the binding affinities for ATP of the wild-type and mutant Ire1p fusion proteins.

The recent discovery of an Ire1p-specific phosphatase, Ptc2p, provides us with a new and powerful tool to test the role of phosphorylation (Welihinda et al., 1998). We consider it likely that the lack of a requirement of a hydrolyzable adenosine nucleotide for nuclease activity may result in part from the fact that a fraction of the Ire1p molecules are already phosphorylated. We can directly test this by adding recombinant Ptc2p phosphatase to the cleavage reaction. If such treatment results in a decrease in the amount of cleaved *HAC1* RNA, it would provide strong evidence for a role of phosphorylation in its activation as an RNase.

If we can establish that the kinase activity of Ire1p is required *in vitro* for its nuclease activity, we would then like to understand how phosphorylation of Ire1p results in its activation. The data presented here are consistent with the idea that addition of ATP leads to oligomerization of Ire1p (k+t). The appearance of a faster migrating band in the fraction corresponding to the putative dimer population suggests than in the process of oligomerization, Ire1p becomes phosphorylated. In analogy to other transmembrane kinases, we favor a model in which phosphorylation of Ire1p (k+t) stabilizes the dimer conformation. The generation of Ire1p-fusion proteins that contain dimerization modules such as FKBP2 may be very useful in analyzing the effects of dimerization on its function as a nuclease. The advantage of this approach is that we can manipulate the dimerization state experimentally, irregardless of the nucleotide occupancy or phosphorylation state of Ire1p.

The *HAC1^u* mRNA splicing phenotype observed in a strain expressing Ire1p-K702A was very surprising. The uncoupling observed between the cleavage and ligation steps suggests a new role for the kinase activity of Ire1p. We favor a model in which phosphorylation of Ire1p is required for association with tRNA ligase. Recently, we expressed and purified an active GST fusion of tRNA ligase and should be able to test this possibility directly. If this model is correct, *in vitro* binding of Ire1p (k+t) to tRNA ligase should require pre-incubation with ATP. Conversely, addition of the Ptc2p phosphatase to the binding reaction should block their association. Moreover, if the splicing phenotype of the *ire1-K702A* mutant is due to Ire1p's lack of phosphorylation, the expectation is that the *HAC1^u* mRNA splicing phenotype of a strain over-expressing Ptc2p should be the same.

Activation of Ire1p's nuclease activity has to be tightly regulated by conditions in the ER lumen. It is becoming evident that in order to understand how this activation occurs, we need to elucidate how the different states of this protein affect its nuclease activity. Oligomerization, phosphorylation, and nucleotide occupancy are all likely to play a role in this process. As shown in this chapter, assays employing a combination of wild-type and mutant kinases with ATP and non hydrolyzable analogs should allow us to gain insights regarding the molecular mechanism of activation of this novel transmembrane kinase/nuclease. The ultimate goal is to understand how Ire1p senses the signal from unfolded proteins in the ER lumen and how it transduces this information across the membrane.

Materials and Methods

Plasmids and expression of GST-Ire1p fusions

The GST-Ire1p (k+t) (Ire1p-C-terminal domain, amino acids 556-1115) construct (pCF-210) was made by subcloning the Ire1p fragment from pCS116 (Sidrauski and Walter, 1997) into pGEX-6P-2. This construct contains a precission protease cleavage site for removal of the GST moiety. The GST-Ire1p (k+t)–K702A and GST-Ire1p (k) constructs were generated by PCR, and the PCR fragments were subcloned into pGEX-6P-2 (pCF-211 and pCF213 respectively). These plasmids were transformed into the DH-5-α strain of *E.coli*. Cells were induced for 4 hr at 35°C with 0.1 mM IPTG, and the fusion proteins were purified using glutathione-Sepharose beads from Pharmacia. Ire1p (k+t) was eluted from the beads by addition of 40units of GST-Precission Protease (Pharmacia). Only Ire1p (k+t) was found in the eluate of an overnight incubation (4°C), as this protease-fusion remains bound to the beads.

Kinase Assays

The *in vitro* kinase assays are performed for 30 min. at 30°C in kinase buffer (20 mM Hepes, 250 mM KOAc, 1 mM DTT, 10 mM $Mg(OAc)_2$) in the presence of 100 μM ATP and 50 μCi of [γ-^{32}p]ATP (Amersham). When assaying trans-phosphorylation of GST-Ire1p (k+t)-K702A, no KOAc was added to the reaction. We found that in low salt, the level of phosphorylation of the mutant protein increases.

In vitro transcription and cleavage of *HAC1^u* RNA

In vitro transcription and *HAC1^u* RNA cleavage reactions are carried out as described by Sidrauski and Walter, 1997. Instead of using a linearized plasmid, the template for synthesis of *HAC1^u* 3′ splice-junction RNA is a gel-purified oligonucleotide containing the 3′ splice junction sequence fused to the T7 promoter (5′TGAGGTCAAACCTGACTGCGCTTCGGACAGTACA

AGCTTGACCTATAGTGAGTCGTATTA3'). A second oligonucleotide containing only the complementary T7 promoter sequence (5'TAATACGACTCACTAT3') is used as a primer for transcription from this template. Fifteen picomoles of the T7 oligonucleotide and 0.25 picomoles of the 3' splice junction-containing oligonucleotide were hybridized by heating to 65°C for 3 minutes and subsequently transfering them to ice.

Sucrose gradient ultracentrifugation

Ten µg of purified Ire1p (k+t) fusion was loaded on a 5-20% sucrose gradient in kinase buffer (250mM KOAc) and spun for 16 hr at 55,000 rpm at 4°C in a Beckman L8-M ultracentrifuge (SW60 rotor). The pre-incubation step with 2 mM ATP was performed in kinase buffer at 30°C for 30 min.

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Table IV-1. Kinase and nuclease activities of different forms of Ire1p

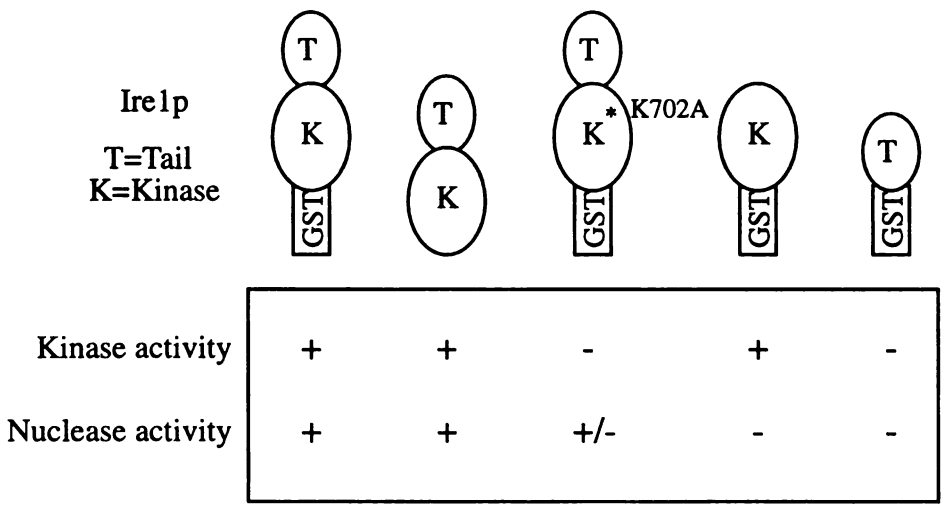


Figure IV-1. Similarities between Ire1p and RNase L

Both RNase L, a soluble protein, and Ire1p, a transmembrane protein, contain a kinase domain (K) followed by a C-terminal tail domain (T) that is required for nuclease activity. These two domains show significant amino-acid sequence similarity. The N-terminal regions of both proteins, which show no homology, function to sense the upstream signal in their respective pathway. Binding of 5′ phosphorylated, 2′-5′linked oligoadenylates (2-5A) to two ankyrin repeats in the RNase L molecule induces oligomerization and activation of the nuclease domain. Similarly, accumulation of unfolded proteins in the ER lumen leads to oligomerization and activation of the kinase and nuclease activities of Ire1p.

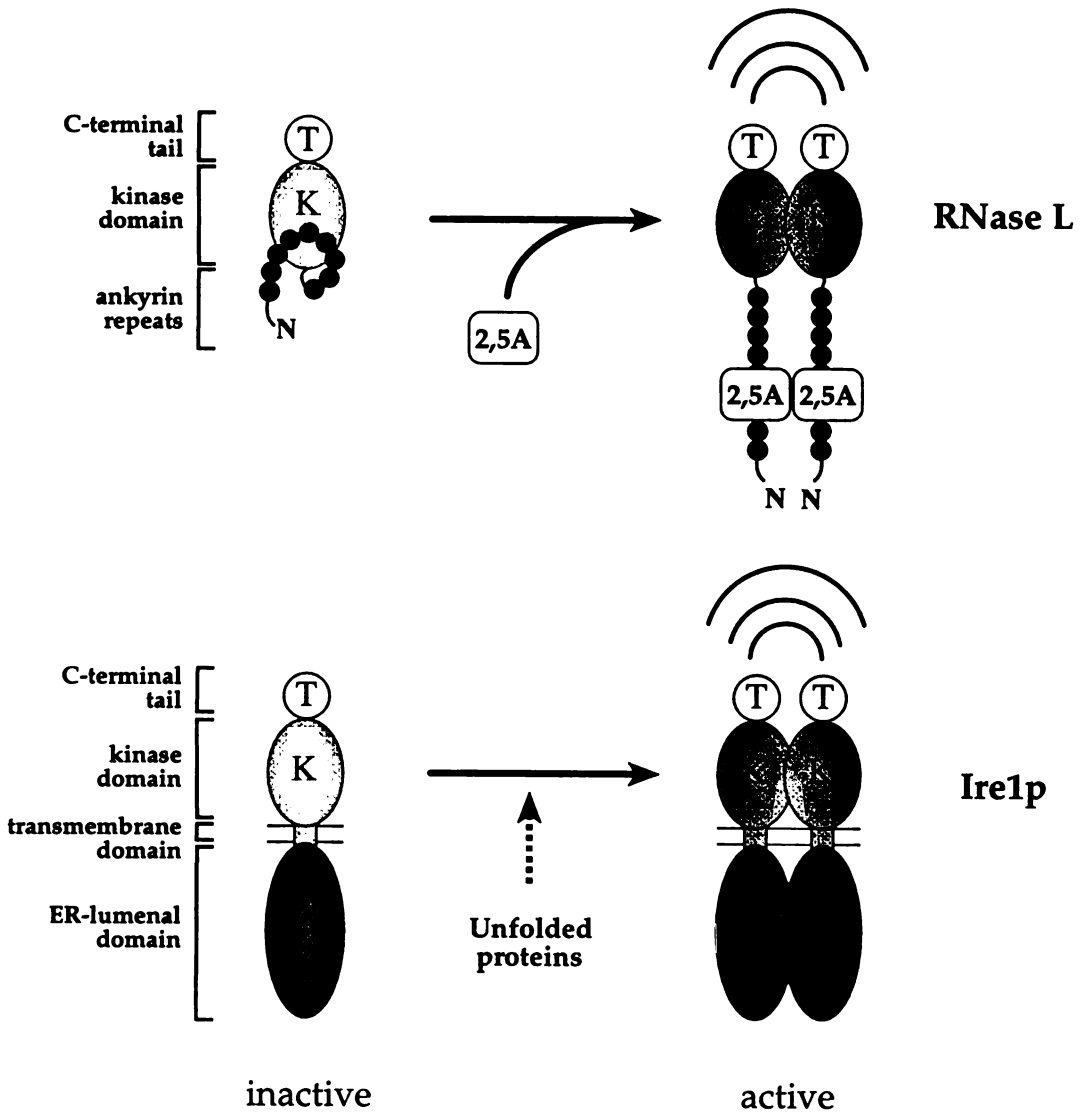

C-terminal tail
kinase domain
ankyrin repeats
T
K
N
2,5A
T
T
2,5A
2,5A
N N
RNase L
C-terminal tail
kinase domain
transmembrane domain
ER-lumenal domain
T
K
Unfolded proteins
T
T
Ire1p
inactive
active

Figure IV-2. The predicted 3′ splice junction stem-loop is sufficient for Ire1p-mediated cleavage *in vitro.*

GST-Ire1p (k+t) (lanes 2 and 4) or GST-Ire1p (k+t) K702A (lanes 1 and 3) were incubated with radioactively labeled *HAC1*^{u} 3′ splice junction RNA in the absence (lanes 1-2) and presence of ATP (lanes 3-4). The products of the reaction were fractionated on a denaturing 15% polyacrylamide gel. The icons indicate the different products of the cleavage reaction.

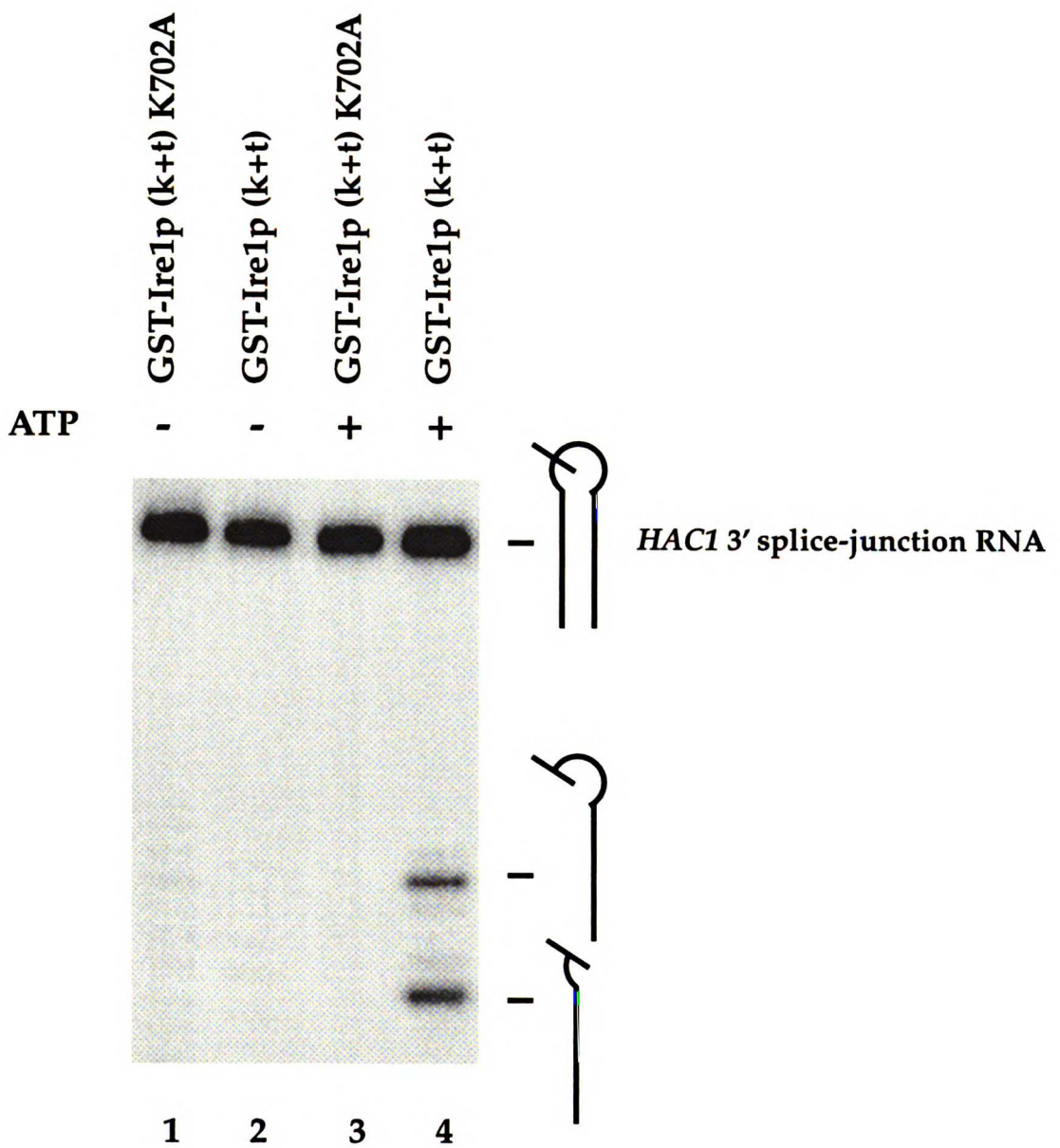

GST-Ire1p (k+t) K702A
GST-Ire1p (k+t)
GST-Ire1p (k+t) K702A
GST-Ire1p (k+t)
ATP
-
-
+
+
HAC1 3′ splice-junction RNA
1
2
3
4

Figure IV-3. GST- Ire1p (k+t)-K702A has partial nuclease activity

Different amounts of GST-Ire1p (k+t) (lanes 2-4 and 9) or GST-Ire1p (k+t) K702A (lanes 5-8) were incubated with *in vitro* transcribed $HAC1^{u}$ 508 RNA. The products of the reaction were fractionated on a denaturing 5% polyacrylamide gel.

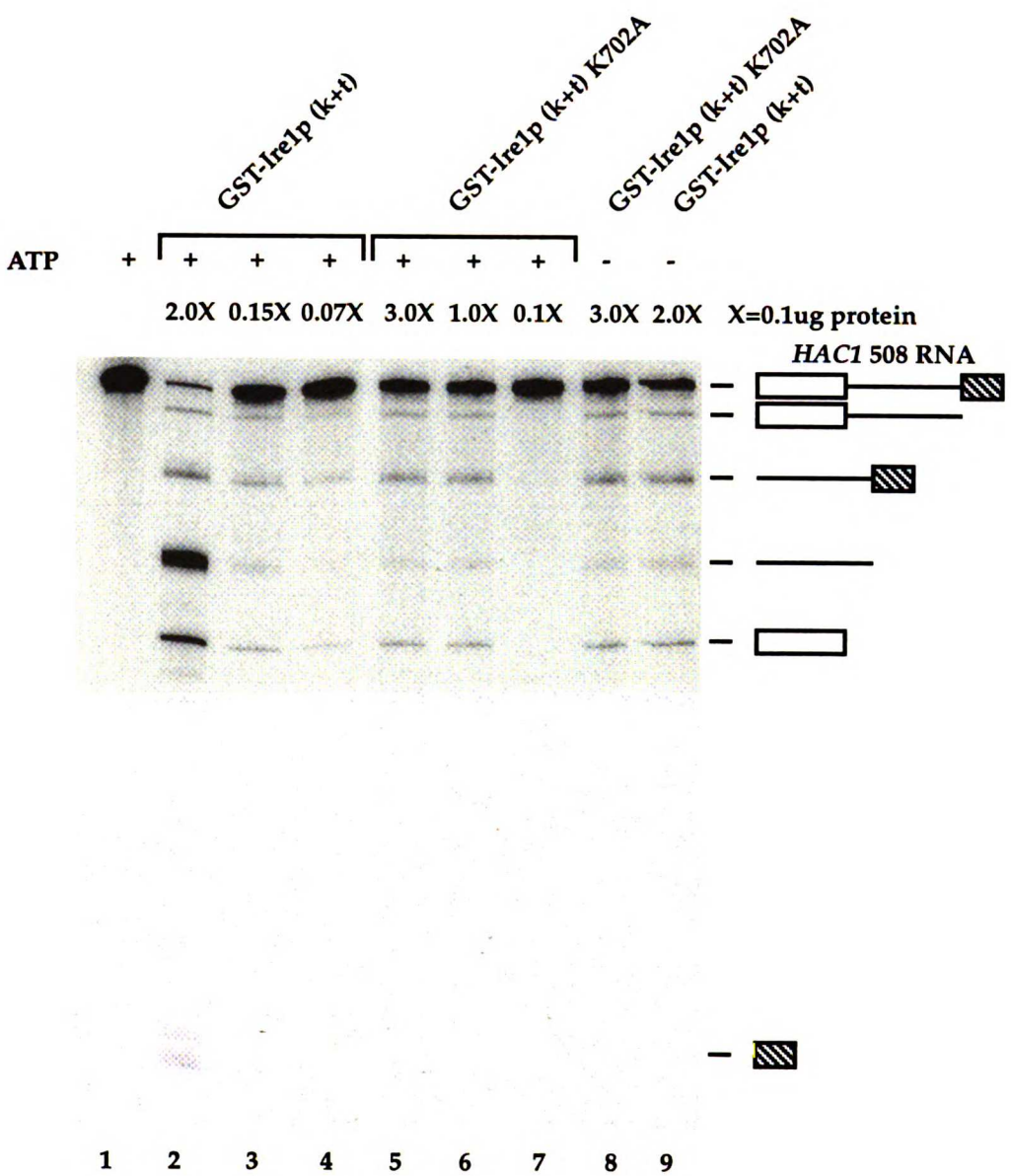

GST-Ire1p (k+t)
GST-Ire1p (k+t) K702A
GST-Ire1p (k+t) K702A
GST-Ire1p (k+t)
ATP + + + + + + + - -
2.0X 0.15X 0.07X 3.0X 1.0X 0.1X 3.0X 2.0X X=0.1ug protein
HAC1 508 RNA
1 2 3 4 5 6 7 8 9

Figure IV-4. The nuclease activity of GST-Ire1p (k+t) K702A is not stimulated by addition of adenosine nucleotides.

GST-Ire1p (k+t) K702A (lanes 1-3) or GST-Ire1p (k+t) (lanes 4-6) were incubated with *HAC1*^{u} 508 RNA in the absence of cofactor (lanes 2 and 5), in the presence of 2 mM ATP (lanes 1 and 4), or 2 mM ADP (lanes 3 and 6). The products of the reaction were fractionated on a denaturing 5% polyacrylamide gel.

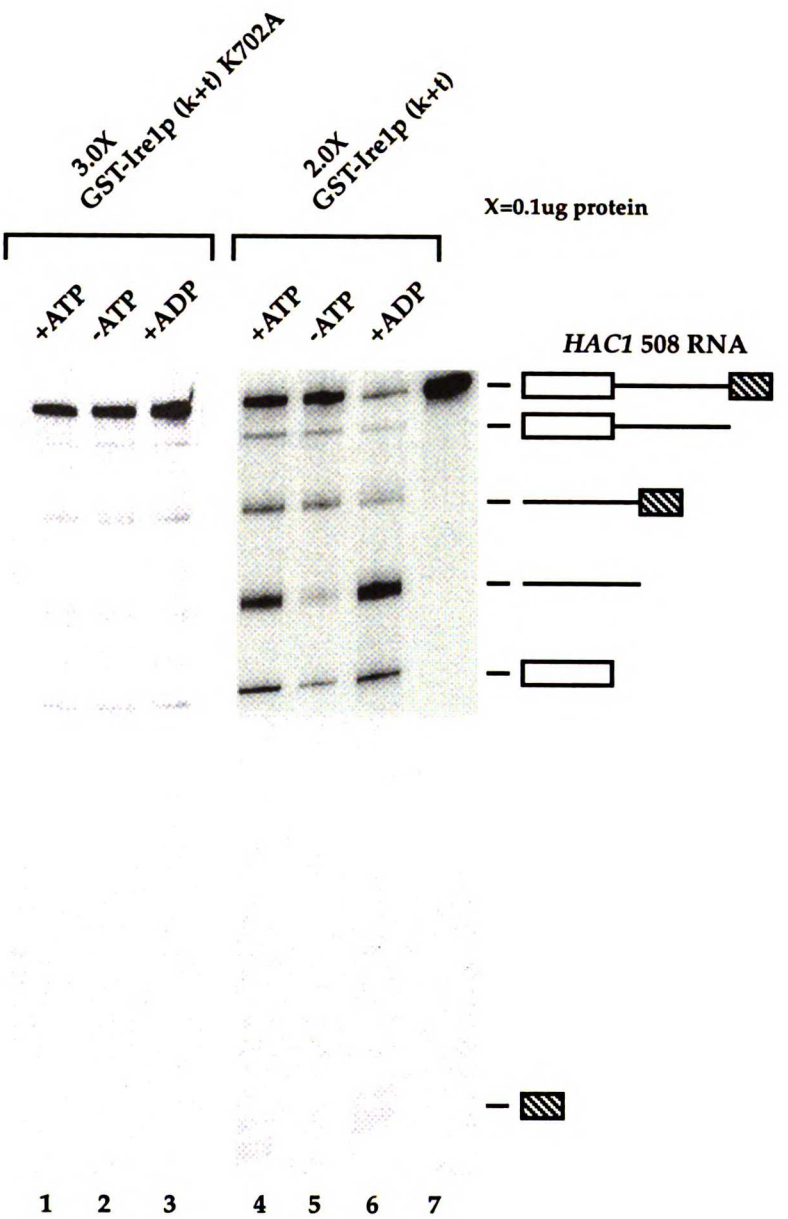

3.0X
GST-Ire1p (k+t) K702A
2.0X
GST-Ire1p (k+t)
X=0.1ug protein
+ATP
-ATP
+ADP
+ATP
-ATP
+ADP
HAC1 508 RNA
1
2
3
4
5
6
7

Figure IV-5. Trans-phosphorylation of GST-Ire1p (k+t) K702A

Trans-phosphorylation was assayed by incubating equivalent amounts of purified Ire1p (k+t) and GST-Ire1p (k+t) K702A bound to glutathione-sepharose beads in kinase buffer (without salt) in the presence of [γ^{32}P] ATP (lane 1). The proteins were then TCA precipitated and loaded on an SDS-polyacrylamide gel. Lanes 2-5 correspond to the eluates of three consecutive high-salt washes of the beads containing this mixture. In lane 5, GST-Ire1p (k) was added to the GST-Ire1p (k+t) K702A-containing beads.

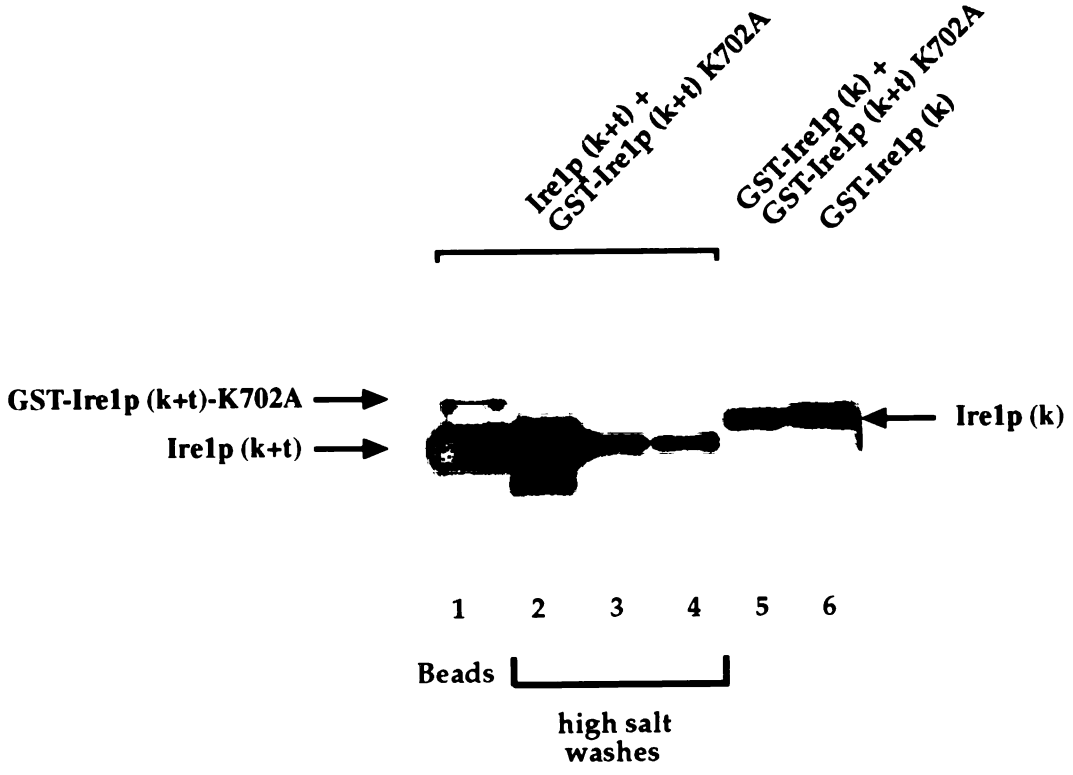

Ire1p (k+t) +
GST-Ire1p (k+t) K702A
GST-Ire1p (k) +
GST-Ire1p (k+t) K702A
GST-Ire1p (k)
GST-Ire1p (k+t)-K702A
Ire1p (k+t)
Ire1p (k)
1
2
3
4
5
6
Beads
high salt
washes

Figure IV-6. Ire1p (k+t) oligomerizes in the presence of ATP

Purified Ire1p (k+t) was loaded unto a 5-20% sucrose gradient in kinase buffer in the absence (panel A) or presence (panel B) of 2mM ATP. The protein fractions were TCA precipitated, loaded on an SDS-polyacrylamide gel, and stained with Coomassie Blue.

A


-ATP

B


+ATP
*

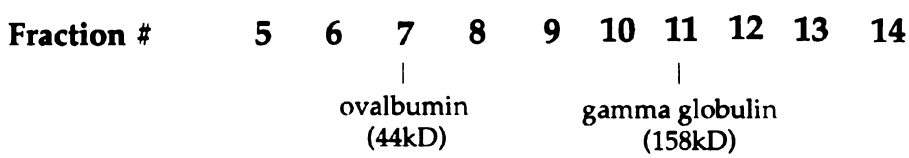

Fraction # 5 6 7 8 9 10 11 12 13 14
ovalbumin
(44kD)
gamma globulin
(158kD)

Figure IV-7. Partial cleavage of *HAC1*^{u} mRNA but no appearance of spliced *HAC1*^{i} mRNA is detected in *ire1-K702A* cells

(A) Northern hybridization was performed on RNA isolated from the following strains: wt, lanes 1 and 2; *Δire1* lanes 3 and 4; *Δire1* carrying a high copy plasmid with *ire1-K702A* , lanes 5 and 6; and *Δire1* carrying a high copy plasmid with *ire1-K702R* , lanes 7 and 8. All these strains also carry a centromeric plasmid containing *HAC1*. Each strain was grown to mid-log phase and incubated in the presence (even-numbered lanes) or absence (odd-numbered lanes) of tunicamycin for 30 min. Total RNA was extracted and probed with *HAC1*. (B) The blot was stripped and rehybridized with specific probes for *KAR2* and *ACT1*. The data were quantitated, and *KAR2* mRNA levels were normalized to *ACT1* mRNA levels.

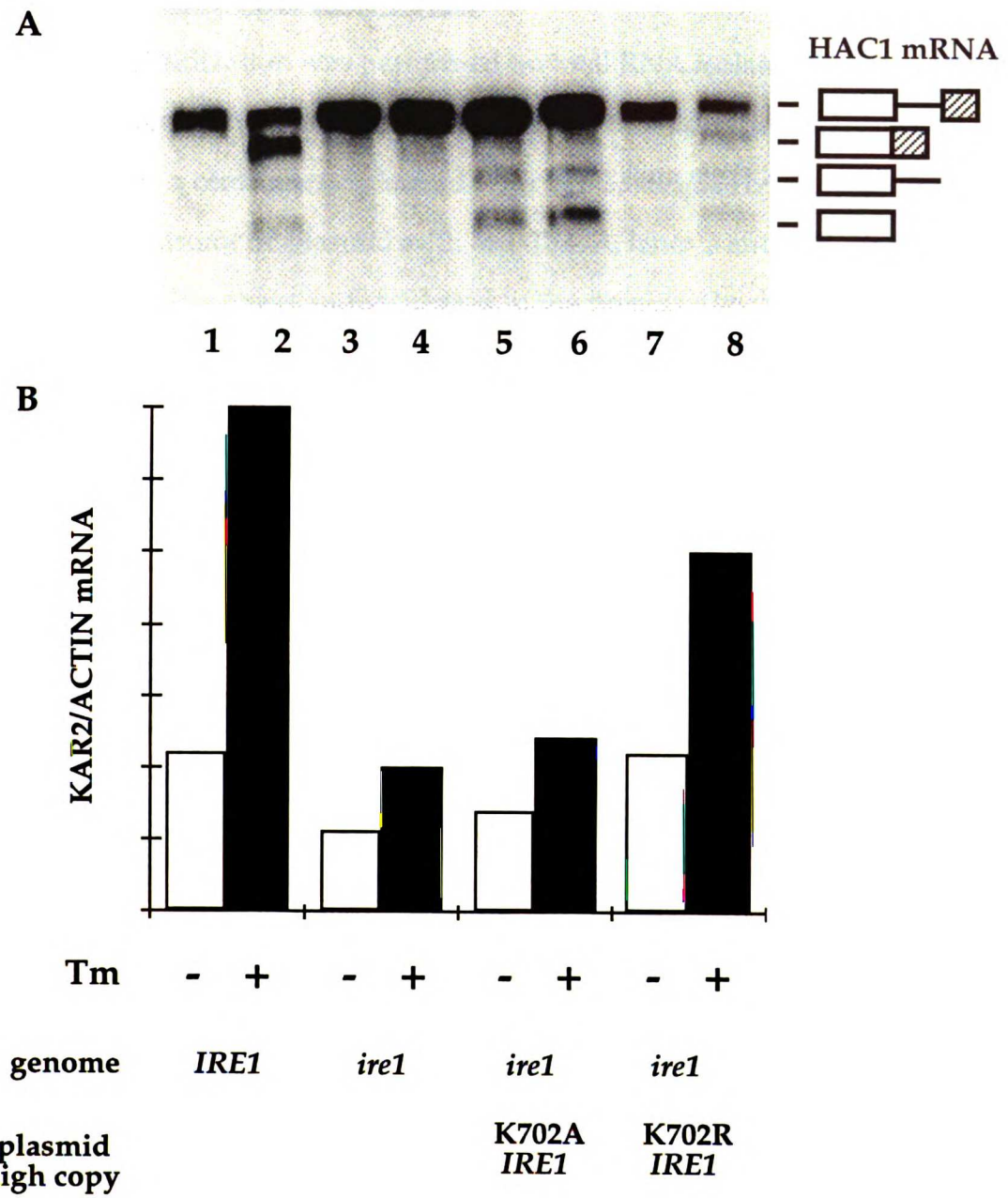
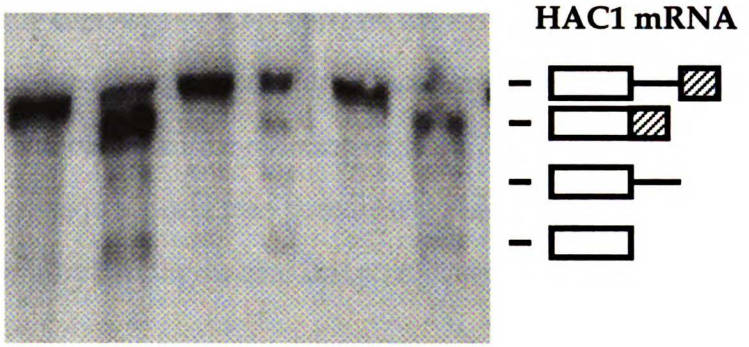

A
HAC1 mRNA
1 2 3 4 5 6 7 8
B
KAR2/ACTIN mRNA
Tm - + - + - + - +
genome IRE1 ire1 ire1 ire1
plasmid high copy
K702A IRE1
K702R IRE1

Figure IV-8. Appearance of spliced *HAC1*^{i} mRNA is detected in cells expressing Ire1p-S840A/S841A and Ire1p-T844A

Northern hybridization was performed on total RNA isolated from the following strains: *Δire1*carrying a centromeric plasmid with wild-type *IRE1*, lanes 1 and 2; *Δire1* carrying a centromeric plasmid with *ire1-S840A/S841A*, lanes 3 and 4; *Δire1* carrying a centromeric plasmid with *ire1-T844A*, lanes 5 and 6. Each strain was grown to mid-log phase and incubated in the presence (even-numbered lanes) or absence (odd-numbered lanes) of tunicamycin for 30 min.

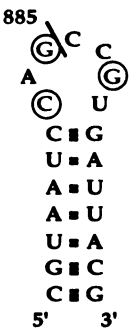


	1	2	3	4	5	6
Tm	-	+	-	+	-	+
genome	*ire1*		*ire1*		*ire1*	
centromeric plasmid	wild-type *IRE1*		S840A S841A *IRE1*		T844A *IRE1*	

Figure IV-9. Secondary structure predictions of *HAC1*^{u} mRNA

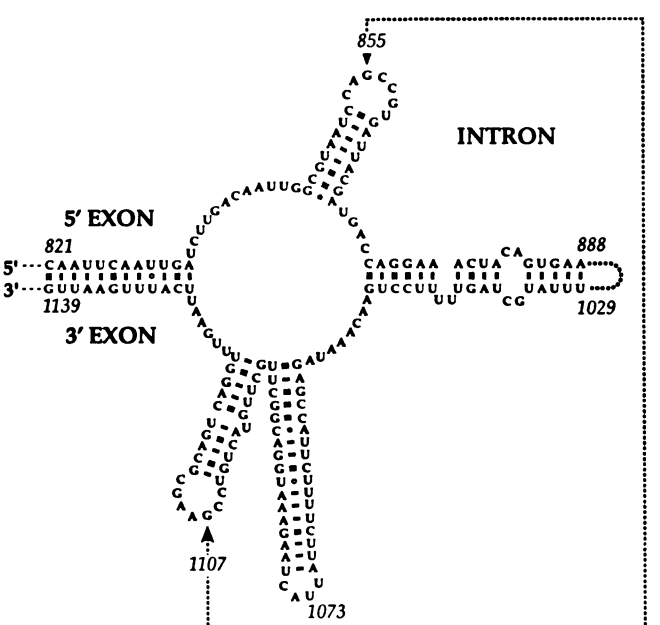
(A) The 5′ and 3′ splice junctions are predicted to form similar stem-loop structures. At each junction, cleavage occurs 3′ of the conserved G residue that is located in the third position of a seven-membered loop. Two other nucleotides are conserved in the loop (marked with circles). All conserved nucleotides in the loop are required for cleavage *in vivo*. (B) The intron is also predicted to contain two stem-loops. The smaller stem contains a spliceosome branchpoint-like sequence which is not required for splicing of *HAC1*^{u} mRNA. The exons pair, bringing the two splice junctions into proximity, possibly positioning them for concerted processing by an oligomeric Ire1p complex.

HAC1^u mRNA
5′ splice junction



HAC1^u mRNA
3′ splice junction

Putative *HAC1^u* mRNA Secondary Structure



Chapter V

Summary and Future Prospects

When this work was began, it was not known how Ire1p activation led to induction of downstream target events. By analogy to other serine-threonine kinase receptors, the expectation was that Ire1p would activate a kinase cascade that would lead to phosphorylation and activation of a downstream transcription factor. The discovery that regulated splicing of *HAC1* mRNA modulates the induction of downstream events revealed a remarkably different signal transduction pathway. This, however, was not the only unexpected result. Splicing occurs by a non-conventional splicing mechanism that bypasses the need for the spliceosome.

This thesis describes the identification of a splicing machinery composed of a transmembrane kinase/nuclease, Ire1p, and tRNA ligase. This complex plays a key role in regulating the activity of the UPR. Its identification has been a crucial step in unraveling how the unfolded protein signal is transmitted from the ER lumen to the transcriptional machinery in the nucleus (Figure V-1). Although the progress made by us and others in the past three years has provided the basic outline of the pathway, several questions remain.

How Ire1p senses the accumulation of unfolded proteins in the ER lumen is not known. All the insights gained so far pertain to downstream events in the pathway. This remains an open and exiting question. Several models for how protein folding may be monitored by Ire1p have been suggested, but there are currently no convincing data that characterizes the activation step (reviewed by Shamu et al., 1994). In addition to the accumulation of unfolded proteins, inositol starvation and the overproduction of ER membrane proteins also activate the UPR (Cox et al.,

1997; Wright, 1993). It is not clear at which step these different stimuli converge. Biochemical approaches, such as characterization of components that are crosslinked to the lumenal domain of Ire1p, may prove successful in identifying the ligand of Ire1p. Alternatively, mutagenesis of Ire1p's lumenal domain and isolation of extragenic suppressor mutations that alleviate *IRE1* defects may reveal interacting components.

The discovery that Hac1p expression is regulated at the level of translation revealed another unique regulatory mechanism in the UPR pathway (Chapman and Walter, 1997). Although it is clear that the intron of *HAC1* mRNA functions as an efficient tranlational attenuator, how it prevents ribosomes from synthesizing the uninduced form of the protein ($Hac1p^u$) is not known. Secondary-structure predictions show that the intron is likely to form stable stem-loop structure, which could be inhibitory for the progression of ribosomes. Deletion analysis can identify the elements, sequence or structure, that impose this translational block. The discovery that cells attenuate translation of $Hac1p^u$ prompts the speculation that the elongation step may be an additional level of regulation. Overcoming the block may enable cells to produce $Hac1p^u$. This could provide an alternative way of inducing the UPR. Alternatively, the elongation block may respond to a signal(s) other than that of unfolded proteins in the ER. The transcriptional activity of $Hac1^up$ may differ from that of $Hac1p^i$; they are structurally distinct both with respect to their C-terminal tail and their post-translational modifications. In this way, production of $Hac1p^u$ and $Hac1p^i$ may correspond to two distinct signaling pathways.

The availability of an *in vitro* system for splicing of *HAC1* mRNA provides us with an invaluable tool to study the Ire1p/tRNA ligase-mediated splicing event. As described in Chapter IV, how the oligomerization and the

kinase activity of Ire1p influence its RNase activity is not understood. By manipulating the dimerization and phosphorylation states of the protein, we should be able to elucidate the mechanism by which Ire1p's ribonucleolytic activity is induced. According to one model, binding of a ligand to Ire1p leads to its dimerization and trans-autophosphorylation. The phosphorylation of Ire1p helps stabilize a dimeric conformation necessary for nuclease activity. In another model, the kinase domain directly phosphorylates the nuclease domain, thus activating it. Ire1p belongs to a new class of hybrid kinase/nuclease signaling molecules, the only other known member of this class being RNase L, a component of the interferon signaling pathway that is activated upon viral infection of animal cells. It would not be surprising if similar hybrid protein kinase/RNases are identified as components of other signaling pathways.

Finally, a new and exiting aspect of the UPR is the study of the pathway in mammalian cells. Yeast *HAC1* mRNA is accurately spliced in HeLa cells, suggesting that the nonconventional splicing mechanism is evolutionarily conserved (M.Niwa, unpublished results). Moreover, two human homologues of Ire1p have recently been identified (M.Niwa, unpublished results; Tirasophon et al., 1998). Interestingly, incubation of yeast *HAC1* mRNA with one of these human recombinant proteins results in accurate cleavage of the 5′ splice junction. However, no cleavage of the 3′ splice junction is detected. This result suggests that human Ire1p is composed of more than one subunit and implies that different subunits catalyze cleavage of the 5′ and 3′ splice junctions. Such is the case with yeast tRNA endonuclease, which contains two functionally independent active sites for cleavage of the 5′ and 3′ splice sites. Thus, the mammalian UPR is starting to reveal new intricacies. In contrast to Ire1p, no homologue of *HAC1* has been

identified. The study of the promoter elements of the UPR target genes in mammalian cells indicates that their regulation is more complex than that of the yeast counterparts. The identification of more than one UPR-specific transcription factor in animal cells would not be surprising. An exciting extension of the work in mammalian cells is to determine whether the pathway contributes to cellular differentiation. The UPR has been proposed to be involved in ER proliferation in cells that have to expand their secretory capacity as part of their developmental program, such as plasma cells and hepatocytes (Kanai et al., 1986; Wiest et al., 1990). With the identification of mammalian UPR components, we are now in a position to test this hypothesis directly.

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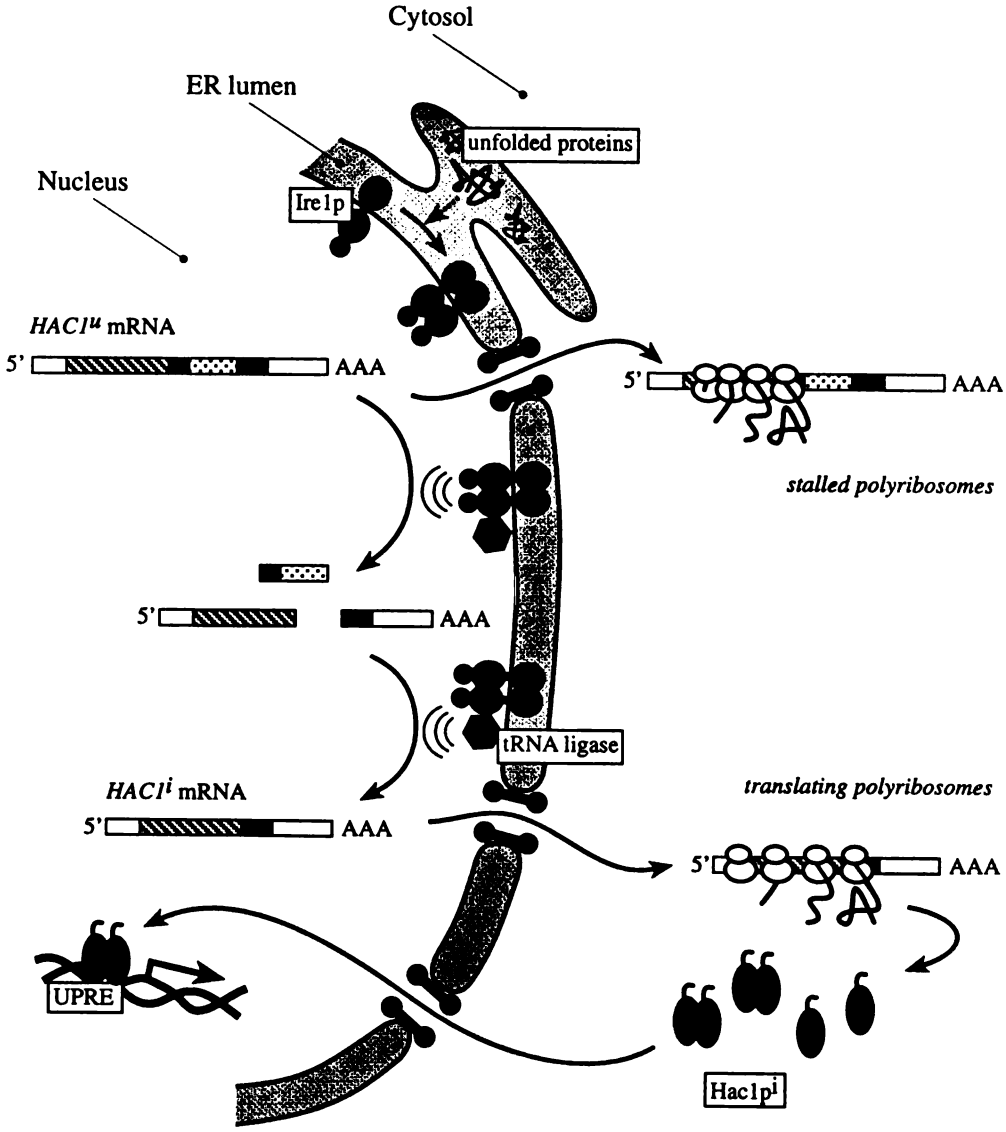
Figure V-1. Model of the UPR signaling pathway

Accumulation of unfolded proteins in the endoplasmic reticulum (ER) triggers activation of inner-nuclear membrane-localized Ire1p, which cleaves *HAC1*^{u} mRNA at both splice junctions. tRNA ligase then joins the exons to produce spliced *HAC1*^{i} mRNA. Both forms of *HAC1* mRNA exit the nucleus and associate with polyribosomes. However, only the spliced form gives rise to protein, Hac1^{i}p, which then enters the nucleus and up-regulates UPRE-containing genes.


Cytosol
ER lumen
Nucleus
unfolded proteins
Ire1p
HAC1u mRNA
5'
AAA
5'
AAA
stalled polyribosomes
5'
AAA
tRNA ligase
HAC1i mRNA
translating polyribosomes
5'
AAA
5'
AAA
UPRE
Hac1pi