

Ire1p: A Kinase and Site-Specific Endoribonuclease

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1. Introduction

1.1. *Ire1p* in the Unfolded Protein Response

The lumen of the endoplasmic reticulum (ER) is a highly specialized compartment in eukaryotic cells. Here, secretory and most membrane proteins are folded, covalently modified, and oligomerized with the assistance of specialized ER resident proteins (1). Perturbation of the ER lumen interferes with the production of many essential cellular components and can thus be highly deleterious. Indeed, in humans, defects in protein folding in the ER can lead to devastating diseases, such as cystic fibrosis, alpha₁-antitrypsin deficiency, and osteogenesis imperfecta (2). One way in which cells cope with the accumulation of unfolded proteins in the ER is by activating the unfolded protein response (UPR), an ER-to-nucleus signal transduction pathway (3–5). In the yeast *Saccharomyces cerevisiae*, Ire1p is an essential component of this pathway.

Ire1p is a transmembrane protein localized to ER membranes. When Ire1p was initially identified as a component of the UPR, it was thought to function primarily as a serine/threonine kinase because of its homology to other kinases and its demonstrated kinase activity (6–8). Subsequent studies revealed that Ire1p functions as a site-specific endoribonuclease as well (9). Both the kinase and endoribonuclease activities map to the carboxy-terminal half of Ire1p. The amino-terminal portion of Ire1p lies in the ER lumen (6,7), where it senses increases in the concentration of misfolded proteins. Since the ER and nuclear membranes are continuous, the carboxy-terminal portion of Ire1p is predicted to be located in either the cytoplasm and/or nucleus, where it induces downstream events in the UPR pathway. Immunoprecipitation of Ire1p from yeast-cell extracts has demonstrated that oligomerization of Ire1p coincides with its phosphorylation, suggesting that, as is the case for transmembrane receptor kinases, Ire1p can trans-autophosphorylate (10). Oligomerization of Ire1p coincides with induction of its endoribonuclease activity *in vivo* (9,10). The only cellular substrate known to be cleaved by Ire1p is the mRNA encoding the UPR-specific transcription factor, Hac1p.

1.2. Splicing of HAC1 mRNA by Ire1p and tRNA Ligase

Hac1p upregulates transcription of genes encoding ER resident proteins by binding to a common regulatory sequence in their promoters (11–14). Cellular levels of Hac1p are controlled by the regulated splicing of its mRNA (13,15). In the absence of splicing, *HAC1* mRNA translation is inhibited by a 252 nucleotide intron located at the 3'-end of the Hac1p coding region (15,16). Unlike the spliceosomal catalyzed splicing of all other pre-mRNAs known to date, removal of the *HAC1* intron is catalyzed by the combined actions of Ire1p and tRNA ligase (9). When unfolded proteins accumulate in the ER, Ire1p initiates splicing by cleaving *HAC1^u* mRNA (*u* for *uninduced* or *unspliced*) to liberate the intron and 5' and 3' exons. The exons are then joined by tRNA ligase, an enzyme previously thought to function exclusively in the splicing of pre-tRNAs. The ligated product, *HAC1ⁱ* mRNA (*i* for *induced*), goes on to be efficiently translated to produce Hac1p. Thus the regulated splicing of *HAC1* mRNA is a key step in the UPR signaling pathway.

The splicing of *HAC1* mRNA by Ire1p and tRNA ligase is unprecedented. Significantly, Ire1p and tRNA ligase are sufficient to splice *HAC1^u* mRNA *in vitro* (9,17). This is in striking contrast to the splicing of all other studied pre-mRNAs, which require the more than 100 proteins and small nuclear RNAs which constitute the spliceosome and its associated components (18). The splicing of *HAC1^u* mRNA mechanistically resembles the splicing of pre-tRNAs (19,20). Ire1p and tRNA endonuclease both cleave to produce 5'-OH and 2',3'-cyclic phosphate RNA termini. Exon ligation by tRNA ligase in both splicing reactions follows the same chemical steps (17). Aside from these similarities, intriguingly, Ire1p and tRNA endonuclease are quite different. The two endoribonucleases lack any significant similarity in amino acid sequence or subunit composition, and recognize different structural features in their RNA substrates. Stem-loop structures predicted to form at the *HAC1^u* mRNA splice junctions are required and sufficient to direct Ire1p cleavage. The stem-loop substrates that define the minimal Ire1p cleavage site are described elsewhere (17).

1.3. Ire1p Is Similar to RNase L

The kinase and nuclease domains of Ire1p share significant homology with RNase L (8). RNase L cleaves RNA nonspecifically in cells infected with double-stranded RNA viruses, and has no known role in pre-mRNA processing (20,21). *In vitro* dimerization of RNase L induces its nuclease activity (23). Analogously, oligomerization of Ire1p correlates with its nuclease activity *in vivo* (9,10). In contrast to Ire1p, the kinase domain of RNase L appears to be catalytically inactive and has been shown to be involved in mediating dimerization of the protein (23). The relationship between the kinase domain and nuclease activity of Ire1p has yet to be determined.

We can follow the Ire1p kinase (9) and *HAC1^u* RNA-splicing activities (9,17) *in vitro* by using a recombinant fragment carrying the Ire1p kinase and putative nuclease tail domains, Ire1p(k+t). The reconstituted splicing reaction has been used to define the minimal substrate requirements for Ire1p cleavage, determine the chemical nature of the RNA termini produced by Ire1p cleavage, and map Ire1p cleavage sites. Overall, we found that small stem-loop minisubstrate RNAs are sufficient to direct cleavage by Ire1p; that, like tRNA endonuclease, Ire1p endonucleolytic cleavage produces RNA

fragments with 2',3'-cyclic phosphate and 5'-OH termini; and that Ire1p cleaves 3' of the guanosine residue found at each splice site (17). This chapter focuses on the methods we have used during our studies of the kinase and nuclease activities of yeast Ire1p.

2. Materials

2.1. Equipment, Chemicals, and Molecular Biology Reagents

1. Equipment: Microfluidizer (Microfluidics, Newton, MA), scintillation counter.
2. Chemicals: Glycogen (Promega, Madison, WI), *N*-butanol, phenylmethylsulfonyl flouride (PMSF), tRNA, diethyl pyrocarbonate (DEPC), 10% Triton X-100, carbenicillin, isopropyl-1-thio- β -D-galactoside (IPTG), glutathione, LiCl, phenol, chloroform, formamide.
3. Nucleotides: ADP, 5'pN, 5'pN2', 5'pN 3'p, and pN2'3'(cyclic)p (Sigma); [α -³²P]NTP and [γ -³²P]ATP (3000 Ci/mM; Amersham, Arlington Heights, IL); NTPs; *N* = A, C, G, or U.
4. Enzymes: T7 RNA polymerase, nuclease P1, AMV reverse transcriptase, and mutant T4 polynucleotide kinase lacking 3' phosphatase activity (Roche Molecular Biochemicals); T4 polynucleotide kinase and *Sac*I restriction enzyme (New England Biolabs, MA); PreScission Protease and ribonuclease U2 (Pharmacia, Uppsala, Sweden).
5. Miscellaneous: glutathione-Sepharose (Pharmacia, Uppsala, Sweden); RNasin ribonuclease inhibitor (Promega, Madison, WI); Centricon-50 ultrafiltration units (Amicon, Beverly, MA); PEI cellulose thin-layer chromatography plates (EM Science, Gibbstown, NJ); X-ray film; Glogos II Autorad Markers (Stratagene, La Jolla, CA); plastic wrap (i.e., Saran Wrap), bovine serum albumin (BSA).

2.2. Solutions (see Note 1)

1. Crude extract buffer: 20 mM HEPES, pH 7.6, NaCl (0.35 or 1 M), 2 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X-100.
2. 100 mM PMSF in ethanol.
3. Ire1p elution buffer: 20 mM HEPES, pH 7.6, 0.35 mM NaCl, 2 mM EDTA, 20 mM glutathione, pH 7.6.
4. Ire1p kinase buffer: 20 mM HEPES, pH 7.6, 250 mM K-acetate, 10 mM Mg(acetate)₂, 1 mM DTT, 100 mM ATP.
5. Ire1p cleavage buffer: 20 mM HEPES, pH 7.6, 50 mM K-acetate, 1 mM Mg(acetate)₂, 1 mM DTT, 2 mM ADP, 40 U RNasin.
6. PreScission protease buffer: 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol.
7. Transcript elution buffer: 0.3 M Na-acetate, pH 5.2, 10 mM Mg(acetate)₂.
8. Phenol/chloroform: mix equal parts phenol and chloroform.
9. TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0.
10. Stop solution: 50 mM Na-acetate, pH 5.2, 1 mM EDTA, 0.1% SDS.
11. RNA gel-loading buffer: 99% formamide, 1 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 0.1% SDS, xylene cyanol FF, Bromophenol blue.
12. Ribonuclease U2 buffer: 20 mM Na-citrate, pH 3.5, 1 mM EDTA, 7 M urea, 0.5 μ g/ μ L tRNA.
13. Alkaline hydrolysis buffer: 50 mM NaHCO₃, pH 9.0, 1 mM EDTA, 0.5 μ g/ μ L tRNA.
14. Nuclease P1 buffer: 20 mM Na-acetate, pH 5.2, 50 ng/ μ L tRNA.
15. 1 M LiCl.
16. Primer extension buffer: 20 mM NaCl, 15 mM HEPES, pH 7.6.

2.3. Plasmids and Oligonucleotides

1. Vector for expression of recombinant *S. cerevisiae* Ire1p(k+t) in *Escherichia coli*: We expressed and purified a fragment of Ire1p fused at its amino-terminal end to glutathione-S-transferase (GST). This fragment (Ire1p amino acids 556–1115) carries the kinase (k) and putative nuclease tail (t) domains of Ire1p, and is termed GST-Ire1p(k+t). The expression vector pCF210 (9) was made by subcloning the Ire1p(k+t) coding region into pGEX-6p-2 (Pharmacia, Uppsala, Sweden). In this construct, a PreScission Protease cleavage site is encoded in the region that links GST and Ire1p(k+t) in the fusion protein.
2. *HAC1^U* 508 RNA in vitro transcription vector: The vector pCF187 (9) was constructed by subcloning a PCR fragment carrying *HAC1^U* mRNA sequences into pBluescript IISK(–) (Stratagene). Using linearized pCF187 as a template for T7 RNA polymerase transcription produces a *HAC1^U* RNA carrying 181 nucleotides of the 5' exon, the 252 nucleotide intron, and 75 nucleotides of the 3' exon, for a total of 508 nucleotides. Full-length *HAC1^U* mRNA is 1560 nucleotides.
3. Oligonucleotides for *HAC1^U* stem-loop minisubstrate in vitro transcriptions: Make *HAC1^U* stem-loop minisubstrates by in vitro transcription using annealed oligonucleotides as templates. Use the following oligonucleotides: T7 promoter, 5'TAATACGACT CACTATAG; hactng-38 (encoding the wild-type 5' splice-site stem-loop RNA), 5'TGAGCCGGTC ATCGTAATCA CGGCTGGATT ACGCCAACCG GCTATAGTA GTCGTATTA; and hactng-10 (encoding the wild-type 3' splice-site stem-loop RNA), 5'TGAGGTCAAA CCTGACTGCG CTTCGGACAG TACAAGCTTG ACCTATAGTG AGTCGTATTA (17).
4. Oligonucleotides for primer extension reactions: Use the sequencing primers TGSP-3 (5'GAAGAAATCA TTCAATTCAA ATGAATTC) and TGSP-1 (5'GCTAGTGTTC TTGTTCACTG) to map the 5' and 3' Ire1p(k+t) cleavage sites in *HAC1^U* 508 RNA, respectively (17).

3. Methods

3.1. Expression and Purification of Ire1p(k+t)

The portion of *S. cerevisiae* Ire1p containing its kinase and C-terminal tail domains fused to glutathione-S-transferase (GST-Ire1p(k+t)) is expressed in and purified from *E. coli*. A 1-L culture yields about 0.5 mg GST-Ire1p(k+t). Ire1p(k+t) is produced by proteolytic cleavage of GST-Ire1p(k+t) with PreScission Protease (see Note 2).

1. Grow *E. coli* strain DH5 α carrying plasmid pCF210 at 37°C with shaking (~200 rpm) in 1 L LB media plus 100 μ g/mL carbenicillin. When the culture reaches an OD₆₀₀ of 0.5, induce expression of GST-Ire1p(k+t) by adding IPTG to a final concentration of 0.1 mM and grow cells for another 5 h.
2. Harvest cells by centrifugation and quick-freeze the pellet on dry ice. At this point, the cell pellet can be stored at –80°C until ready to continue. Thaw and resuspend the cell pellet on ice in 40 mL Ire1p crude extract buffer (0.35 M NaCl) with 1 mM PMSF (see Note 3). Break cells by passing three times through the microfluidizer. Pellet cell debris by centrifugation at 30,000g for 20 min.
3. Capture GST-Ire1p(k+t): In a 50-mL conical tube, incubate the supernatant at 4°C for 1 h with 0.5 mL glutathione-Sepharose pre-equilibrated in Ire1p crude-extract buffer (0.35 M NaCl). Rotate the tube to gently mix its contents.
4. Wash resin: Spin down the resin (5 min at 1500g), discard the supernatant, and add 50 mL cold Ire1p crude-extract buffer (1 M NaCl). Spin down the resin and discard the supernatant. Add 50 mL of the same buffer, and rotate gently for 30 min at 4°C. Spin down the resin and discard the supernatant.

5. Elute GST-Ire1p(k+t): Add 3 mL cold Ire1p elution buffer to the resin and rotate at 4°C for 15 min. Spin and remove eluate to a tube on ice. Repeat elution 2–3 more times. Pool the eluates and dialyze in PreScission Protease buffer to remove the glutathione. Dialyze at 4°C in an amount of buffer that is 1000 times the volume of the eluate you are dialyzing. After 3–12 h, replace the dialysis buffer and dialyze for an additional 3–12 h.
6. Cleave GST-Ire1p(k+t) to produce Ire1p(k+t): Add 20 U PreScission Protease (~4 U/100 µg fusion protein) and rotate for 4 h at 4°C. Remove PreScission Protease (see **Note 4**), GST, and residual GST-Ire1p(k+t) by addition of 0.5 mL glutathione-Sepharose pre-equilibrated in PreScission Protease buffer. Rotate for 1 h at 4°C.
7. Spin down the resin and remove the supernatant containing Ire1p(k+t). Wash the resin with Ire1p crude-extract buffer (0.35 M NaCl), and pool the supernatants. Dialyze into Ire1p cleavage buffer *lacking* ADP, and concentrate the protein by ultrafiltration in Centricon-50 concentrators following the manufacturer's recommendations (see **Note 5**). Determine the purity of your Ire1p(k+t) preparation by electrophoresis of the protein through a polyacrylamide sodium dodecyl sulfate (SDS) gel followed by Coomassie blue staining. Ire1p(k+t) resolves to a position between the 50 and 60 kDa protein markers on a 12% polyacrylamide gel. Estimate the Ire1p(k+t) concentration by comparing the intensity of the Ire1p(k+t) band to that of BSA of known concentration separated on the same gel. Aliquot Ire1p(k+t) and store frozen at -80°C (see **Note 6**).

3.2. Ire1p(k+t) RNA Substrates (see Note 7)

3.2.1. In Vitro Transcription of HAC1^U 508 RNA

1. Linearize plasmid pCF187 by digestion with *SacI* restriction enzyme.
2. Set up transcription reactions (20 µL) containing 1 mM each of ATP, CTP, GTP, 0.1 mM UTP, 25 mCi of [α -³²P]UTP, 1 µg linearized pCF187 DNA, 40 U RNasin, and 20 U T7 RNA polymerase. Incubate at 37°C for 1.5 h. To generate unlabeled RNA, omit [α -³²P]UTP and add 1 mM UTP to your reactions. Add 180 µL water and extract with phenol-chloroform. Ethanol precipitate the RNA using 40 µg glycogen as a carrier.
3. Prepare transcripts for electrophoresis by addition of 25 µL RNA gel-loading buffer followed by heating at 95–100°C for 3 min. Separate the transcription reaction products by electrophoresis through denaturing 5% polyacrylamide gels. Visualize the transcripts by exposing the gel to X-ray film for 3–5 min. When exposing the gel, use glow-in-the-dark stickers (Glogos II Autorad Markers) to help align the gel with the film later. On the developed X-ray film, cut out the most prominent band. Use this X-ray film mask to guide you as you cut out a slice of gel containing your transcript. Elute the RNA from the gel slice by shaking vigorously overnight at 4°C in 400 µL transcript elution buffer plus 400 µL phenol-chloroform. Ethanol precipitate and resuspend the RNA in 10 µL water. Determine the counts per minute (cpm) for 1 µL of the radiolabeled RNA.

3.2.2. In Vitro Transcription of HAC1^U Minisubstrates

HAC1^U minisubstrates are transcribed using single-stranded DNA oligonucleotide templates to which the “T7 promoter” oligonucleotide is annealed to create a double-stranded T7 RNA polymerase promoter (24). These minisubstrates should form stable stem-loop structures that mimic the structures predicted to form at the 5' and 3' splice sites of HAC1^U mRNA (17). Mutant minisubstrate RNAs can be made simply by using DNA oligonucleotide templates that carry the mutations of interest.

1. Gel-purify each oligonucleotide by electrophoretically fractionating each through a denaturing 12% polyacrylamide gel. Cover the gel with plastic wrap and place it on top of a

PEI cellulose thin-layer chromatography (TLC) plate. Visualize the oligonucleotide containing band by illuminating the gel from above with long-wavelength UV light. The oligonucleotide containing band will cast a shadow on the TLC plate. On the plastic wrap, mark the location of the most prominent band for each oligonucleotide and cut the bands out of the gel. Elute the oligonucleotides from the gel slices by shaking vigorously overnight in TE. Ethanol precipitate the oligonucleotides. Resuspend them in water or TE and determine the oligonucleotide concentration by measuring the absorbance at 260 nm. At 260 nm, an absorbance of 1 is equal to 40 $\mu\text{g}/\text{mL}$ of single stranded DNA.

2. Anneal the oligonucleotides by heating 15 μM T7 promoter oligonucleotide with 0.25 μM template oligonucleotide (i.e., hactng-10) at 95–100°C for 3 min. Immediately place on ice. The amount of [α - ^{32}P]NTP and NTPs to use in each 20 μL transcription reaction is given in **Table 1**. Add NTPs, [α - ^{32}P]NTP, 40 U RNasin, and 20 U T7 RNA polymerase to the annealed oligonucleotides. Incubate for 1.5 h at 37°C. To make unlabeled stem-loop RNAs, omit [α - ^{32}P]NTP and include 1 mM of each NTP. Phenol-chloroform extract and ethanol precipitate the RNA. Add 20 mL RNA gel-loading buffer and heat at 95–100°C for 3 min.
3. Separate the transcription products on a 15% denaturing polyacrylamide gel. Purify and quantitate the transcripts as in **Subheading 3.2.1**.

3.3. Kinase Assay

Kinase reactions containing 0.5 μg Ire1p(k+t) should produce a band visible by overnight autoradiography.

1. In 20 μL , incubate Ire1p(k+t), and 50 mCi [γ - ^{32}P]ATP in kinase buffer at 30°C for 30 min.
2. Fractionate the protein by electrophoresis through a 12% polyacrylamide gel.
3. Visualize the phosphorylated Ire1p(k+t) by autoradiography of the gel.

3.4. RNA Cleavage Assay

3.4.1. Cleavage Assay

A 30-min cleavage reaction containing 0.5 μg Ire1p(k+t) should result in 100% cleavage of *HAC1^U* 508 RNA. A 2-h cleavage reaction containing 1 μg Ire1p(k+t) should result in about 50% cleavage of a wild-type stem-loop minisubstrate.

1. In 20 μL , combine Ire1p(k+t) and Ire1p cleavage buffer. For cleavage of *HAC1^U* 508 RNA, use 20,000 cpm of labeled *HAC1^U* 508 RNA per reaction. For cleavage of *HAC1^U* stem-loop minisubstrates, use 2000 cpm of labeled *HAC1^U* stem-loop minisubstrate per reaction.
2. Incubate at 30°C for 0.5–2 h. Stop the reaction with 400 μL stop solution, and extract with 400 μL vol of phenol-chloroform. Ethanol precipitate the RNA using 40 μg glycogen as carrier. Add 8 μL of RNA gel-loading gel buffer and heat at 95–100°C for 3 min.
3. Separate the cleavage products of the *HAC1^U* 508 RNA reactions and the *HAC1^U* stem-loop minisubstrate reactions on 5 and 15% gels, respectively.
4. Visualize RNA by autoradiography of the gels.

3.4.2. Marker Preparation

Alkaline hydrolysis of a 5'-end-labeled RNA will produce a ladder of bands differing by one nucleotide each. A partial ribonuclease U2 RNA digest of the same end-labeled RNA will help you align your alkaline hydrolysis ladder with your Ire1p(k+t) cleavage reaction products. This approach works well with small RNAs, such as the *HAC1^U* stem-loop minisubstrates (see **Note 8**).

Table 1
Transcription Reaction Mixtures for Internally Radiolabeled *HAC1^U* Stem-Loop Minisubstrates

Radiolabeled nucleotide	μCi , [α - ^{32}P]NTP	mM ATP	mM CTP	mM GTP	mM UTP
A	300	0.1	1	1	1
C	300	1	0.1	1	1
G	300	1	1	0.1	1
U	50	1	1	1	0.1

1. Make unlabeled minisubstrate RNA as in **Subheading 3.2.2.**, and 5'-end label it using [γ - ^{32}P]ATP and T4 polynucleotide kinase.
2. Alkaline hydrolysis RNA ladder: Incubate 30,000 cpm of 5'-end labeled minisubstrate RNA with 10 μL freshly made alkaline hydrolysis buffer at 90°C for 15 min. Immediately place on ice and add 5 μL of RNA gel-loading buffer. Load one-half of the reaction per lane on your gel.
3. Partial RNA digestion with Ribonuclease U2: Incubate 20,000 cpm 5'-end-labeled minisubstrate in 10–20 μL freshly made Ribonuclease U2 buffer for 1 min at 80°C. Immediately place on ice, and add ribonuclease U2 freshly diluted into water. Incubate at 55°C for 15 min. Add 400 μL stop solution and 400 μL phenol-chloroform. Extract and ethanol precipitate the RNA. Load the entire reaction in one lane on your gel.

3.5. Nearest Neighbor Analysis of the Cleavage Termini (see Fig. 1)

This analysis is made possible for the following reasons. First, the dinucleotide at the Ire1p(k+t) cleavage site occurs only once in the loop of the *HAC1^U* stem-loop minisubstrate. Second, nuclease P1 cleaves to the 3' side of nucleotides to produce 5'pN products. However, nuclease P1 *cannot* cleave 2',3'-cyclic phosphate groups.

3.5.1. Nuclease P1 Digests

1. Make *HAC1^U* stem-loop minisubstrate RNAs internally radiolabeled with either A, C, or G.
2. Cleave these RNAs with Ire1p(k+t).
3. Separate the RNA fragments by electrophoresis through a 12% denaturing polyacrylamide gel. Visualize the bands, and cut out the gel slices containing the 5' cleavage fragments as in **Subheading 3.2.1.**
4. Elute the 5' cleavage fragments from the gel slices by shaking vigorously overnight at 4°C in 400 μL water and 400 μL phenol-chloroform.
5. Precipitate the eluted RNA by extracting with *N*-butanol to dryness (see **Note 9**). Wash the pelleted RNA with ethanol and dry.
6. Digest the pelleted RNA at 37°C for 30 min by addition of 10 μL nuclease P1 buffer and 0.2 U of nuclease P1. Place on ice until ready to spot onto your TLC plate.

3.5.2. Marker Preparation for Thin-Layer Chromatography

1. Make radiolabeled marker 5'pN (where *N* = A, C, G, or U) by phosphorylating N3'p nucleotide, in the presence of [γ - ^{32}P]-ATP and wild-type T4 polynucleotide kinase.
2. Make radiolabeled markers 5'pN2'p, 5'pN3'p, and pN2',3'(cyclic)p by phosphorylating N2'p, N3'p, and N2',3'(cyclic)p nucleotides in the presence of [γ - ^{32}P]-ATP, and mutant T4 polynucleotide kinase lacking the 3' phosphatase activity of the wild-type enzyme.

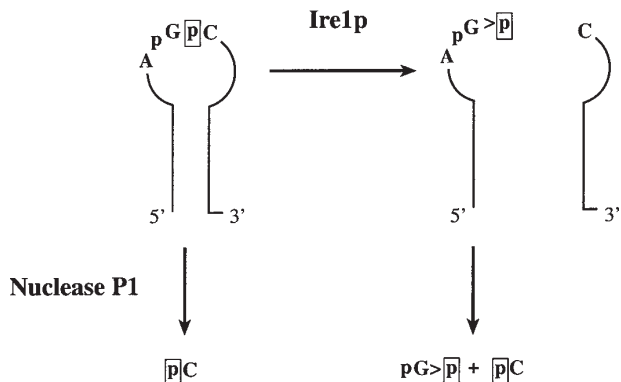


Fig. 1. Experimental scheme for the nearest neighbor analysis. In this scheme, the *HAC1^U* stem-loop minisubstrate was transcribed in the presence of [α - 32 P]CTP. Thus, the phosphate 5' of every C residue is labeled and is here highlighted with a box. When the *HAC1^U* stem-loop minisubstrate is digested with nuclease P1, the only radiolabeled nucleotide produced is [32 P]C. However, if the stem-loop minisubstrate is first cleaved by Ire1p(k+t) and then digested with nuclease P1, [32 P]C as well as pG>[32 P] are produced. Here, “>p” represents a 2',3'-cyclic phosphate group.

3.5.3. Thin-Layer Chromatography

1. Lightly draw a line in pencil across a PEI cellulose thin-layer chromatography (TLC) plate about 3 cm from the bottom of the plate.
2. Spot nuclease P1 digests and radiolabeled markers onto the TLC plate evenly along this line. Spot 1–2 μ L of each sample and let dry. To spot more of each sample, spot another 2 μ L at the same location, and let dry. Repeat until the entire sample is loaded.
3. Develop the plates with 1 M LiCl (see Note 10).
4. Visualize the separated nuclease P1 digestion products by autoradiography of the TLC plates.

3.6. Band Mobility Shift Analysis of the Cleavage Termini (see Fig. 2)

This assay is quick relative to the nearest neighbor analysis. Also, it allows one to determine that Ire1p(k+t) cleavage produces 2',3'-cyclic phosphate groups as well as 5'-OH groups. This approach takes advantage of the fact that the presence or absence of a terminal phosphate group will increase (because of negative charge) or decrease (because of loss of negative charge) the mobility of a *small* RNA electrophoresed through a polyacrylamide gel. Calf intestinal phosphatase (CIP) is used to remove noncyclic terminal phosphates. T4 polynucleotide kinase (PNK) is used to remove 2',3'-cyclic terminal phosphates in the absence of ATP. In the presence of ATP, it is used to phosphorylate 5'-OH groups.

1. Set up a 80- μ L Ire1p(k+t) cleavage reaction containing Ire1p cleavage buffer, 10,000 cpm *HAC1^U* stem-loop minisubstrate RNA, and Ire1p(k+t).
2. Incubate at 30°C for 2 h. Add stop solution and phenol-chloroform extract. Set one-quarter of the reaction aside. This is your untreated sample.

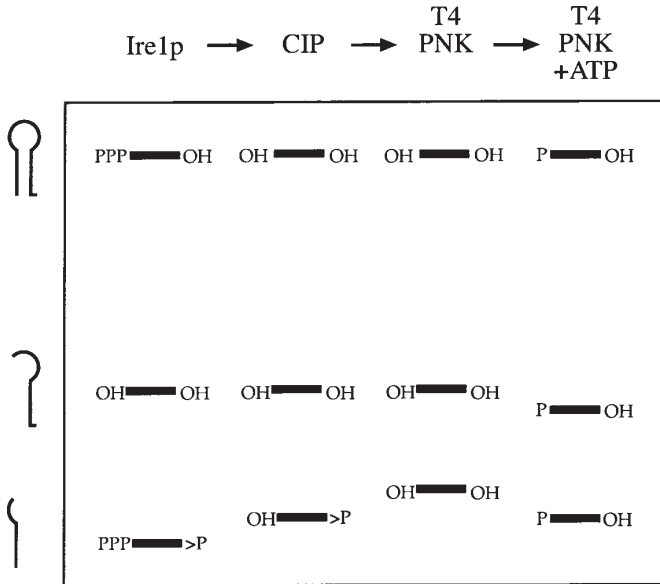


Fig. 2. Experimental scheme for the band mobility shift analysis of the RNA termini produced following cleavage of a *HAC1^U* stem-loop minisubstrate by Ire1p(k+t). The “>p” represents a 2',3'-cyclic phosphate termini.

3. Ethanol-precipitate the remaining 3/4 of the reaction. Treat the precipitated RNA with 30 U of CIP for 1.5 h at 37°C.
4. Phenol-chloroform extract the CIP reaction and set one-third of it aside. This is your CIP-treated sample.
5. Ethanol precipitate the remaining 2/3 of the CIP reaction. Treat the precipitated RNA with 30 U T4 PNK in the absence of ATP for 1.5 h at 37°C.
6. Phenol-chloroform extract the T4 PNK reaction and set one-half of it aside. This is your T4 PNK (minus ATP)-treated sample.
7. Ethanol precipitate the remaining one-half of the T4 PNK reaction. Treat the precipitated RNA with 30 U T4 polynucleotide kinase plus 2 mM ATP for 1.5 h at 37°C.
8. Phenol-chloroform extract and ethanol precipitate the RNA. This is your T4 PNK (plus ATP)-treated sample.
9. Ethanol precipitate all samples that you have set aside.
10. Add 8 μ L RNA gel-loading buffer to the precipitated RNA samples and heat them at 95–100°C for 3 min.
11. Separate the RNA fragments on a 15% denaturing polyacrylamide gel.
12. Visualize the RNA fragments by autoradiography of the gel.

3.7. Mapping Ire1p(k+t) Cleavage Sites

3.7.1. Primer Extension of Cleaved *HAC1^U* 508 RNA

1. Use the sequencing primers TGSP-3 and TGSP-1 to map the 5' and 3' Ire1p(k+t) cleavage sites in *HAC1^U* 508 RNA, respectively. Each of these primers lies within 20–30 nucleotides of the 5' or 3' Ire1p(k+t) cleavage site.

2. Set up primer extension reactions containing 1 pM 5' end-labeled sequencing primer and 10 ng Ire1p(k+t) cleaved or uncleaved *HAC1^U* 508 RNA in primer extension buffer. Primer extension reactions on uncleaved *HAC1^U* 508 RNA provide a control for the pausing of AMV reverse transcriptase that naturally occurs during primer extension on the template.
3. Heat at 100°C for 3 min in a metal block. Anneal the primer to the *HAC1^U* 508 RNA by slowly cooling the metal block to 40°C on your benchtop.
4. Start the extension reaction by adding dNTPs to a final concentration of 0.1 mM and 3 U AMV reverse transcriptase.
5. Incubate at 40°C for 30 min.
6. Add RNA gel-loading buffer.
7. Analyze samples by electrophoresis through a 10% denaturing polyacrylamide gel followed by autoradiography.
8. Generate sequencing ladders using the primer extension reaction protocol, but include 0.1 mM of ddATP, ddCTP, ddGTP, or ddTTP in each reaction.

3.7.2. Nearest Neighbor Analysis of Cleaved Stem-Loop *HAC1^U* Minisubstrates

Mapping of the Ire1p(k+t) cleavage site on each stem-loop minisubstrate can be unambiguously achieved by the nearest neighbor analysis outlined in **Subheading 3.5.** as long as the dinucleotide at the cleavage site only occurs once in the loop of the stem-loop minisubstrate.

4. Notes

1. Your solutions need to be RNase-free. RNases can be inactivated by addition of diethyl pyrocarbonate (DEPC), which is carcinogenic. Wear gloves and avoid its fumes. Add 1 mL DEPC/L of solution and stir the mixture for 15 min in a fume hood. Autoclave the solution for 20 min to inactivate the DEPC. Buffers containing Tris-HCl cannot be treated directly with DEPC. Instead, use DEPC-treated water when making them.
2. You can use Ire1p(k+t) or GST-Ire1p(k+t) in all of the assays outlined here. However, the Ire1p(k+t) preparations tend to be of higher purity when compared to the GST-Ire1p(k+t) preparations.
3. PMSF is highly unstable in water, and it is very toxic. Wear gloves and avoid its fumes.
4. PreScission Protease is also a GST fusion protein. This allows for removal of the protease from solution by incubating it with glutathione-sepharose resin.
5. Occasionally Ire1p(k+t) will form a white, flaky precipitant during the concentration step. To avoid this, concentrate the protein as the last step before aliquoting it for storage at -70 to -80°C. Also, to reduce the amount of time it takes to concentrate the protein, use an ultrafiltration unit with the highest molecular weight cut-off possible. You should be able to concentrate Ire1p(k+t) down to 0.5 µg/mL without it precipitating out of solution.
6. To retain Ire1p(k+t) activity, flash-freeze aliquots in liquid nitrogen and store at -70 to -80°C. Ire1p(k+t) loses activity over time when stored at 4°C.
7. Gel purification of RNA transcripts, especially the stem-loop minisubstrates, leads ultimately to cleaner RNA cleavage assays. T7 RNA polymerase tends to add nontemplate nucleotides to the 3' end of transcripts.
8. Ribonuclease U2 cleaves after adenosine residues to produce 3'-phosphate termini. You will need to determine the optimal enzyme to RNA ratio for your partial digestion reactions. Alkaline hydrolysis produces 2'- and 3'-phosphate termini.
9. *N*-butanol extraction allows you to precipitate small quantities of RNA in the absence of carriers such as glycogen or tRNA. Glycogen interferes with sample migration during thin-layer chromatography.

10. To reduce smudging and smearing of samples during thin-layer chromatography, prerun the PEI cellulose TLC plates in distilled water and allow them to dry. Store the plates at 4°C until ready for use. Further improvements in sample separation can sometimes be achieved by soaking the TLC plate in methanol for 10 min following spotting of your samples onto the plate. Allow the plate to dry, and develop it as usual.

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References

1. Gething, M.-J. and Sambrook, J. (1992) Protein folding in the cell. *Nature* **355**, 33–45.
2. Kuznetsov, G. and Nigam, S. K. (1998) Folding of secretory and membrane proteins. *N. Engl. J. Med.* **339**, 1688–1695.
3. Shamu, C. E., Cox, J. S., and Walter, P. (1994) The unfolded protein response pathway in yeast. *Trends Cell Biol.* **4**, 56–60.
4. Chapman, R., Sidrauski, C., and Walter, P. (1998) Intracellular signaling from the endoplasmic reticulum to the nucleus. *Annu. Rev. Cell Dev. Biol.* **14**, 459–485.
5. Sidrauski, C., Chapman, R., and Walter, P. (1998) The unfolded protein response: an intracellular signalling pathway with many surprising features. *Trends Cell Biol.* **8**, 245–249.
6. Cox, J. S., Shamu, C. E., and Walter, P. (1993) Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* **73**, 1197–1206.
7. Mori, K., Ma, W., Gething, M. J., and Sambrook, J. (1993) A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus. *Cell* **74**, 743–756.
8. Bork, P. and Sander, C. (1993) A hybrid protein kinase-RNase in an interferon-induced pathway? *FEBS Lett.* **334**, 149–152.
9. 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* **90**, 1–20.
10. Shamu, C. E. and Walter, P. (1996) Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *EMBO J.* **15**, 3028–3039.
11. Mori, K., Sant, A., Kohno, K., Normington, K., Gething, M. J., and Sambrook, J. F. (1992) A 22 bp cis-acting element is necessary and sufficient for the induction of the yeast KAR2 (BiP) gene by unfolded proteins. *EMBO J.* **11**, 2583–2593.
12. Kohno, K., Normington, K., Sambrook, J., Gething, M. J., and Mori, K. (1993) The promoter region of the yeast KAR2 (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. *Mol. Cell Biol.* **13**, 877–890.
13. Cox, J. S. and Walter, P. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* **87**, 391–404.
14. Nikawa, J., Akiyoshi, M., Hirata, S., and Fukuda, T. (1996) *Saccharomyces cerevisiae* IRE2/HAC1 is involved in IRE1-mediated KAR2 expression. *Nucleic Acids Res.* **24**, 4222–4226.
15. Kawahara, T., Yanagi, H., Yura, T., and Mori, K. (1997) Endoplasmic reticulum stress-induced mRNA splicing permits synthesis of transcription factor Hac1p/Ern4p that activates the unfolded protein response. *Mol. Biol. Cell* **8**, 1845–1862.

16. Chapman, R. E. and Walter, P. (1997) Translational attenuation mediated by an mRNA intron. *Curr. Biol.* **7**, 850–859.
17. Gonzalez, T. N., Sidrauski, C., Dörfler, S., and Walter, P. (1999) Mechanism of non-spliceosomal mRNA splicing in the unfolded protein response pathway. *EMBO J.* **18**, 3119–3132.
18. Moore, M. J., Query, C. C., and Sharp, P. A. (1993) Splicing of precursors to mRNAs by the spliceosome, in *The RNA World* (Gesteland, R. F. and Atkins, J. F., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 303–357.
19. Westaway, S. K. and Abelson, J. (1995) Splicing of tRNA precursors, in *tRNA: Structure, Biosynthesis, and Function* (Söll, D. and RajBhandary, U., eds.), ASM Press, Washington, DC, pp. 79–92.
20. Abelson, J., Trotta, C. R., and Li, H. (1998) tRNA splicing. *J. Biol. Chem.* **273**, 12,685–12,688.
21. Dong, B. and Silverman, R. H. (1997) A bipartite model of 2-5A-dependent RNase L. *J. Biol. Chem.* **272**, 22,236–22,242.
22. Dong, B., Xu, L., Zhou, A., Hassel, B. A., Lee, X., Torrence, P. F., and Silverman, R. H. (1994) Intrinsic molecular activities of the interferon-induced 2-5A-dependent RNase L. *J. Biol. Chem.* **269**, 14,153–14,158.
23. Dong, B. and Silverman, R. H. (1999) Alternative function of a protein kinase homology domain in 2', 5'-oligoadenylate dependent RNase L. *Nucleic Acids Res.* **27**, 439–445.
24. Milligan, J. F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* **15**, 8783–8798.