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A Novel Mechanism for Regulating Activity of a Transcription Factor That Controls the Unfolded Protein Response

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Summary

Cells respond to an accumulation of unfolded proteins in the endoplasmic reticulum (ER) by increasing transcription of genes encoding ER–resident proteins. The information is transmitted from the ER lumen to the nucleus by an intracellular signaling pathway, the unfolded protein response (UPR). We have identified a basic–leucine zipper transcription factor, Hac1p, that is required for the UPR and binds to the UPR element in the promoter of UPR–regulated genes. Surprisingly, Hac1p is found in UPR–activated cells only, and its level is controlled by regulated splicing of its mRNA. Splicing replaces the C-terminal tail of Hac1p with a different peptide that renders Hac1p more resistant to an otherwise extremely rapid ubiquitin-dependent degradation. We propose that the complex regulation of Hac1p expression serves to provide multiple levels at which the UPR can be controlled.

Introduction

The unfolded protein response (UPR) pathway allows eukaryotic cells to respond to changing conditions in the endoplasmic reticulum (ER) by regulating the synthesis of ER–resident proteins. The accumulation of unfolded proteins in the ER leads to increased transcription of genes encoding ER–localized chaperones that assist in protein folding (Kozutsumi et al., 1988; Lee, 1987). The ER and nucleus are distinct membrane-bounded compartments of the cell. The unfolded protein signal, therefore, must be sensed in the lumen of the ER, be transferred across a membrane (either the ER or the inner nuclear membrane with which it is contiguous), and be received by the transcriptional apparatus in the nucleus, where it modulates gene expression (reviewed in Shamu et al., 1994; McMillan et al., 1994).

Although present in all eukaryotic cells examined, the UPR is best understood in the yeast Saccharomyces cerevisiae. Experimentally, the UPR can be activated in yeast by preventing glycosylation, e.g., by treating cells with tunicamycin (Tm) or by preventing disulfide-bond formation with reducing agents such as 2-mercaptoethanol. Genes known to be up-regulated by such treatments include *KAR2* (encoding Kar2p or BiP), *PDI1* (encoding protein disulfide isomerase), *EUG1* (encoding a PDI–like protein), and *FKB2* (encoding a peptidyl–prolyl *cis*–trans isomerase). These genes encode proteins that help catalyze the correct folding of proteins that transit through the ER (reviewed by Gething and Sambrook, 1992; Shamu et al., 1994; Sweet, 1993).

To date, only two components of the UPR have been identified. One of these is the transmembrane kinase Ire1p, encoded by the nonessential gene *IRE1*. Cells in which *IRE1* has been deleted do not increase transcription of ER–resident proteins in response to induction of the UPR (Cox et al., 1993; Mori et al., 1993). Ire1p is located in the ER or inner nuclear membrane or both and is likely to be the component that transmits the unfolded protein signal across the membrane. Its N-terminal domain lies in the lumen of the ER, where it presumably detects the accumulation of unfolded proteins, and its kinase domain lies in the cytoplasm (or nucleus), where it is presumably responsible for transmitting the unfolded protein signal to the appropriate downstream component or components. Ire1p, like most receptor transmembrane kinases, is thought to be activated by oligomerization and phosphorylation by neighboring Ire1p molecules (Shamu and Walter, 1996).

Interestingly, *ire1* mutants do not grow in the absence of inositol in the growth medium (Nikawa and Yamashita, 1992). Models to explain the link between inositol metabolism and the UPR suggest the intriguing possibility that the de novo synthesis of phospholipids and the up-regulation of ER content proteins are linked (Cox et al., 1993; Mori et al., 1993; J. S. C., R. Chapman, and P. W., unpublished data). Thus, the ER membrane may coordinately expand to accommodate an increase in ER content proteins. The unfolded protein response may therefore be more than a stress response pathway: it may execute, or be intimately linked to, a cellular program or programs that allow cells to maintain the appropriate abundance of ER during homeostasis (Nunnari and Walter, 1996).

A second known component of the pathway is the unfolded protein response element (UPRE), a 22 bp sequence present in the promoters of genes that are activated by the UPR. When situated within a heterologous promoter, the UPRE is sufficient to activate transcription in response to the accumulation of unfolded proteins (Kohno et al., 1993; Mori et al., 1992). Mutational analysis has defined the nucleotides within the UPRE that are essential for its ability to activate transcription (Mori et al., 1992).

The putative UPRE binding factor that is responsible for altering gene expression has proven elusive. Gel-shift experiments have identified an activity present in cell extracts that binds specifically to a UPRE probe, regardless of the activity of the UPR (Mori et al., 1992). However, the gene or genes encoding this factor have not been identified, and its participation in the UPR remains unclear. Furthermore, genetic screens to isolate mutants incapable of activating the UPR have isolated different alleles of *IRE1* yet have not identified a gene encoding a DNA-binding protein.

We describe here the identification of a transcription factor, Hac1p, that regulates the UPR by binding to the UPRE only when the signaling pathway is activated. Our studies reveal a complex and unusual regulation of Hac1p activity that distinguishes the UPR from other known signaling pathways.

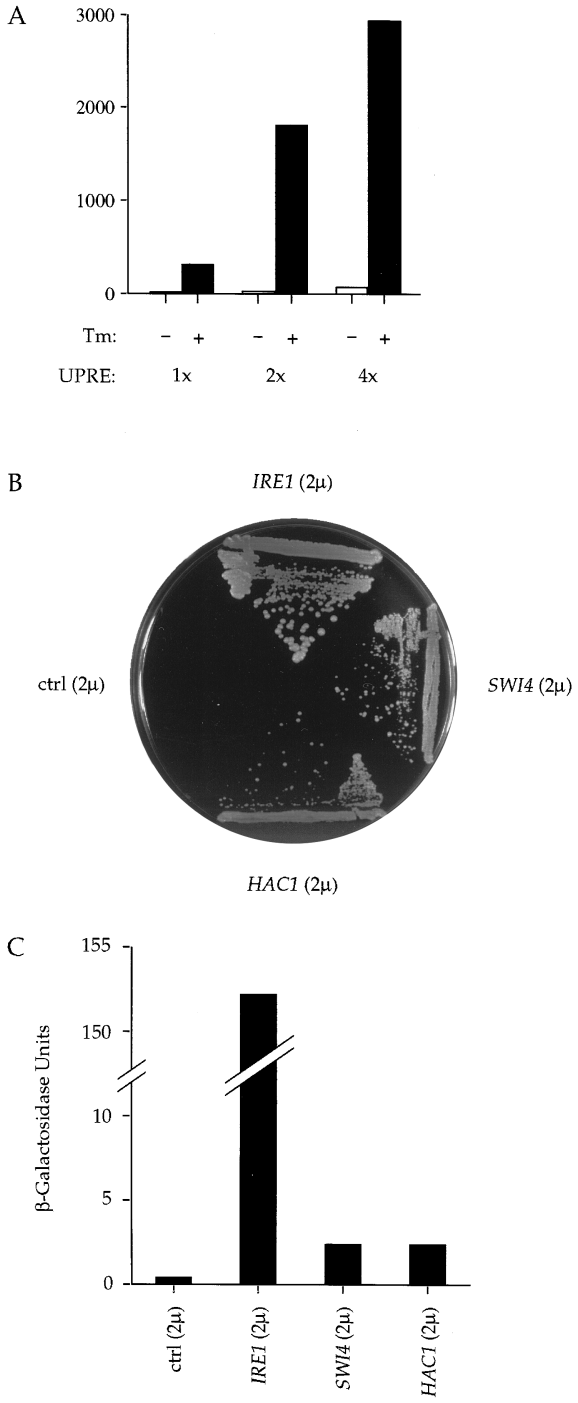

Figure 1. Identification of High Copy Activators of the UPR

(A) Tandem repeats of the UPRE increase the sensitivity of the UPRE-*lacZ* reporter construct. Copies (1, 2, and 4) of an oligonucleotide containing the 22 bp UPRE were inserted upstream of a disabled *CYC1* promoter-*lacZ* gene fusion. Wild-type strain W303-1A bearing these constructs on 2 μ plasmids were grown in the absence ("−Tm," empty bars) or presence ("+Tm," filled bars) of 1 μg/ml of tunicamycin for 4 hr before β-galactosidase levels were determined.

(B) Isolation of plasmids that activate the UPRE-*HIS3* reporter gene in the absence of *IRE1*. Diploid reporter strain JC329 (Δ*ire1*/Δ*ire1*) contains an integrated 4 × UPRE-*HIS3* construct as the only copy of *HIS3* and is unable to grow on media lacking histidine ("ctrl"). Transformants carrying each of the indicated genes on high copy plasmids were streaked for single colonies and grown at 30°C for 3 days.

(C) The levels of activation of the UPR by the high copy plasmids in (B) were quantitated by measuring the activity of a separate 4 × UPRE-*lacZ* reporter construct present in JC329.

Results

HAC1 Encodes an Essential Component of the UPR Pathway

We used a genetic approach in S. cerevisiae to identify new components of the UPR signaling pathway. In particular, we sought components that function downstream of the Ire1p transmembrane kinase. To this end, we screened a multicopy genomic library to isolate genes that, when overexpressed in cells deleted for *IRE1*, turn on the UPR constitutively. The UPR can be monitored using reporter enzymes whose synthesis is placed under control of the UPRE. As shown in Figure 1A, multimerization of the UPRE considerably increased the sensitivity of a reporter system. When one copy is present in the promoter of a *lacZ* reporter gene, the UPRE mediated an 8-fold increased level in β-galactosidase activity upon addition of Tm, an inducer of the UPR (Figure 1, lanes 1 and 2). In contrast, 43-fold and 40-fold increases were observed in cells carrying constructs that contained two and four tandem repeat copies of the UPRE, respectively (Figure 1, lanes 3–6). Because of the enhanced overall signal and the signal-to-background ratio, we used a promoter containing four copies of the UPRE to optimize the sensitivity of the screening procedure.

The Δ*ire1* strain used in our screen contained a $(UPRE)_4$–driven *HIS3* construct that was integrated into the chromosome and provided the only functional copy of *HIS3* in the cell. A multicopy genomic library was screened for plasmids that induced sufficient expression of *HIS3* to allow cells to grow on plates lacking histidine. Plasmids (20) containing different inserts were identified in the library. All 20 inserts contained one of three genes, *IRE1*, *SWI4*, and *HAC1* which, when individually subcloned into multicopy vectors, were sufficient to induce the UPR as shown by growth of the reporter strain in the absence of histidine (Figure 1B). The induction of the UPR was confirmed and quantitated using $(UPRE)_4$–driven *lacZ* that was integrated into the genome as a second independent reporter construct. Overexpression of *HAC1* and *SWI4* caused a 5-fold increase in β-galactosidase activity, whereas overexpression of *IRE1* caused a 500-fold increase (Figure 1C).

Overexpression of *IRE1* was previously shown to induce the UPR constitutively (Shamu and Walter, 1996). Identification of *IRE1* in our screen was therefore expected, validating the approach. Both *SWI4* and *HAC1* encode transcription factors and are therefore candidates to be directly or indirectly involved in the transcriptional activation of genes encoding ER–resident proteins. *SWI4* encodes a general transcription factor that regulates a wide variety of genes (Koch and Nasmyth, 1994; Ogas et al., 1991). Much less is known about *HAC1*. Based on sequence comparisons, Hac1p is a member of the basic–leucine zipper (bZIP) family of transcription factors that contain a basic DNA-binding region followed by a leucine zipper motif (see Figure 3A)

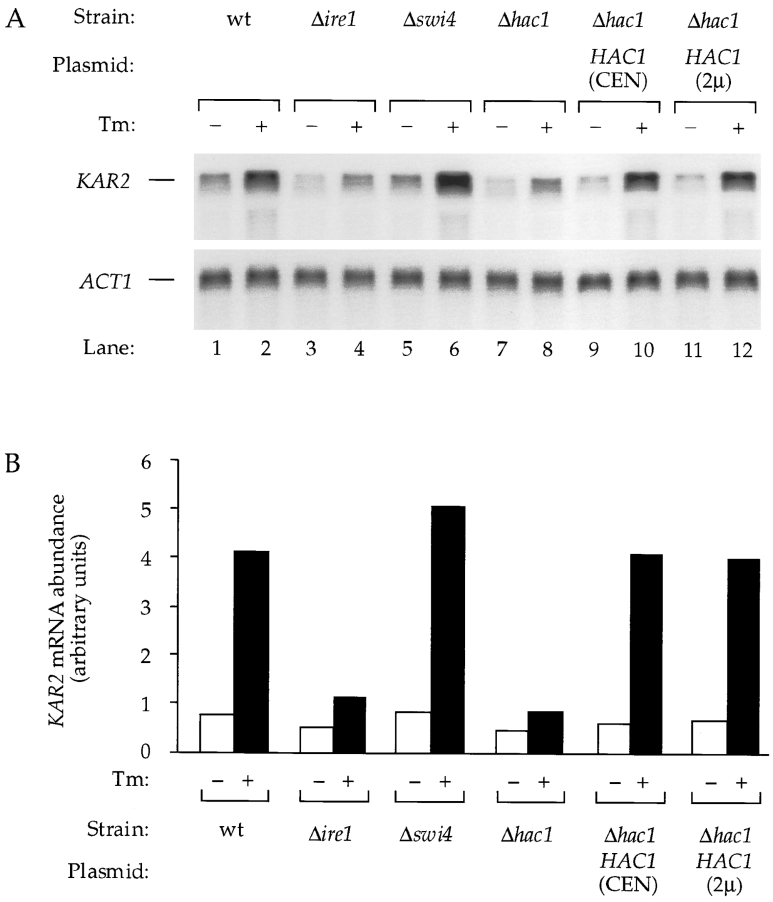

Figure 2. *HAC1* Is Required for the UPR

(A) Northern hybridization was performed on RNA isolated from cells with the indicated genotypes incubated for 1 hr either in the absence or presence of 1 μg/ml of tunicamycin. The same blot was probed for *KAR2* mRNA, stripped, and then probed for *ACT1* mRNA.

(B) The *KAR2* levels from (A) were quantitated and normalized to *ACT1* mRNA levels.

and was accordingly named (HAC, homologous to ATF and CREB [two other members of the bZIP family]; Nojima et al., 1994).

If *SWI4* or *HAC1* or both are bona fide components of the signaling pathway from the ER to the nucleus, then mutations in these genes should impair the UPR. Because neither of the two genes is essential for viability in haploid strains, we tested the ability of cells deleted for either gene to up-regulate ER–resident protein genes in response to Tm treatment. As previously described, wild-type cells induced transcription of *KAR2* mRNA (Figure 2, lanes 1 and 2), whereas the response was reduced to background levels in cells deleted for *IRE1* (Figure 2, lanes 3 and 4; Cox et al., 1993; Mori et al., 1993). Interestingly, Δ*swi4* mutant cells were indistinguishable from wild-type cells (Figure 2, lanes 5 and 6), indicating that *SWI4* is not required for the UPR pathway. In striking contrast, Δ*hac1* cells, like Δ*ire1* cells, were unable to elicit the UPR (Figure 2, lanes 7 and 8), suggesting that Hac1p is an essential component of the UPR. Because of these results, we focused our studies on *HAC1* and did not pursue further the effects that led to the identification of *SWI4*.

As expected, *HAC1* on a CEN/ARS plasmid restored the UPR in Δ*hac1* cells (Figure 2, lanes 9 and 10). Overexpression of *HAC1* from a 2 μ plasmid caused no significant increase above the basal level of *KAR2* transcription (Figure 2, lanes 11 and 12), consistent with the results in Figure 1C that show only a low level of induction of the $(UPRE)_4$–driven reporter construct. Thus, it is unlikely that we would have identified *HAC1* as a multicopy suppressor of an *IRE1* deletion without the sensitive reporter system.

Hac1p Binds Specifically to the UPRE

The results presented so far have identified *HAC1* as a required component of the UPR and, taken together with its similarity to other transcription factors, suggest that Hac1p may bind to the UPRE directly to activate transcription of genes encoding ER–resident proteins. We tested this possibility using gel-shift experiments. To this end, we incubated cell extracts with a ^{32}P-labeled 48 bp oligonucleotide containing the UPRE sequence and flanking DNA from the *KAR2* promoter. After incubation, the mixture was fractionated on nondenaturing polyacrylamide gels. Cell extracts contained a factor that bound to the oligonucleotide and retarded its migration in the gel, resulting in the formation of a new band (Figure 3B, lanes 2 and 3, band A). Importantly, the presence of this DNA-binding activity correlated with the activity of the UPR: DNA-binding activity was induced when cells were treated with Tm (Figure 3B, lane 3) and was undetectable in extracts prepared from Δ*hac1* cells, even if these cells were treated with Tm (Figure 3B, lanes 4 and 5). These results are consistent with the conjecture that Hac1p itself is the factor binding the UPRE.

To test this possibility directly, we expressed an epitope-tagged version of Hac1p. We inserted a DNA segment encoding the HA epitope derived from influenza

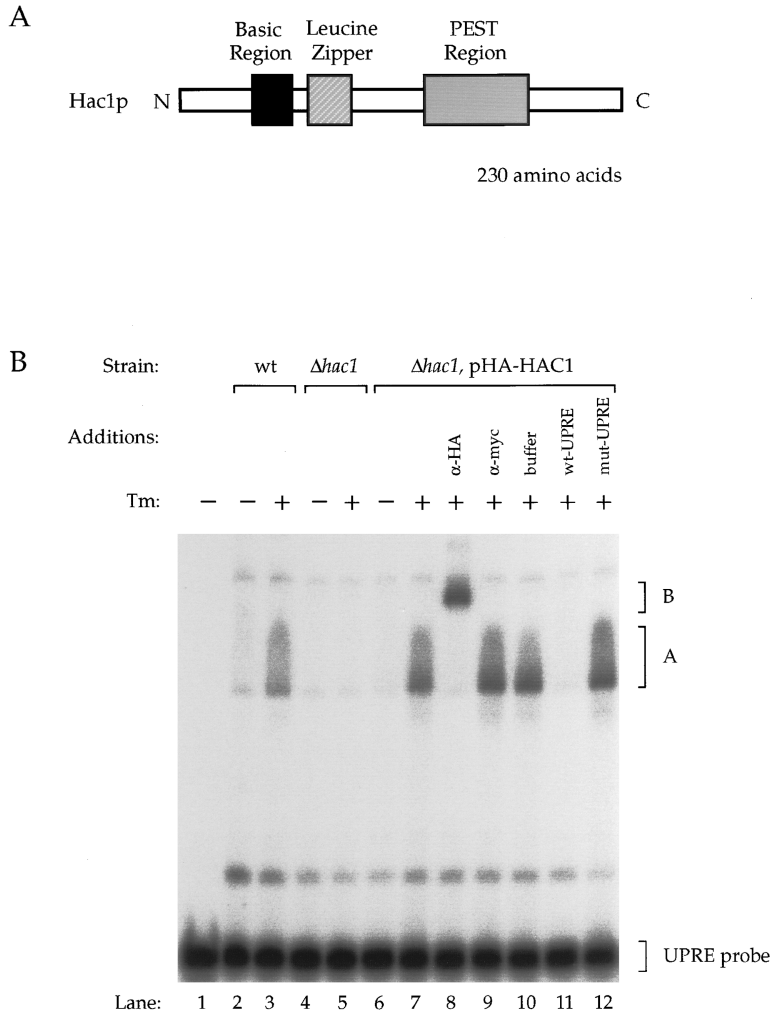

Figure 3. Hac1p, a bZIP Transcription Factor, Binds to the UPRE Only When the UPR Is Activated

(A) A schematic representation of the primary sequence of Hac1p. The PEST region includes a 24 amino acid sequence that the PEST-FIND program identified as a likely degradation signal (Rogers et al., 1986).

(B) Gel-shift analysis. Native extracts (see Experimental Procedures) prepared from indicated strains were incubated with a radiolabeled UPRE probe for 15 min and fractionated on nondenaturing polyacrylamide gels. The plasmid pHA-*HAC1* (pJC316) carries a gene encoding for a version of Hac1p with the HA epitope inserted near the N-terminus of the protein. For lanes 8 and 9, equal amounts of α-HA and α-myc antibodies were added 5 min prior to gel loading. For lanes 11 and 12, a 100-fold molar excess of unlabeled UPRE oligonucleotide to probe was added prior to addition of extract. The particular method of extract preparation was critical. Extracts prepared according to Mori et al. (1992) were devoid of Hac1p (presumably owing to degradation) and any regulated UPRE binding activity.

virus hemagglutinin into *HAC1* near the 5′ end of the coding region. The epitope-tagged gene (*"HA-HAC1"*) fully complemented a *HAC1* deletion, indicating that the presence of the epitope tag did not impair the function of Hac1p (data not shown). Extracts prepared from cells expressing HA-Hac1p also showed an identical UPRE binding activity compared with wild-type extracts (Figure 3B, compare lanes 6 and 7 with lanes 2 and 3). Most importantly, addition of anti-HA antibodies to the binding reaction resulted in the disappearance of band A and the appearance of a new band of slower mobility (Figure 3B, lane 8, band B). In contrast, addition of a control antibody had no effect (Figure 3B, lane 9). The supershift of band A after addition of the anti-HA antibody provides strong evidence that HA-Hac1p, and by extension Hac1p, is a component of the DNA-binding activity that is induced upon activation of the UPR.

We assessed the specificity of Hac1p binding to the UPRE by adding an excess of different unlabeled oligonucleotides into the binding reaction. Hac1p binding was competed by an oligonucleotide consisting of the minimal 25 bp UPRE sequence (Figure 3B, lane 11). In contrast, an identical oligonucleotide containing a single point mutation that abolishes the transcriptional activity of the UPRE in vivo (Mori et al., 1992) did not compete (Figure 3B, lane 12).

Induction of the UPR Correlates with Hac1p Abundance

From the data presented so far, we conclude that Hac1p is the transcription factor that activates the expression of genes encoding ER–resident proteins. As Hac1p functions downstream of the Ire1p kinase, we thought that the intracellular localization of Hac1p in uninduced cells might provide clues as to the route along which the UPR is propagated from the ER lumen to the nucleus. To determine its intracellular localization, we visualized HA-Hac1p by indirect immunofluorescence using anti-HA antibodies. After induction of the UPR with Tm, a strong mostly nuclear signal was detected (Figure 4A, panel labeled "HA-Hac1p; +Tm") that coincided with nuclear DNA as detected by DAPI staining (Figure 4A, panel labeled "DNA; +Tm"). Surprisingly, we observed no staining above background levels in uninduced cells (Figure 4A, panel labeled "HA-Hac1p; −Tm"), suggesting that Hac1p levels are significantly lower in these cells.

This conjecture was supported by Western blot analysis, in which whole cell extracts were probed with anti-HA antibodies. A band corresponding to HA-Hac1p was detected in extracts of cells that were treated with Tm but not in uninduced cells (Figure 4B, lanes 1 and 2). HA-Hac1p was also detected in cells in which the UPR

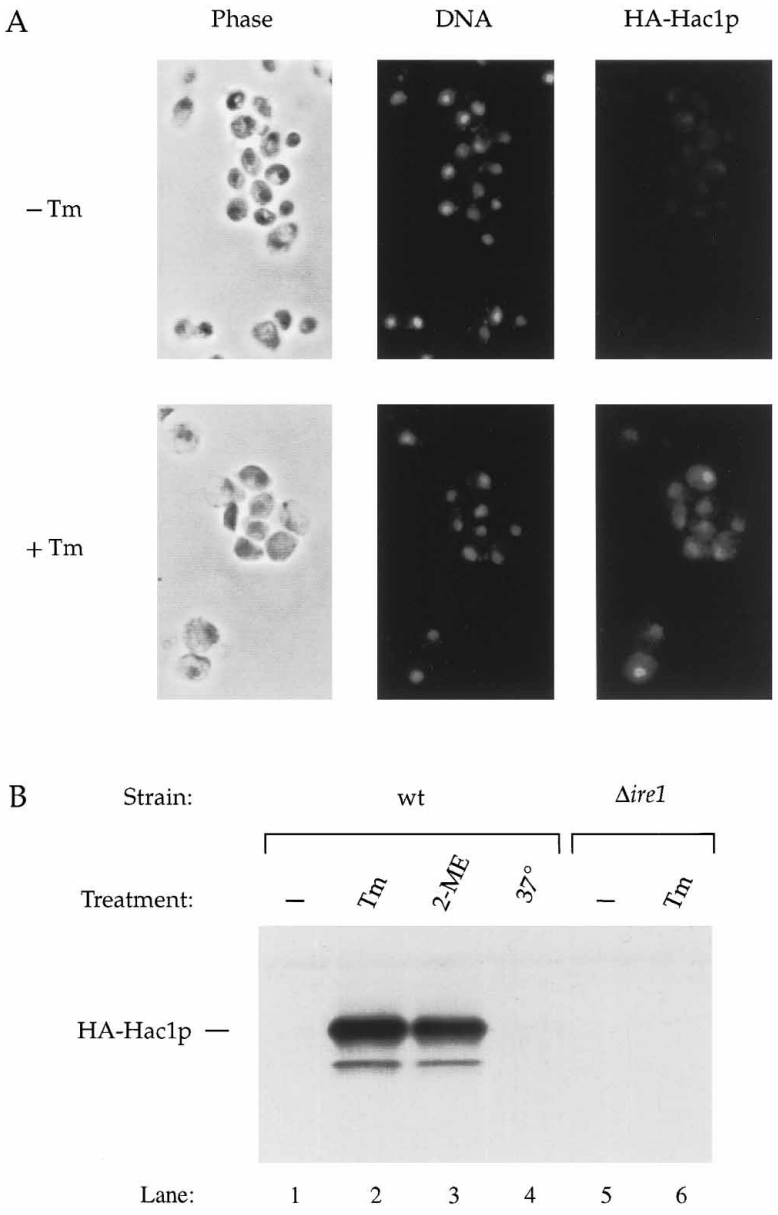

Figure 4. Hac1p Is Detected Only When Unfolded Proteins Accumulate in the ER

(A) HA-Hac1p localizes to the nucleus in Tm-treated cells. Δ*hac1* mutant cells (JC408) harboring the HA-tagged version of the *HAC1* gene were grown either in the absence or presence of Tm and treated as described in Experimental Procedures. Cells were visualized by light microscopy (Phase), and DNA was stained with DAPI. HA-Hac1p was visualized by indirect immunofluorescence using monoclonal α-HA antibodies and FITC–labeled secondary antibodies. Both the untreated and Tm-induced cells were fixed and stained identically, and equal exposure times for each column are shown.

(B) Hac1p induction is specific for ER–localized protein misfolding and requires functional Ire1p. Tm treatment of wild-type cells (JC408 harboring the plasmid pJC316) and Δ*ire1* cells (JC417 harboring pJC316) was performed as in Figure 2A. 15 mM β-mercaptoethanol (2-ME) and 37°C treatments were for 1 hr prior to harvest. Cell extracts were probed by Western blot analysis using α-HA antibodies.

was induced by treatment with β-mercaptoethanol (Figure 4B, lane 3), but not in cells that were temperature-shifted to induce the heat-shock response (Figure 4B, lane 4), indicating that the change in the cellular Hac1p level was specific for the UPR. Consistent with this interpretation, no HA-Hac1p was detected in Δ*ire1* cells even after Tm treatment (Figure 5B, lanes 5 and 6).

HAC1 mRNA Is Spliced When the UPR is Induced

The changes in Hac1p abundance upon induction of the UPR could be due to transcriptional or posttranscriptional events or both. This prompted us to test by Northern blot analysis whether the amount of *HAC1* mRNA was altered when the UPR was induced. As shown in Figure 5A, the overall amount of *HAC1* mRNA detected was unchanged upon induction of the UPR (Figure 5A, lanes 1 and 2). To our surprise, however, we observed a new, faster migrating species of *HAC1* mRNA in cells in which the UPR was induced (Figure 5A, "*HAC1*^{i}"). The mobility shift was a direct consequence of Ire1p kinase activation as it was not observed in cells deleted for *IRE1* (Figure 5A, lanes 3 and 4). We refer to the smaller *HAC1* mRNA species in induced cells as *HAC1*^{i} mRNA and to the larger *HAC1* mRNA species in uninduced cells as *HAC1*^{u} mRNA.

To determine the molecular structure of *HAC1*^{i} mRNA and *HAC1*^{u} mRNA, we first examined the 5′ ends of the two species by primer extension. RNA isolated from induced and uninduced cells gave rise to identical primer extension products (data not shown). We therefore conclude that the different mobilities of the two *HAC1* mRNA species were not due to use of different transcription start sites. The bands representing both primer extension products were of equal intensity, thereby providing independent confirmation of the results in Figure 5A that the total level of *HAC1* mRNA was not significantly changed in either cell.

We next synthesized a cDNA pool from RNA fractions

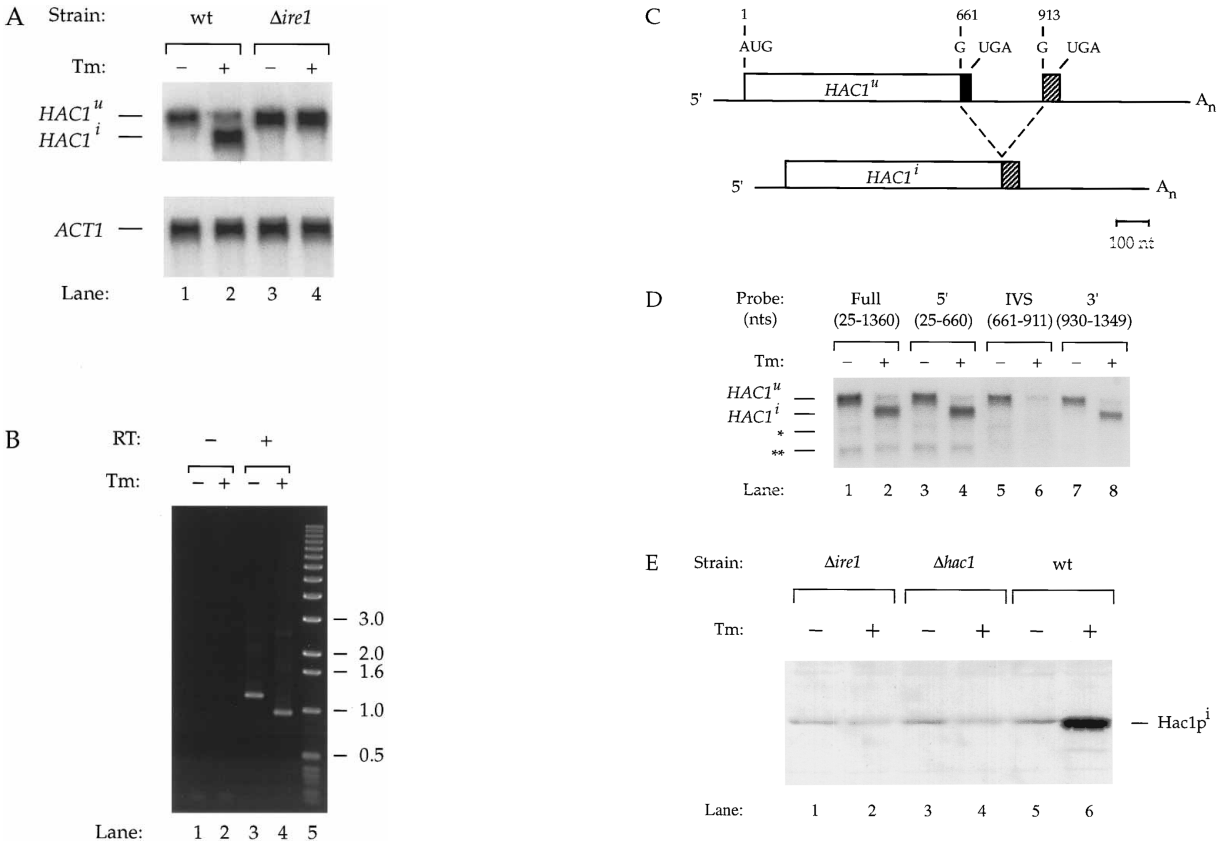

Figure 5. *HAC1* mRNA Is Spliced in Response to UPR Activation

(A) Northern analysis was performed as in Figure 2A except that the blot was probed for *HAC1* mRNA. The uninduced (*HAC1*^{u}) and faster migrating induced (*HAC1*^{i}) forms of *HAC1* mRNA are denoted.

(B) RT–PCR analysis using primers to amplify from the 5′ end of *HAC1* to the poly-adenosine tail of the transcript (see Experimental Procedures) was performed on RNA extracted from wild-type cells (JC103) grown in the absence or presence of 1 μg/ml of Tm. Reverse transcriptase was either omitted ("−RT") or added ("+RT") to the first-strand cDNA synthesis reaction. An aliquot of the final PCR reaction was loaded onto a 1% agarose gel and electrophoresed in the presence of ethidium bromide.

(C) Linear representation of *HAC1* mRNA splicing deduced from sequencing the PCR products from (B). The dotted line represents excision of the 252 nt intron from *HAC1*^{u}, giving rise to *HAC1*^{i} . Boxes above the lines represent Hac1p coding information and depict the removal of 10 codons from *HAC1*^{u} (black) with the concomitant addition of 18 codons (diagonally striped) to *HAC1*^{i} . *HAC1*^{i} mRNA contains one G residue at the exon–exon junction. We cannot distinguish whether this residue is derived from G661 or G913 of *HAC1*^{u} mRNA. The IVS therefore corresponds either to nucleotides 661–912 or to nucleotides 662–913.

(D) Northern analysis probing for different portions of *HAC1* mRNA. DNA probes were generated that contain only the sequences denoted (nts) and correspond to those in (C). Faint bands (denoted with asterisks) were consistently detected and may represent the 5′ exon alone (double asterisks) and the 5′ exon plus the IVS (single asterisk). It is not clear whether these bands correspond to splicing intermediates or dead-end products.

(E) Hac1p^i has a new C-terminus. Polyclonal rabbit serum raised against the predicted tail-peptide of Hac1p^i (diagonally striped box in [C]) was used in a Western blot of cell extracts prepared as in (B).

isolated from uninduced and induced cells using reverse transcriptase primed with oligo-dT containing DNA oligonucleotide primers. Since both *HAC1* mRNA species contain the identical 5′ end, we amplified *HAC1* cDNAs by polymerase chain reaction (PCR), using an oligonucleotide corresponding to the common 5′ end in combination with a second oligonucleotide corresponding to a sequence that was introduced with the oligo-dT primer during first-strand synthesis. PCR products obtained from RNA isolated from induced cells were approximately 250 nt smaller than those obtained from RNA isolated from uninduced cells (Figure 5B, compare lane 4 with lane 3), consistent with the difference in size observed in the Northern analysis in Figure 5A.

We next cloned and sequenced the PCR fragments. DNA sequencing showed that the 3′ ends of *HAC1*^{i} mRNA and *HAC1*^{u} mRNA were very similar, although the precise poly-A addition site differed by a few nucleotides in individual clones. *HAC1*^{i} mRNA, however, lacked an internal stretch of 252 nt (Figure 5C), indicating that it had undergone an RNA-splicing reaction. This result was surprising, since the sequences surrounding the presumptive splice junctions do not conform to the consensus sequences for mRNA splicing in yeast (Sidrauski et al., 1996 [this issue of *Cell*]). We therefore confirmed the prediction from the cDNA cloning by repeating the Northern analysis shown in Figure 5A but using probes that exclusively monitor either the 5′ exon, the 3′ exon,

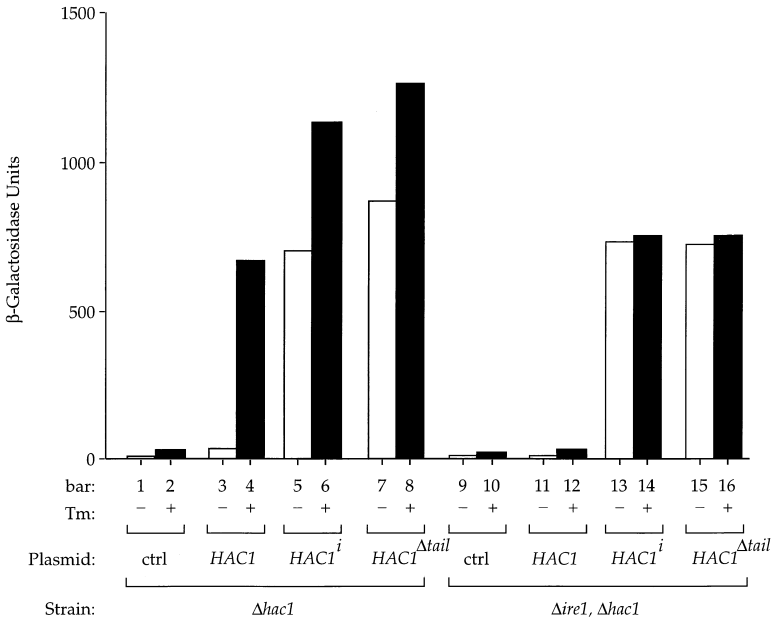

Figure 6. *HAC1* mRNA Splicing Is Sufficient to Activate the UPR

lacZ expression from an integrated 1 × UPRE-*lacZ* reporter construct was determined in Δ*hac1* mutant cells (JC408) or from Δ*ire1*, Δ*hac1* double-mutant cells (JC417). Strains harbored plasmids containing either no insert ("ctrl"), wild-type *HAC1* ("*HAC1*"), a modified *HAC1* gene in which the sequence corresponding to the IVS of the *HAC1* mRNA was removed ("$HAC1^{i}$"), or a version of *HAC1* that encodes for a truncated version of Hac1p in which a stop codon was inserted at the exon–exon junction ($HAC1^{\Delta tail}$). The different forms of Hac1p all contained the HA-tag at their N-termini.

or the intron (intervening sequence [IVS[). As expected, the 5′ and 3′ exons were present in both *HAC1* mRNA species (Figure 5D, lanes 3 and 4, 7 and 8), whereas the 252 bp IVS could only be detected in $HAC1^{u}$ mRNA that is predominant in uninduced cells (Figure 5D, lanes 5 and 6).

We isolated and sequenced seven independent cDNA clones of spliced *HAC1* mRNA. We found that the seven isolates varied by a few nucleotides at their 3′ ends, suggesting that they were derived from individual mRNA molecules that differed from one another because poly-A addition occurred at slightly different sites. Importantly, all seven clones lacked the identical 252 nt IVS. We therefore conclude that splicing of *HAC1* mRNA is precise, i.e., that the 5′–3′ exon junction determined by cDNA sequencing is the major, if not the only, product of the splicing reaction in induced cells.

Splicing of *HAC1* mRNA Is Sufficient to Induce the UPR

Because the splicing of *HAC1* mRNA correlates directly with induction of the UPR, we considered it an attractive hypothesis that the splicing reaction is a required step in the signaling pathway. The IVS is positioned towards the 3′ end of the open reading frame of *HAC1*, so that splicing is predicted to change the C-terminal tail of Hac1p (Figure 5C). In particular, as the result of splicing, the most C-terminal 10 amino acids of Hac1p are replaced by a sequence of 18 different amino acids, thus predicting a distinct protein product that henceforth will be referred to as $Hac1p^{i}$. To ascertain that $Hac1p^{i}$ is actually synthesized, we raised an antibody against a peptide comprising the C-terminal 10 amino acids of the predicted $Hac1p^{i}$ sequence. As shown by Western blot analysis, the anti-$Hac1p^{i}$ tail antibody recognizes a band of the correct size in cells treated with Tm (Figure 5E, lane 6) but fails to detect a band in cells in which the UPR was not induced (lane 5) or in which *HAC1* or *IRE1* were disrupted (lanes 1–4). In addition to showing directly that $Hac1p^{i}$ is synthesized in vivo, these data provide additional support for the notion that *HAC1* mRNA splicing accurately produces the exon–exon junction that was determined by nucleotide sequencing.

We next tested directly whether splicing of *HAC1* mRNA is sufficient to cause induction of the UPR. For this purpose, we removed the IVS from the *HAC1* gene, thereby creating a gene that only encodes $Hac1p^{i}$. Indeed, expressing the intron-less $HAC1^{i}$ from its own promoter in Δ*hac1* cells resulted in constitutive induction of the UPR that reached about the same level as Tm-treated cells expressing wild-type *HAC1* (Figure 6, compare bars 4 and 5). Whereas induction of the UPR in wild-type cells required *IRE1* (Figure 6, bars 4 and 12), the constitutive induction of the UPR in $HAC1^{i}$-expressing cells was *IRE1*-independent (Figure 6, bars 5 and 13), indicating that the requirement for *IRE1* to induce *HAC1* mRNA splicing has been bypassed. These results, therefore, establish a causal relationship between splicing and the UPR. We also observed that cells expressing $HAC1^{i}$ gave rise to significantly smaller colonies, suggesting that a permanent strong induction of the UPR is detrimental to cell growth.

Interestingly, a further 1.5-fold induction of the UPR was observed upon Tm treatment of $HAC1^{i}$-expressing cells (Figure 6, compare bars 5 and 6). In contrast to the constitutive induction of the UPR caused by expression of $HAC1^{i}$ alone, this further increase was *IRE1*-dependent (Figure 6, compare bars 6 and 14). This result suggests that in addition to inducing *HAC1* mRNA splicing, there is another way in which *IRE1* can affect the level of the UPR.

Both $Hac1p^{u}$ and $Hac1p^{i}$ Are Synthesized, But Only $Hac1p^{i}$ Is Stable

As shown above, Hac1p levels are significantly greater in cells in which the UPR is induced. Splicing of *HAC1* mRNA could increase the levels of Hac1p by two principally different mechanisms. First, only $HAC1^{i}$ mRNA may

be efficiently translated. According to this scenario, *HAC1*^{u} mRNA could be retained in the nucleus unless it becomes spliced or, if it reaches the cytosol, translation may be impaired owing to the presence of the unspliced IVS. Alternatively, both *HAC1*^{u} mRNA and *HAC1*^{i} mRNA may be efficiently translated, but changing the C-terminal tail of Hac1p could result in a protein with increased stability.

To begin to address these possibilities, we asked first whether *HAC1*^{i} mRNA and *HAC1*^{u} mRNA were translated with similar efficiencies. To this end, we prepared cell extracts from induced and uninduced cells and fractionated them by sucrose density centrifugation to display polyribosome profiles. The ultraviolet absorbance profiles of the gradients showed that intact polyribosomes were well preserved during the fractionation procedure (Figures 7A and 7B). RNA was isolated from individual fractions and analyzed by Northern blotting. Actin mRNA was recovered exclusively in the polyribosome peak near the bottom of the gradients (Figures 7A and 7B; "*ACT1*"). Interestingly, *HAC1*^{i} mRNA in Tm-induced cells (Figure 7B) and most *HAC1*^{u} mRNA in uninduced cells (Figure 7A) were also recovered in the polyribosome region, indicating that both RNAs are actively translated. These results suggest that neither mRNA stability nor access to or recruitment onto ribosomes are significantly different between the two *HAC1* mRNA species.

It is interesting to note, however, that in contrast to *HAC1*^{i} mRNA and the actin mRNA control, a small portion (approximately 20%) of *HAC1*^{u} mRNA in uninduced and about half of *HAC1*^{u} mRNA in induced cells always sedimented near the top of the sucrose gradient. This result indicates that *HAC1*^{u} mRNA is not quite as efficiently recruited onto ribosomes as *HAC1*^{i} mRNA or other cellular mRNAs.

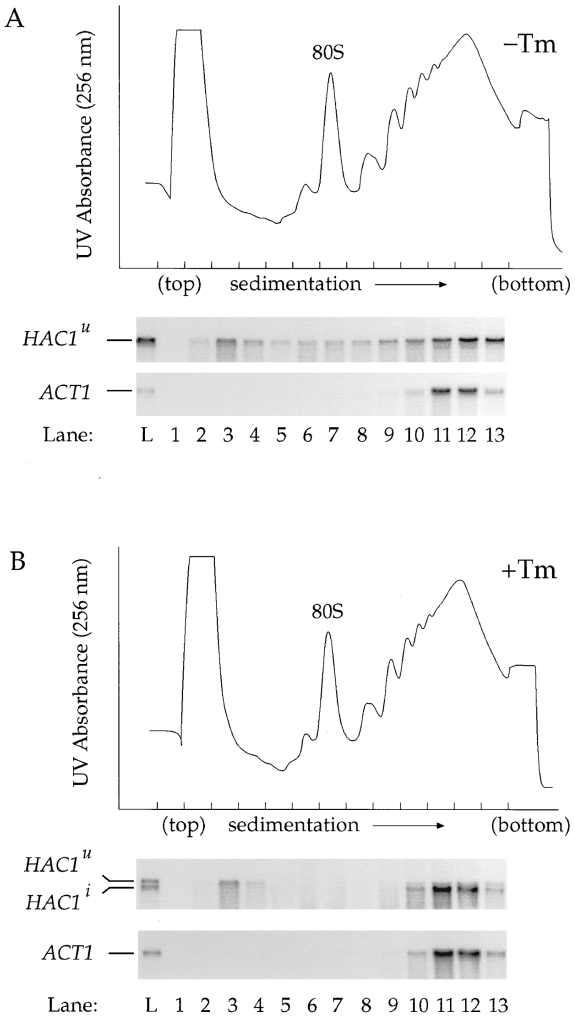

Figure 7. Both Forms of *HAC1* mRNA Are Translated

Northern analysis of the distribution of *HAC1* and *ACT1* mRNAs in polysomes from extracts of wild-type cells incubated either (A) in the absence of Tm or (B) in the presence of Tm. Cell extracts were centrifuged on 7%–47% sucrose gradients and fractionated using an ISCO gradient fractionator. An ultraviolet absorbance profile was recorded by scanning at 254 nm to display the polysome profile. RNA was isolated from individual gradient fractions, and equal volumes of each fraction were subjected to Northern analysis. The position of the 80S monosome peak and the direction of sedimentation are indicated. L, load fraction applied to gradient.

Hac1p^u Activates the UPR If Its Ubiquitin-Dependent Degradation Is Prevented

If both *HAC1*^{u} and *HAC1*^{i} mRNA are indeed translated to a similar extent, then Hac1p^u must be degraded significantly faster than Hac1p^i. Inspection of the Hac1p sequence reveals clues about the mechanism of Hac1p degradation; the C-terminal half of the protein (see Figure 3A, amino acids 119–230) contains multiple clusters of the amino acids Pro, Glu, Asp, Ser, and Thr, which overall constitute 47% of the amino acids that make up this domain. Regions rich in these amino acids, known as PEST sequences, have been shown to destabilize proteins by targeting them into ubiquitin-dependent proteolysis pathways. The first step in ubiquitin-dependent protein breakdown is recognition of the substrate protein by one of the ubiquitin-conjugating enzymes, a family of structurally and functionally related proteins, encoded in yeast by the *UBC* genes, which catalyze the initial covalent attachment of ubiquitin (reviewed by Hochstrasser, 1995). Mutations in different *UBC* enzymes affect the stability of different substrate proteins. We therefore asked if we could identify *ubc* mutants in which Hac1p^u would be stabilized, reasoning that stabilization by itself might be sufficient to activate the UPR constitutively.

Strains bearing mutations in different *UBC* genes were transformed with the UPRE-*lacZ* reporter construct. Indeed, we found that β-galactosidase was induced in different mutant strains (Figure 8A). Highest levels of induction were observed in strains deleted for both *UBC4* and *UBC5*, whereas the deletion of either gene alone had more marginal effects. *UBC4* and *UBC5* encode ubiquitin-conjugating enzymes that are closely related in their primary structure and have an overlapping function (Seufert and Jentsch, 1990). β-galactosidase was also induced in *ubc7* and *ubc8* strains, indicating that multiple UBC proteins may cooperate to degrade Hac1p (Figure 8A). β-galactosidase levels in Δ*ubc4,5* strains were 39% of those obtained after induction of the UPR with Tm (Figure 8B, compare bars 3 and 4),

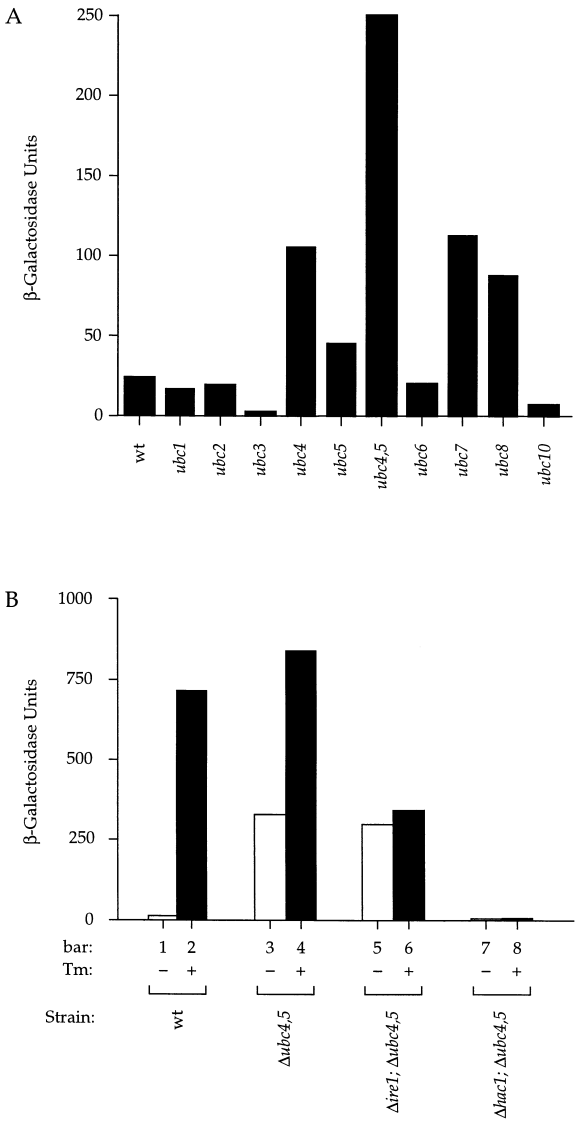

Figure 8. The UPR Is Activated in Mutants Deficient for Ubiquitin-Dependent Proteolysis

(A) A panel of *ubc* mutants were tested for their ability constitutively to activate a 1 × UPRE-*lacZ* reporter construct present on plasmid pJC31. The strain bearing the temperature-sensitive *ubc3* allele, also called *cdc34*, was shifted to the nonpermissive temperature for 4 hr before β-galactosidase levels were determined.

(B) The Δ*ubc4* Δ*ubc5* mutant strain (MHY508) was further tested for its ability to respond to Tm as well as for its ability to activate the UPR constitutively in the absence of *IRE1* and *HAC1* (strains JC472 and JC460, respectively).

indicating that the increased stability of Hac1p in these cells led to a significant level of induction of the UPR. Importantly, a similar induction was measured in Δ*ubc4,5* strains that also lacked *IRE1* (Figure 8B, bar 5), although, as expected, this response could not be further induced upon addition of Tm (Figure 8B, bar 6). Moreover, induction of the UPR was abolished in Δ*ubc4,5* strains that also lacked *HAC1*, indicating that activation of the UPR in Δ*ubc4,5* cells is not due to indirect effects that bypass *HAC1* function altogether (Figure 8B, bars 7 and 8). These results are therefore consistent with the notion that Ubc4p and Ubc5p are directly involved in the degradation of Hac1p.

Hac1p^u and Hac1p^i differ only by their C-terminal tails. Because Hac1p^i appears to be considerably more stable than Hac1p^u, the Hac1p^i tail must exert a stabilizing influence on the protein or, alternatively, the Hac1p^u tail must be destabilizing. To distinguish between these possibilities, we constructed a deletion mutant that inserted a stop codon at the exon–exon splice junction. This tail-less version of *HAC1* ("*HAC1*$^{\Delta tail}$") was expressed in Δ*hac1* cells. We found that expression of *HAC1*$^{\Delta tail}$ induced the UPR to the same degree as expression of *HAC1*^{i} (see Figure 6, bars 7, 8, 15, and 16). We therefore conclude that the tail of Hac1p^u is required for its extreme instability in uninduced cells.

Discussion

We have identified Hac1p as the transcription factor that controls the UPR in yeast. Hac1p binds directly to the UPRE, and disruption of *HAC1* results in the complete loss of the UPR. Δ*hac1* cells grow at wild-type rates and require inositol for growth (Sidrauski et al., 1996); i.e., their phenotype is identical to that of Δ*ire1* cells. We therefore propose that the function of Hac1p is dedicated to the UPR pathway.

As a member of the bZIP family of transcription factors, it is likely that Hac1p functions as a dimer in which two subunits are linked via a leucine zipper. We cannot presently distinguish whether the active transcription factor is a homodimer or a heterodimer in which Hac1p assembles with an unknown partner. *HAC1* has been previously shown to be transcribed at constant levels throughout the yeast cell cycle (Nojima et al., 1994). Expression was increased, however, during late phases of meiosis. In light of the newly proposed role of Hac1p as regulator of the UPR, it is interesting to speculate that this up-regulation reflects an additional need for ER membrane, possibly required to envelop the developing spores. Intriguingly, meiotic up-regulation of *HAC1* was blocked in Δ*swi4* cells, indicating that *SWI4*, directly or indirectly, controls *HAC1* transcription (Nojima et al., 1994). This observation provides a possible explanation for the identification of *SWI4* in our screen.

In the same study, Hac1p was also proposed to bind to cAMP regulatory elements. Binding, however, was assayed using high concentrations of purified glutathione S-transferase–Hac1p fusion proteins, raising some doubts about specificity and the physiological significance of the observed interaction. Although we consider it unlikely, we cannot rule out from the data presented here that Hac1p binds to other DNA targets in addition to the UPRE.

Most importantly, we have shown here that the UPR is controlled by regulating the cellular level of Hac1p, which, in turn, coincides with splicing of the *HAC1* mRNA. Our current model for these regulatory events is shown in Figure 9. When unfolded proteins accumulate in the ER, Ire1p is activated and induces the processing of *HAC1* mRNA (Figure 9, left branch). We have shown that splicing is strictly Ire1p-dependent and produces a unique new exon–exon junction. The consequence is a change in the *HAC1* open reading frame so

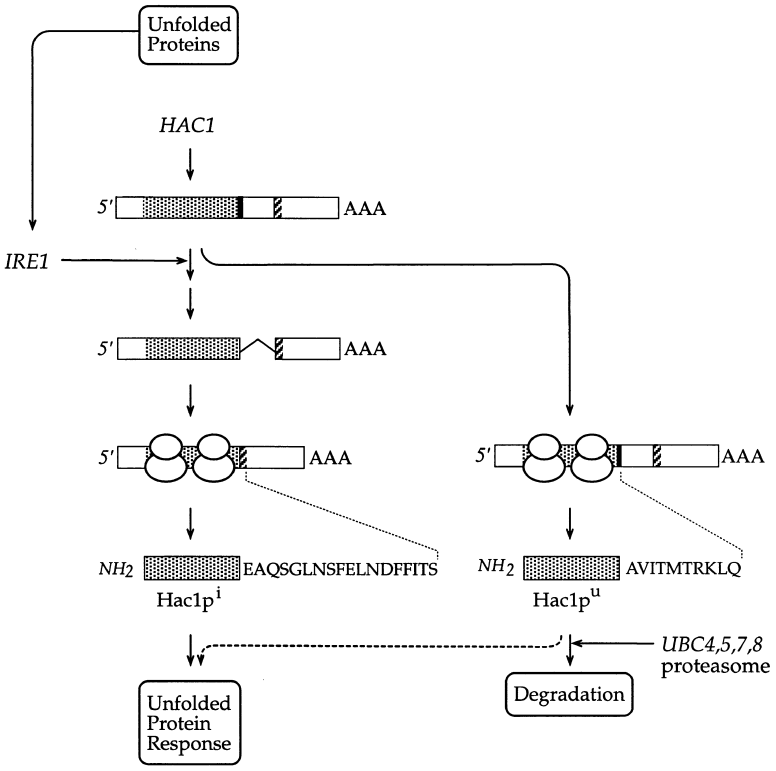

Figure 9. Model for Regulation of Hac1p

The individual steps are described in the Discussion. The left branch depicts the Ire1p-activated splicing pathway of *HAC1* mRNA, leading to the production of Hac1p^{i}; the right branch depicts Hac1p^{u} synthesis and degradation in uninduced cells. The C-terminal peptide sequences of Hac1p^{i} and Hac1p^{u} are spelled out in single-letter code. The dashed black arrow (bottom) indicates the putative activation of the UPR by stabilizing or activating Hac1p^{u} that is discussed in the text.

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}, is readily detectable in cells (Figure 3). Indeed, expression from its own promoter of an intron-less construct encoding Hac1p^{i} induces the full UPR. Thus, as the result of splicing, Hac1p^{i} becomes available in sufficient concentrations to mediate the transcriptional activation of its target genes. We therefore consider it likely that splicing of *HAC1* mRNA is an obligate event in eliciting the response under physiological conditions.

In S. cerevisiae, RNA splicing generally occurs constitutively. In addition to *HAC1* mRNA, the splicing of only one other transcript, *MER2* mRNA coding for a meiosis-specific protein, is known to be positively regulated (Engebrecht et al., 1991). Efficient *MER2* mRNA splicing requires a dedicated factor, Mer1p, which is only expressed during meiosis and helps engage *MER2* mRNA with the spliceosome. Thus, similar to many known examples of regulated splicing in higher eukaryotic cells, *MER2* mRNA splicing is induced after cells have made the irreversible decision to enter a different developmental state. In contrast, *HAC1* mRNA splicing is part of a bona fide intracellular signaling pathway that is operative in vegetatively growing cells. To our knowledge, splicing has not been described as a regulatory step in signaling pathways in yeast or other cells.

The details of *HAC1* mRNA splicing are remarkable. In particular, first, the splice junctions do not conform to the consensus of spliceosome-mediated splicing, and second, splicing is not blocked in mutants affecting spliceosome function (Sidrauski et al., 1996). We therefore propose that *HAC1* mRNA bypasses spliceosome-mediated processing and is spliced in a nonconventional manner. Furthermore, whereas yeast introns are commonly found close to the 5′ end of open reading frames, the IVS is positioned near the 3′ end of the *HAC1* open reading frame. Early stop codons introduced by introns are thought to limit the recruitment of ribosomes onto unspliced mRNAs, thereby contributing to the destabilization of unspliced pre-mRNAs such as pre-*MER2* mRNA (He et al., 1993). In contrast, we find that unspliced *HAC1^{u}* mRNA is found on polyribosomes of similar size as is *HAC1^{i}* mRNA (Figure 7) and that both versions of *HAC1* mRNA have similar half-lives (data not shown). Splicing of *HAC1* mRNA therefore represents a switch between two functional mRNAs.

As we show in Figure 7, *HAC1^{u}* mRNA is actively recruited to polyribosomes. Thus, in contrast to most other intron-containing mRNAs that are retained in the nucleus as hnRNA until they become spliced, the majority of *HAC1^{u}* mRNA exits the nucleus to become translated. In uninduced cells, about 20% of *HAC1^{u}* mRNA, however, is not found in polyribosomes. This portion of the mRNA molecules may represent a pool that has not exited the nucleus and would therefore be available as splicing substrate when Ire1p becomes activated upon changing conditions in the ER. Thus, we predict that the rate of exit of *HAC1^{u}* mRNA from the nucleus is slowed, possibly owing to interactions with the components mediating its splicing.

Formally, we have not yet demonstrated that *HAC1^{u}* mRNA is indeed translated constitutively to produce Hac1p^{u}, because we cannot detect Hac1p^{u} in uninduced cells (Figure 3). Indirect evidence suggests, however, that Hac1p^{u} is indeed synthesized but then degraded as rapidly as it is made (Figure 9, right branch). First, most *HAC1^{u}* mRNA is found on polyribosomes. To our knowledge, there is no example of an mRNA found on

polyribosomes in vivo that is not being actively translated. However, we cannot rule out the possibility that, for some reason, ribosomes may traverse the unspliced *HAC1* mRNA less efficiently than the spliced *HAC1* mRNA. An increase in the translation efficiency of the spliced *HAC1* mRNA could thus contribute to the high levels of Hac1p^{i} detected in induced cells. Second, the UPR is partially induced in Δ*ubc4,5* cells, indicating that blocking ubiquitin-dependent protein breakdown leads to the accumulation of sufficient Hac1p levels to activate the response. Even then, however, we have not been able to detect HA-Hac1p^{u} in Δ*ubc4,5* cells using anti-HA antibodies, unless overexpressed from a 2 μ plasmid. Thus, if our conjecture is correct and Hac1p^{u} is constitutively synthesized, only small levels of Hac1p^{u}, i.e., levels below our detection limit, are sufficient to induce the UPR significantly. It follows that the Hac1p^{i} levels that are produced upon induction of the UPR via splicing and that are readily detectable in Western blots must represent a significant excess over this minimal amount. Importantly, the UPR is also constitutively induced in Δ*ire1* Δ*ubc4,5* triple mutant cells (Figure 8B), suggesting that Hac1p^{u} is, in principle, a functional transcription factor that does not require any further activation by Ire1p.

The data from the *ubc* mutant strains indicate that Hac1p is degraded by the proteosome after ubiquination. It is likely that the extended PEST region found in the C-terminal halves of both Hac1p^{u} and Hac1p^{i} target either protein for degradation. Other bZIP transcription factors, such as Gcn4p, also contain pronounced PEST regions that determine their short half-lives (Kornitzer et al., 1994). In Hac1p, the additional amino acid sequences that constitute the Hac1p^{u} tail must further stimulate degradation, because Hac1p$^{\Delta tail}$, a mutant Hac1p bearing no C-terminal tail, exhibited the same activity as Hac1p^{i}. The molecular basis for this effect remains to be investigated.

In addition to initiating *HAC1* mRNA splicing, we observed a second point of regulation that is exerted by Ire1p. When we compared the activity of Hac1p^{i} in wild-type and Δ*ire1* cells, we noticed that activation of Ire1p with Tm caused an additional 1.5-fold increase in the levels of the UPRE–controlled reporter enzyme. We consider it likely that Ire1p can render Hac1p^{i} more active, possibly by phosphorylating Hac1p^{i} itself or, more indirectly, by modifying other components required for its synthesis, stability, or activity. This additional activation of Hac1p^{i} by Ire1p may also be exerted on the activity of Hac1p^{u}.

This possibility may provide a rationale as to why cells seemingly squander energy on degrading Hac1p^{u} as quickly as it is synthesized. If Hac1p^{u} is synthesized as a functional transcription factor and can be activated by Ire1p, then the constitutively synthesized Hac1p^{u} may provide a physiologically important pool that could be made instantly available to mount a transcriptional response. In contrast, Ire1p-activated splicing would induce a slower but longer-lasting activation by producing the intrinsically more stable Hac1p^{i}. *HAC1^{i}* mRNA decays with a half-time of about 20 min (Sidrauski et al., 1996), so that each splicing event is committed to memory for a significant period of time. Thus, splicing allows the UPR to be sustained even after the stimulus has been removed. The two branches of the model in Figure 9 may therefore represent two different means of temporal regulation, allowing cells to fine-tune the UPR. Furthermore, although the components that mediate the signaling in mammalian cells are still unknown, the general features of the UPR are conserved among eukaryotic cells. It will therefore be interesting to determine if similar mechanisms of transcriptional regulation operate in higher eukaryotic cells.

Experimental Procedures

Media and General Methods

YPD (complete) and synthetic minimal media are described by Sherman (1991). All synthetic media were supplemented with inositol (myo-inositol; Sigma) to a final concentration of 50 μg/ml. When added, Tm (Boehringer Mannheim) was at a final concentration of 1 μg/ml (for W303-derived strains) or 10 μg/ml (for all other strain backgrounds) and β-mercaptoethanol was at 15 mM. X-Gal indicator plates are described by Chien et al. (1991) and contained 40 mg/ml of X-Gal (Biosynth International). Yeast transformations were performed by lithium acetate procedures (Elble, 1992; Ito et al., 1983), and plasmids were isolated from yeast as described by Strathern and Higgins (1991).

Yeast Strains and Plasmids

Yeast strains used in this study are listed in Table 1. The *hac1::URA3* disruption was created by homologous recombination of BamHI-linearized pHAKO1 plasmid and removes only the Hac1p coding region. pHAKO1 was created using PCR–amplified fragments of sequences adjacent to *HAC1* ligated into plasmid pRS306 (*URA3*; Sikorski and Hieter, 1989). Correct integration of pHAKO1 in JC408 was confirmed by Southern blot. Strains JC406 and JC417 were created by crossing JC408 with appropriate congenic strains containing the *pep4::TRP1* and *ire1::URA3* alleles, respectively.

Plasmid pJC5 contains one UPRE oligonucleotide inserted upstream of the disabled *CYC1* promoter-driven *lacZ* reporter gene fusion (Cox et al., 1993). Plasmids containing two and four tandem repeats of the same UPRE oligonucleotide, pJC100 and pJC104, respectively, were created by iterative insertion of the UPRE into the BglII and SalI sites of pJC5. pJC31 was created by placing the entire UPRE–driven *lacZ* gene from pJC5 into pRS314 (*TRP1*, CEN/ARS).

The diploid reporter strain used for the selection of suppressor plasmids, JC329, contains two reporters integrated at the *URA3* loci. One reporter construct, pJCI96, places the *HIS3* gene under the control of the 4 × UPRE–driven crippled *CYC1* promoter followed by the *ACT1* transcriptional terminator. To create pJCI96, the 4 × UPRE-*CYC1* promoter fragment of pJC104 replaced the *GAL1/10* promoter of pTSI210. pTSI210 is a derivative of pTS210 (gift of Tim Stearns, Stanford University) in which the yeast origin of replication and the centromere have been removed. Finally, a PCR fragment containing the *HIS3* gene was then inserted between the 4 × UPRE-*CYC1* promoter and the *ACT1* terminator. pJCI96 was linearized with NcoI and integrated at the *URA3* locus. The other reporter construct, pJCI186, has the *lacZ* gene under the control of the 4 × UPRE–driven crippled *GAL1* promoter. pJCI186 was created by inserting the tetramer UPRE XhoI–SalI fragment of pJC104 into the XhoI site of pJL638 (Li and Herskowitz, 1993). pJCI186 was linearized with StuI and integrated at the *URA3* locus. The *ire1:TRP1* disruption present in JC329, which removes all Ire1p coding sequence as well as 675 bp of the promoter region of *IRE1*, was created by homologous recombination of SmaI-linearized pJC73. pJC73 was created using PCR–amplified fragments of sequences adjacent to *IRE1* ligated into plasmid pRS304 (*TRP1*). Correct integration of pJC73 was confirmed by Southern blot.

Most of the *HAC1*-containing plasmids isolated from the pGAD libraries contained short inserts and were missing either 5′ or 3′ flanking sequences. Therefore, a plasmid containing a genomic

Table 1. Yeast Strains

Strain	Genotype	Source/Reference
W303-1A	*MATa; ura3-1; leu2-3,-112; his3-11,-15; trp1-1; ade2-1; can1-100*	R. Rothstein
W303-1Apep4$^-$	same as W303-1A, except *pep4::TRP1*	R. Rothstein
JC103	same as W303-1A, except *leu2-3,-112::LEU-UPRE-lacZ; his3-11,-15::HIS3-UPRE-lacZ*	Cox et al., 1993
CS165	same as JC103, except *ire1::URA3*	Cox et al., 1993
JC408	same as W303-1A, except *leu2-3,-112::LEU-UPRE-lacZ; hac1::URA3*	this study
JC406	same as JC408, except *pep4::TRP1*	this study
JV417	same as JC408, except *ire1::TRP1*	this study
RT228	same as W303-1A, except *swi4::HIS3*	I. Herskowitz
JC329	*MATa/MATα; ire1::URA3/ire1::TRP1; leu2-3,-112/leu2-3,-112; his3-11,-15/his3-11,-15; trp1-1/trp1-1; ade2-1/ade2-1; can1-100/can1-100; ura3-1::URA3-4xUPRE-HIS3/ura3-1::URA3-4xUPRE-lacZ*	this study
MHY501	*MATα; ura3-52; leu2-3,-112; his3-Δ200; trp1-1; lys2-801*	Chen et al., 1993
MHY495	same as MHY501 except *ubc6-Δ1::HIS3*	Chen et al., 1993
MHY497	same as MHY501 except *ubc1-Δ1::HIS3*	Seufert et al., 1990
MHY498	same as MHY501 except *ubc4-Δ1::HIS3*	Seufert & Jentsch, 1990
MHY499	same as MHY501 except *ubc5-Δ1::LEU2*	Seufert & Jentsch, 1990
MHY507	same as MHY501 except *ubc7::LEU2*	Jungmann et al., 1993
MHY508	same as MHY501 except *ubc4-Δ1::HIS3; ubc5-Δ1::LEU2*	Chen et al., 1993
MHY599	*MATα; ura3-52; leu2-3; trp; ade1; ubc10(pas2)*	Chen et al., 1993
MHY601	same as W303-1A except *MATα; ubc8::URA3*	Chen et al., 1993
MHY612	same as MHY501 except *ubc2::LEU2*	M. Hochstrasser
MHY680	*MATa; ura3-52; leu2-Δ3; bas1-2; bas2-2a; gcn4-Δ1; ade8-GCN4; cdc34-2(ubc3)*	Kornitzer et al., 1994
JC460	*MATa; ura3-52; leu2-3,-112; his3-Δ200; trp1-1; hac1::URA3; ubc4-Δ1::HIS3; ubc5-Δ1::LEU2*	this study
JC472	*MATα; ura3; leu2-3,-112; his3; trp1-1; ire1::URA3; ubc4-Δ1::HIS3; ubc5-Δ1::LEU2*	this study

copy of *HAC1* with ample surrounding sequence, pJC314, was isolated from a different library (Nasmyth) by colony hybridization with a *HAC1* DNA probe. A 3.2 kb ClaI–NgoMI fragment that contained *HAC1* was subcloned into pRS313 (*HIS3*, CEN/ARS), creating pJC315. pJC322 and pJC328 were created by subcloning the ClaI–NgoMI fragment of pJC315 into pRS423 (*HIS3*, 2 μ) and pRS425 (*LEU2*, 2 μ) respectively. pJC316 was created by inserting a double-stranded oligonucleotide coding for the HA epitope (YPYDVPDYA) into the unique SpeI site of pJC315, placing the epitope between the tenth and eleventh codon of wild-type *HAC1*.

Genetic Selection

JC329 was unable to grow on plates lacking histidine owing to low expression of the *HIS3* reporter. We confirmed that the *HIS3* reporter was functional because plasmids containing *IRE1* allowed for JC329 to grow on plates lacking histidine. The high copy genomic libraries pGAD1-3 (Chien et al., 1991) were screened for plasmids that allowed JC329 to form colonies on −his plates. The resulting colonies were replica-plated to X-Gal indicator plates and allowed to develop overnight. Only plasmids that activated both reporters were harvested and then amplified in DH5α Escherichia coli cells, and the ends of the genomic inserts were sequenced.

RNA Analyses

Northern Blots

Cells were treated and total RNA prepared as described previously (Cox et al., 1993). RNA was quantitated and equal amounts were loaded on 6.7% formaldehyde and 1.5% agarose gels and run in 1 × E buffer (20 mM MOPS [pH 7.0], 5 mM NaOAc, 0.5 mM EDTA). The RNA was transferred to Duralon-UV membranes (Stratagene) and probed overnight at 65°C in Church hybridization buffer (0.5 M $NaPO_4$ (pH 7.2), 7% SDS, 1 mM EDTA). The membranes were subsequently washed in 0.5–1 × SSC and exposed. Quantitation of Northern blots was performed on a Molecular Imager System GS-363 (BioRad).

Probes

All probes were labeled with [α-^{32}P]dCTP using the Ready-To-Go DNA-labeling kit (Pharmacia). The *KAR2* and *ACT1* probes were generated by PCR of 1 kb fragments of the respective coding regions. The "Full" and "3′" *HAC1* probes were made by isolating the 1.3 kb SpeI–EcoNI and the 419 bp fragments of pJC315, respectively. Both the "5′" and "IVS" probes were generated by PCR.

Reverse Transcriptase–PCR

RNA used for reverse transcriptase–PCR (RT–PCR) analysis was extracted several times with $CHCl_3$ prior to ethanol precipitation. First-strand cDNA synthesis and tagging was performed by annealing a mixture of three oligonucleotides [GCCGATCGATAAGCTTCC GC[T]$_{18}$[G,A,C]) with 10 μg of total RNA and extending for 1 hr using AMV reverse transcriptase at 48°C. This cDNA template was subjected to two rounds of nested PCR using as the 3′ primer an oligonucleotide identical to the tag incorporated during first-strand synthesis. The first round of PCR was performed using a 5′ primer (*HAC16*) that anneals at +1 to +24 of *HAC1*. This PCR reaction was diluted and used as a template for a second round of PCR using a 3′ primer (HAC23) that anneals to +210 to +231. A 1 μl sample of this reaction was cloned using the TA cloning kit (Invitrogen), and individual clones were sequenced.

Gel-Shift Assay

The synthetic double-stranded UPRE30/31 oligonucleotide used as a probe in all binding experiments is as follows: 5′-GATCTCG CGGCACCCGAGGAACTGGACAGCGTGTCGAAAAAGTTGCTTTTT TTAC-3′ 3′-AGCGCCGTGGGCTCCTTGACCTGTCGCACAGCTTTTT CAACGAAAAAATGAGCT-5′ UPRE30/31 was end-labeled and gel-purified. The UPRE1/2 oligonucleotide used as an unlabeled competitor is a shorter version of UPRE30/31 and is described in Cox et al. (1993). UPRE24/25, a single point mutant derivative of UPRE1/2, changes the sequence CAGCG to CATCG and has been shown previously to abolish UPRE activity in vivo (Mori et al., 1992).

Extracts were prepared as described by Einerhand et al. (1993) with slight modifications. Cells were harvested by centrifugation and washed once with ice-cold breakage buffer (200 mM Tris [pH 8.0], 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 5 μg/ml of leupeptin, 2 μg/ml of pepstatin). Cell pellets were resuspended in an equal volume of breakage buffer and disrupted by vortexing in the presence of zirconia/silica beads (Biospec Products, Inc.). After centrifugation for 5 min in a microfuge, the supernatant was recentrifuged in a TL-100 ultracentrifuge using a TLA100.2 rotor at 90,000 rpm for 20 min. The supernatant was frozen in liquid nitrogen and stored at −80°C.

Binding reactions (20 μl) included 0.2 ng of radiolabeled UPRE oligonucleotide probe, 1 μg of poly(dI-dC) (Pharmacia), and 1 × binding buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 2.5 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethanesulfonyl

fluoride). Crude extract (15 μg) was added last, and binding proceeded at room temperature for 15 min. The reactions were loaded on a 4% polyacrylamide gel containing 5% glycerol and electrophoresed at 150 V for 2.5 hr in 0.5 × TBE at room temperature. Where appropriate, addition of antibodies to the binding reaction occurred 5 min prior to gel loading. For competition assays, a 100-fold molar excess of unlabeled oligonucleotide as compared with the probe was added prior to addition of the extract.

Antibodies, Western Blots, and Immunofluorescence

The anti-HA ascities fluid was purchased (BAbCO), and the anti-myc monoclonal antibody is described by Evan et al. (1985). Rabbit anti-Hac1p^{i} serum was raised against a peptide corresponding to its last 10 C-terminal amino acids (CFELNDFFITScooh) that was covalently coupled to keyhole limpet hemocyanin.

Denatured protein extracts were prepared by bead-beating cells directly into 10% TCA, 10 mM Tris (pH 8.0), 25 mM NH_4OAc, 1 mM EDTA. TCA precipitates were resuspended in 100 mM Tris (pH 11.0), 3% SDS, and heated to 100°C for 5 min. SDS–polyacrylamide gel electrophoresis was performed on 10–15% gradient gels, and Western blots were visualized by enhanced chemiluminescence (ECL, Amersham) according to the instructions of the manufacturer. Immunofluorescence was performed exactly as described by Harlow and Lane (1988) using anti-HA ascites fluid. Polysome gradients were prepared exactly as described in Baim et al. (1985).

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References

Baim, S.B., Pietras, D.F., Eustice, D.C., and Sherman, F. (1985). A mutation allowing an mRNA secondary structure diminishes translation of *Saccharomyces cerevisiae* iso-1-cytochrome *c*. Mol. Cell. Biol. *5*, 1839–1846.

Chien, C., Bartel, P.L., Sternglanz, R., and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA *88*, 9578–9582.

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.

Einerhand, A.W.C., Kos, W.T., Distel, B., and Tabak, H.F. (1993). Characterization of a transcriptional control element involved in proliferation of peroxisomes in yeast in response to oleate. Eur. J. Biochem. *214*, 323–331.

Elble, R. (1992). A simple and efficient procedure for transformation of yeasts. BioTechniques *13*, 18–20.

Engebrecht, J., Voelkel, M.K., and Roeder, G.S. (1991). Meiosis-specific RNA splicing in yeast. Cell *66*, 1257–1268.

Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. *5*, 3610–3616.

Gething, M.-J., and Sambrook, J. (1992). Protein folding in the cell. Nature *355*, 33–45.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

He, F., Peltz, S.W., Donahue, J.L., Rosbash, M., and Jacobson, A. (1993). Stabilization and ribosome association of unspliced pre-mRNAs in a yeast *upf1*- mutant. Proc. Natl. Acad. Sci. USA *90*, 7034–7038.

Hochstrasser, M. (1995). Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr. Opin. Cell Biol. *7*, 215–223.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

Jungmann, J., Reins, H. A., Schobert, C., and Jentsch, S. (1993). Resistance to cadmium mediated by ubiquitin-dependent proteolysis. Nature *361*, 369–371.

Koch, C., and Nasmyth, K. (1994). Cell cycle regulated transcription in yeast. Curr. Opin. Cell Biol. *6*, 451–459.

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.

Kornitzer, D., Raboy, B., Kulka, R.G., and Fink, G.R. (1994). Regulated degradation of the transcription factor Gcn4. EMBO J. *13*, 6021–6030.

Kozutsumi, Y., Segal, M., Normington, K., Gething, M.J., and Sambrook, J. (1988). The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature *332*, 462–464.

Lee, A.S. (1987). Coordinated regulation of a set of genes by glucose and calcium ionophores in mammalian cells. Trends Biochem. Sci. *12*, 20–23.

Li, J.J., and Herskowitz, I. (1993). Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science *262*, 1870–1874.

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

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.

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.

Nikawa, J.I., and Yamashita, S. (1992). *IRE1* encodes a putative protein kinase containing a membrane-spanning domain and is required for inositol phototrophy in *Saccharomyces cerevisiae*. Mol. Microbiol. *6*, 1441–1446.

Nojima, H., Sun-Hee, L., Araki, H., Sakai, A., Nakashima, N., Kanaoka, Y., and Ono, Y. (1994). Hac1: a novel yeast bZIP protein binding to the CRE motif is a multicopy suppressor for *cdc10* mutant of *Schizosaccharomyces pombe*. Nucl. Acids Res. *22*, 5279–5288.

Nunnari, J., and Walter, P. (1996). Regulation of organelle biogenesis. Cell *84*, 389–394.

Ogas, J., Andrews, B.J., and Herskowitz, I. (1991). Transcriptional activation of CLN1, CLN2, and a putative new G1 cyclin (HCS26) by SWI4, a positive regulator of G1-specific transcription. Cell *66*, 1015–1026.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science *234*, 364–368.

Seufert, W., and Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. EMBO J. *9*, 543–550.

Seufert, W., McGrath, J. P., and Jentsch, S. (1990). *UBC1* encodes a novel member of an essential subfamily of yeast ubiquitin-conjugating enzymes involved in protein degradation. EMBO J. *9*, 4535–4541.

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.

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

Sherman, F. (1991). Getting started with yeast. Meth. Enzymol. *194*, 3–21.

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

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19–27.

Strathern, J.N., and Higgins, D.R. (1991). Recovery of plasmids from yeast into Escherichia coli: shuttle vectors. Meth. Enzymol. *194*, 319–329.

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