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Transcriptional Induction of Genes Encoding Endoplasmic Reticulum Resident Proteins Requires a Transmembrane Protein Kinase

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Summary

The transcription of genes encoding soluble proteins that reside in the endoplasmic reticulum (ER) is induced when unfolded proteins accumulate in the ER. Thus, an intracellular signal transduction pathway must exist that mediates communication between the ER lumen and the nucleus. We have identified a gene in S. cerevisiae, *IRE1*, that is required for this pathway: *ire1*⁻ mutants cannot activate transcription of *KAR2* and *PDI1*, which encode the ER resident proteins BiP and protein disulfide isomerase. Moreover, *IRE1* is essential for cell viability under stress conditions that cause unfolded proteins to accumulate in the ER. *IRE1* encodes a transmembrane serine/threonine kinase that we propose transmits the unfolded protein signal across the ER or inner nuclear membrane. *IRE1* is also required for inositol prototrophy, suggesting that the induction of ER resident proteins is coupled to the biogenesis of new ER membrane.

Introduction

In all eukaryotic cells, secreted and transmembrane proteins must be folded and assembled correctly in the endoplasmic reticulum (ER) before they can exit the ER. Folding in the ER is assisted by a set of enzymes including BiP, a member of the HSP70 family of molecular chaperones (reviewed in Gething and Sambrook, 1992). BiP is thought to bind transiently to nascent proteins as they are translocated into the ER lumen to assist proper folding and to prevent aggregation of folding intermediates (Pelham, 1986; Haas and Wabl, 1983; Ng et al., 1989; Machamer et al., 1990). BiP also binds to partially assembled and misfolded proteins and thereby may help target dead-end products to degradative pathways in the ER (Ng et al., 1990; Gething et al., 1986; Knittler and Haas, 1992). Another well-characterized ER lumenal protein that is required for proper protein folding is protein disulfide isomerase (PDI), which catalyzes the formation of disulfide bonds (Freedman, 1989).

Interestingly, the synthesis of these ER resident proteins is regulated according to demand for them inside the organelle. In mammalian cells, for example, synthesis of BiP, PDI, and glucose-related protein GRP94, a member of the HSP90 family, is induced when unfolded proteins accumulate in the ER (Lee, 1987; Kozutsumi et al., 1988; Dorner et al., 1992). Experimentally, the accumulation of unfolded proteins in the ER is induced by various treatments: the inhibition of glycosylation either by glucose starvation or by the addition of drugs such as tunicamycin or 2-deoxyglucose; the addition of reducing agents, such as β-mercaptoethanol, which are thought to affect protein folding by preventing proper disulfide bond formation; the expression of folding-defective mutant secretory proteins; or the addition of calcium ionophores that deplete calcium stores in the ER. Induction of the ER resident proteins occurs at the transcriptional level (Lee, 1987). Thus, an unfolded protein response pathway must exist that allows the transduction of a signal from the ER lumen, where unfolded proteins accumulate, to the cell nucleus, where transcription is activated.

Lee and colleagues have identified a promoter element in the BiP gene of mammalian cells that is required for BiP induction in response to unfolded proteins. The sequence of this element is conserved among different mammalian species (Resendez et al., 1988). Genes encoding other ER lumenal proteins are coordinately regulated with the BiP gene. For example, GRP94 contains similar upstream promoter elements, and it has been proposed that the two genes are regulated by a common transcription factor(s) (Chang et al., 1989; Li and Lee, 1991; Li et al., 1992).

In the yeast Saccharomyces cerevisiae, BiP is encoded by the *KAR2* gene, and, as in higher cells, its transcription is induced when unfolded proteins accumulate in the ER (Normington et al., 1989; Rose et al., 1989). However, unlike the mammalian BiP gene, *KAR2* transcription is also induced by heat shock. An analysis of the *KAR2* promoter has revealed that the unfolded protein response is regulated by a 22 bp unfolded protein response element (UPRE) that is distinct from the heat shock element in the *KAR2* promoter (Kohno et al., 1993; Mori et al., 1992). The UPRE is sufficient to activate transcription from a heterologous promoter in response to the accumulation of unfolded proteins in the ER lumen. Here, we describe the results of a genetic screen in which we used a UPRE-controlled reporter gene to isolate yeast mutants defective in the unfolded protein response pathway.

Results

Construction of a *lacZ* Reporter Gene That Is Activated by the Accumulation of Unfolded Proteins in the ER Lumen

To isolate S. cerevisiae mutants defective in *KAR2* induction, we first made a *lacZ* reporter gene that is induced when unfolded proteins accumulate in the ER. Because transcription from the intact *KAR2* promoter occurs at a relatively high basal level even in the absence of *KAR2* induction, we chose to construct a hybrid promoter with a lower basal level. To this end, we took advantage of the observation that the UPRE in the *KAR2* promoter can function as an upstream activating sequence when fused to a heterologous promoter (Mori et al., 1992). Thus, we synthesized a 38 bp DNA fragment bearing the UPRE from *KAR2* and inserted it upstream of a crippled *CYC1* promoter that, in the absence of an upstream activating

Table 1. Yeast Strains

Strain	Genotype	Source/Reference
W303-1A	*leu2-3,-112; his3-11,-15; trp1-1; ura3-1; ade2-1; can1-100; MATa*	R. Rothstein, Columbia University
W303-1B	*leu2-3,-112; his3-11,-15; trp1-1; ura3-1; ade2-1; can1-100; MATα*	R. Rothstein, Columbia University
JC103[a]	same as W303-1A, except *his3-11,-15::HIS⁺UPRE–lacZ; leu2-3;-112::LEU⁺UPRE–lacZ; met⁻*	This study
JC104	same as W303-1B, except *his3-11,-15::HIS⁺UPRE–lacZ;* and *leu2-3;-112::LEU⁺UPRE–lacZ*	This study
CS165	same as JC103, except *ire1::URA3*	This study
CS171[b]	same as JC103, except *ire1-1*	This study
CS172	same as CS171, except *MATα*	This study
CS181	diploid product of CS165 × CS172	This study
BRS1015	*ino1-13; trp1; leu2; ura3; MATα*	Culbertson and Henry, 1975
JRY318	*tun1-1; his4-260,-39; leu2-1; ura3; thr4; met⁻; MATα*	Barnes et al., 1984

[a]Spontaneous *met⁻* derivative of W303.
[b]CS171 is the product of the third backcross of our original *ire1-1* isolate, but for simplicity we designate all of the *MATa* products of *ire1-1* backcrosses by this name. In practice, the data in Figure 2 were obtained using the original *ire1-1* isolate and products of the first backcross, the *ire1-1* strain rescued by pJC012 (from the yeast genomic library) was a product of the second backcross, and the data in Figures 3B, 3C, and 4 were obtained using the product of the third backcross. CS172 is a product of the third backcross.

sequence, is transcriptionally silent (Guarente and Mason, 1983). Single copies of this reporter construct were integrated at two different locations in the genome of yeast strain W303 to create the reporter strain JC103 (see Experimental Procedures; Table 1).

We can detect the induction of *lacZ* expression from the reporter construct using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) plates that contain tunicamycin, a drug that blocks protein glycosylation and causes the accumulation of unfolded proteins in the ER. JC103 colonies turn blue when transferred to X-Gal–tunicamycin indicator plates, but are white when no drug is present (data not shown). This induction is due to the presence of the UPRE, because an isogenic strain bearing a *lacZ* construct that lacks this element fails to turn blue on X-Gal–tunicamycin plates (data not shown). This result indicates that the integrated *lacZ* reporter constructs respond properly to the presence of unfolded proteins in the ER.

To confirm this observation, we analyzed the accumulation of *KAR2* and *lacZ* messenger RNAs (mRNAs) in JC103 cells after treatment with tunicamycin. Figures 1B and 1C show the results from a quantitative S1 nuclease protection assay. When cells were grown in the absence of tunicamycin, the amount of *KAR2* mRNA was constant at all time points analyzed (Figure 1B, lanes 1, 3, and 5; Figure 1C, Tm⁻), and, as expected from the color assay described above, *lacZ* mRNA was barely detectable. In contrast, when cells were grown in the presence of tunicamycin, a 3.5- to 5-fold increase in the amount of *KAR2* mRNA was seen (Figure 1B, compare lanes 2 and 4; Figure 1C, Tm⁺). *lacZ* mRNA levels were induced as well, increasing more than 150-fold after the addition of tunicamycin. This greater induction is due to the lower basal transcription of the UPRE–*lacZ* reporter gene. Both *KAR2* mRNA and *lacZ* mRNA amounts remained elevated at a constant level for at least 6 hr after the addition of tunicamycin (Figure 1B, lane 6). Thus, there is a strict correlation between the induction of the *lacZ* reporter gene and the induction of the endogenous *KAR2* gene upon tunicamycin treatment.

Isolation of Mutants Defective in the Unfolded Protein Response

We used JC103 cells in a genetic screen to isolate mutants that fail to induce *KAR2* transcription in response to the accumulation of unfolded proteins in the ER. We reasoned that the unfolded protein response pathway might not be essential for cell viability under normal, nonstress conditions and that loss-of-function mutations in components that participate in the signaling pathway could be obtained. In the primary screen, JC103 cells were mutagenized with ethyl methanesulfonate (to 30% survival) and plated on rich medium. Colonies were grown at room temperature and replica-plated from the master plates onto X-Gal–tunicamycin indicator plates. Mutant cells unable to induce transcription from the UPRE–*lacZ* reporter gene formed white colonies on the indicator plates. The corresponding colonies were picked from the master plates for further analysis. Approximately 45,000 mutagenized JC103 colonies were screened in this way.

To eliminate mutants that are not defective for *KAR2* induction, we applied a series of secondary criteria to the isolated mutants. First, we eliminated mutants that were unable to take up tunicamycin or were resistant to its activity. Such mutants have been described previously (Barnes et al., 1984). We required that cell growth was still sensitive to tunicamycin and that 2-deoxyglucose, another drug that leads to impaired protein glycosylation in wild-type cells, would fail to induce the reporter gene. Therefore, we discarded mutants that grew well on plates containing tunicamycin and those that turned blue on X-Gal plates containing 2-deoxyglucose.

Next, we eliminated mutants that were unable to induce transcription of *lacZ* from a second regulated promoter. Such mutants might be unable to carry out the β-galactosidase enzymatic color reaction or might fail, in general, to induce transcription. To this end, we took advantage of the rat glucocorticoid receptor (GR), which can function as a hormone-inducible transcriptional activator in yeast (Schena and Yamamoto, 1988). Each prospective mutant

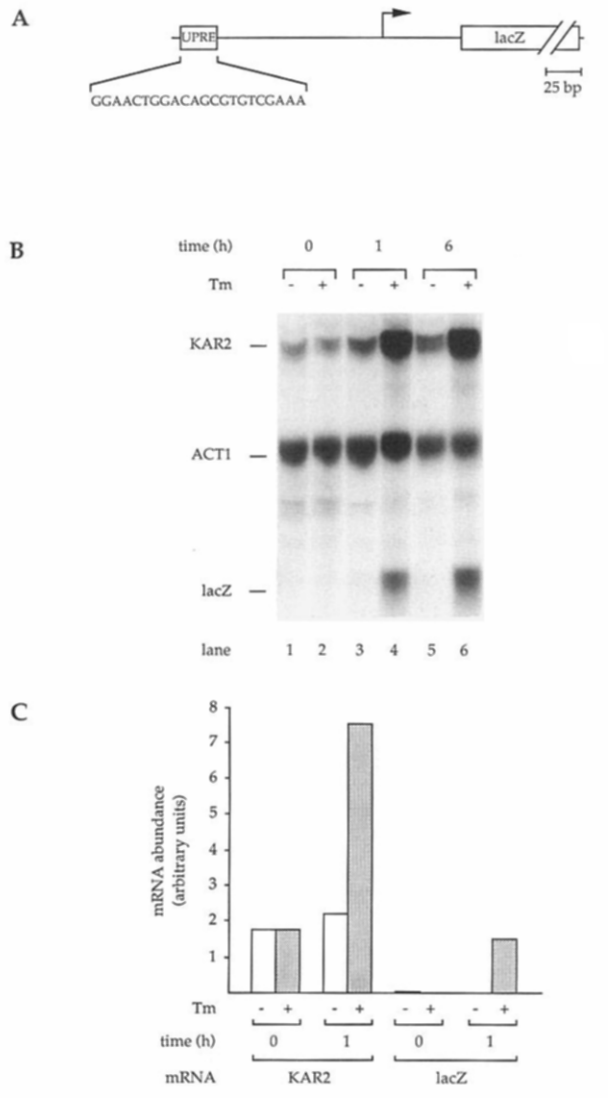

Figure 1. Characterization of the Reporter Strain JC103

(A) The UPRE–*lacZ* reporter construct. An oligonucleotide bearing the 22 bp UPRE defined by Mori et al. (1992) was inserted upstream of a disabled *CYC1* promoter and *lacZ* gene fusion. Two copies of this construct were integrated into the genome to produce reporter strain JC103 (see Experimental Procedures). The approximate position of the transcription start site is indicated by an arrow.

(B) Induction of *lacZ* transcription parallels that of *KAR2*. Midlog phase cultures of JC103 were incubated at room temperature in the absence (minus Tm) or presence (plus Tm) of 1 μg/ml tunicamycin for 0, 1, or 6 hr. Total RNA was harvested, and the abundance of *KAR2*, *lacZ*, and *ACT1* mRNAs was analyzed by S1 nuclease protection assays (see Experimental Procedures).

(C) The data from the 0 and 1 hr time points in (B) were quantitated using the PhosphorImager (Molecular Dynamics, Sunnyvale, California). *KAR2* and *lacZ* mRNA levels were normalized to *ACT1* mRNA levels. The relative induction of *KAR2* and *lacZ* mRNA did not change significantly between the 1 and 6 hr time points.

was transformed with two plasmids, one encoding GR under the control of a constitutively active promoter and a second bearing the *lacZ* gene controlled by glucocorticoid response element upstream activating sequences. GR-dependent transcription from the glucocorticoid response element–*lacZ* reporter construct is induced by steroid hormones, such as deoxycorticosterone, and wild-type yeast cells bearing these constructs are blue on X-Gal plates containing deoxycorticosterone. Mutants that carried these plasmids and produced white colonies on X-Gal–deoxycorticosterone plates were eliminated from further analysis.

Finally, each mutant was tested directly for its ability to induce transcription of the *KAR2* gene. RNA was harvested from cells grown in the presence of tunicamycin, and the level of *KAR2* transcript present was determined by S1 nuclease protection. This test provided the most direct confirmation that the induction pathway of endogenous *KAR2*, and not just the *lacZ* reporter, was compromised in the mutant cells. We obtained two mutant strains that passed all three secondary tests. The mutations in the strains are recessive with regard to their white phenotype on X-Gal–tunicamycin indicator plates, and the mutants fall into different complementation groups (data not

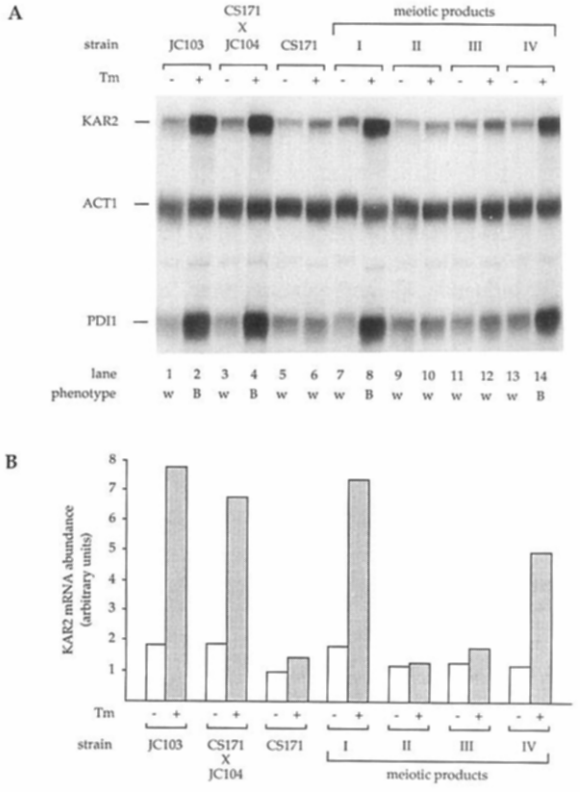

Figure 2. Strain CS171 Carries a Recessive Mutation That Abolishes UPRE-Dependent Induction of the *KAR2* and *PDI1* Genes

(A) S1 protection analysis was performed on RNA isolated from the parent JC103, haploid mutant CS171, the heterozygous diploid product of CS171 crossed with reporter strain JC104, and the four meiotic products of sporulation of the diploid. RNA from two tetrads that were products of different backcrosses of CS171 was analyzed by S1 nuclease protection; the data in lanes 7–14 are a representative result for one tetrad. Each strain was grown for 1 hr in the absence or presence of 1 μg/ml tunicamycin (Tm), and the amount of *KAR2*, *ACT1*, and *PDI1* mRNA was analyzed. The phenotype of each strain on X-Gal indicator plates made without or with 1 μg/ml tunicamycin is indicated under each lane as either blue (B) or white (w).

(B) The data from (A) were quantitated and normalized to actin mRNA levels as in Figure 1C.

shown). In this study, strain CS171 was chosen for further analysis. The second mutant will be described elsewhere.

Characterization of CS171 Cells

When analyzed by S1 nuclease protection, CS171 cells show a severely impaired level of *KAR2* mRNA induction upon tunicamycin treatment as compared with the parent JC103 cells (Figure 2A, compare bands labeled KAR2 in lanes 5 and 6 with lanes 1 and 2). The mutation in CS171 cells is recessive with regard to *KAR2* induction: the diploid formed by mating of CS171 cells with the wild-type reporter strain JC104 showed the same amount of tunicamycin-dependent *KAR2* mRNA induction as did JC103 cells (Figure 2A, compare bands labeled KAR2 in lanes 3 and 4 with lanes 1 and 2). Furthermore, analysis of the four colonies derived from spores of a single tetrad after sporulation of the heterozygous diploid strain (CS171 × JC104) showed a 2:2 segregation of the *KAR2* induction phenotype (Figure 2, lanes 7–14), consistent with the idea that the mutant phenotype results from a mutation in a single gene. As expected, the inability of the mutant to induce *KAR2* transcription correlated with its white colony color on X-Gal indicator plates containing either tunicamycin or 2-deoxyglucose (Figure 2A).

In mammalian cells, the expression of soluble ER resident proteins other than BiP (e.g., PDI) is also induced upon accumulation of unfolded proteins in the ER. We therefore wished to determine whether corresponding yeast ER resident proteins are similarly coregulated and, if this is the case, whether CS171 cells are impaired in their induction. To address this question, we first established with the S1 nuclease protection experiment shown in Figure 2 that yeast PDI, encoded by the *PDI1* gene (LaMantia et al., 1991), is induced in wild-type cells upon tunicamycin treatment (Figure 2A, compare lanes 1 and 2, bands labeled PDI1). Analysis of CS171 cells showed that the tunicamycin-dependent induction of *PDI1* is impaired (Figure 2). These results suggest that the unfolded protein response pathway in yeast regulates a set of genes whose products are involved in protein folding and assembly in the ER and that the product of the gene mutated in CS171 cells is required for the induction of both *KAR2* and *PDI1* transcription.

The *IRE1* Gene Restores Induction of *KAR2* Transcription in CS171 Cells

To identify the gene that is defective in CS171 cells, we screened a yeast genomic library for complementation of the mutant phenotype. CS171 cells were transformed with a high copy plasmid library and replica-plated onto X-Gal–tunicamycin indicator plates. One plasmid, pJC012, containing a 9 kb insert complemented the white phenotype of CS171 cells, yielding blue colonies on indicator plates. The 9 kb DNA insert was moved to a low copy vector that also complemented the CS171 phenotype on X-Gal–tunicamycin plates. This result ruled out the possibility that the observed complementation was the result of high copy suppression.

To identify the region of the 9 kb DNA insert required for complementation, a pool of pJC012 was generated that

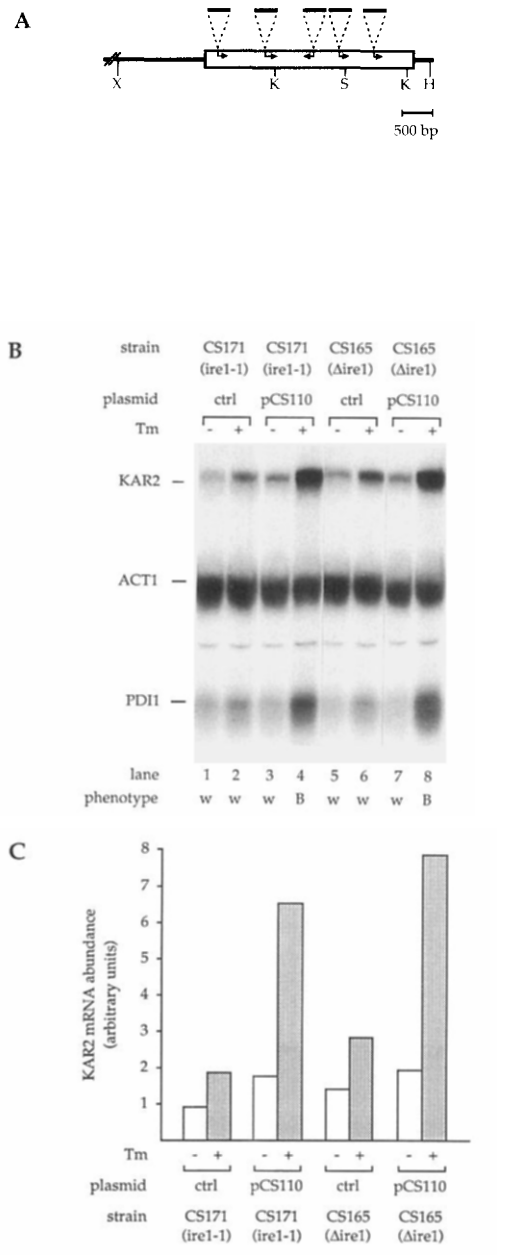

Figure 3. Cloning of *IRE1*

(A) Restriction map of the 9 kb insert of plasmid pJC012, the plasmid from the genomic library that complements the mutant phenotype of CS171. The open reading frame encoding Ire1p is represented by an open box. The positions of Tn10LUK insertions (solid lines above box) that abolish complementation are indicated. Arrows denote direction of sequencing and approximate length of DNA sequence obtained using primers from the ends of the inserted transposons. Restriction sites: HindIII (H), KpnI (K), SacI (S), and XhoI (X). The region between the XhoI and the HindIII site is drawn to scale except for the lines representing Tn10LUK.

(B) *IRE1* restores tunicamycin (Tm)-dependent *KAR2* and *PDI1* mRNA induction to CS171 (*ire1-1*) and CS165 (*Δire1*) cells. *KAR2* and *PDI1* mRNA levels were analyzed in CS165, and CS171 cells that carried either the CEN/ARS control plasmid pRS314 (ctrl) or pRS314 bearing the XhoI–HindIII fragment shown in (A) (pCS110). Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Figure 2A. The phenotype of each strain on X-Gal indicator plates is indicated as in Figure 2B.

(C) The data from (A) were quantitated and normalized to *ACT1* mRNA levels as in Figure 1C.

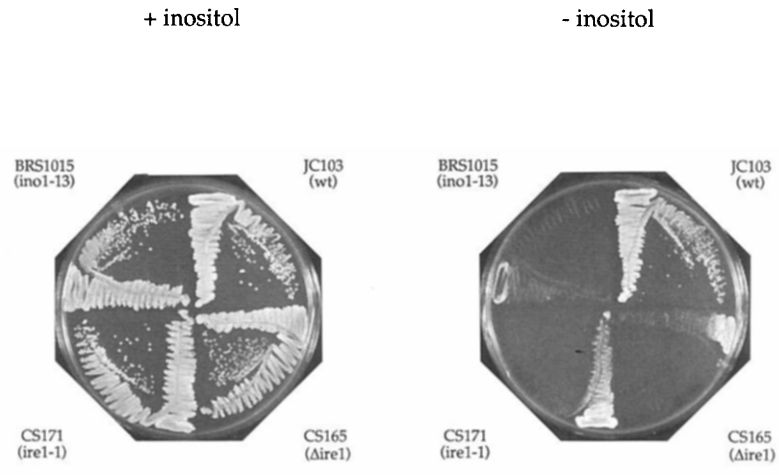

Figure 4. Inositol Auxotrophy of *ire1-1* and *Δire1* Cells

The yeast strains indicated were streaked for single colonies on media containing either 100 μg/ml inositol or no inositol. Plates were incubated at room temperature for 3 days and then photographed.

had a bacterial transposon, Tn10LUK, inserted at random positions (see Experimental Procedures). CS171 cells were transformed with this plasmid pool. Colonies containing plasmid were selected and then screened for complementation of the CS171 phenotype by replica-plating onto X-Gal–tunicamycin indicator plates. Five independent pJC012::Tn10LUK plasmids were isolated that were unable to complement the CS171 defect, presumably because the transposon had inserted within the region of the plasmid insert required for complementation. We mapped the position of these transposon insertions by restriction enzyme digestion and found that all five insertions were clustered in a 2.5 kb segment of the 9 kb insert (Figure 3A). Moreover, when a 5 kb XhoI–HindIII fragment of pJC012 (Figure 3A, X→H) that includes the essential region identified by the transposon insertion was subcloned into a low copy plasmid (generating plasmid pCS110), it was found to be sufficient to restore the tunicamycin-dependent induction of *KAR2* mRNA in CS171 cells (Figure 3B, compare lanes 3 and 4). As expected, the control plasmid lacking the insert had no effect (Figure 3B, lanes 1 and 2).

These results strongly suggested that the transposon insertions disrupted a gene sufficient to complement the defect in CS171 cells. Using DNA primers complementary to regions close to the ends of the transposon, we determined the nucleotide sequences of regions flanking the transposon insertion sites (Figure 3A, arrows). A comparison of these sequences with GenBank entries revealed that all five transposition events disrupted the coding region of a previously sequenced gene, *IRE1* (Nikawa and Yamashita, 1992; GenBank accession number Z11701). The restriction map of the 5 kb insert in pCS110 is also in complete agreement with the published sequence of *IRE1*.

CS171 Cells Are Mutant in the *IRE1* Gene

To confirm that CS171 indeed has a defective *IRE1* gene, we first disrupted the chromosomal copy of *IRE1* in JC103 cells. In the resulting strain, CS165, approximately 70% of the *IRE1* coding sequence was removed and replaced by the *URA3* gene. The integration of the selectable marker at the expected locus and the concomitant disruption of *IRE1* were confirmed by Southern blot. CS165 cells are viable, consistent with the previous finding that *IRE1* is a nonessential gene under normal growth conditions (Nikawa and Yamashita, 1992).

When we analyzed the phenotype of CS165 cells on X-Gal–tunicamycin indicator plates, we found that the colonies remained white, indicating that the UPRE-driven *lacZ* reporter gene was not induced upon tunicamycin treatment. Moreover, analysis by S1 nuclease protection showed that neither *KAR2* nor *PDI1* transcription was induced in CS165 cells after tunicamycin treatment (Figures 3B and 3C). Thus, cells bearing the null allele of *IRE1* are defective in the unfolded protein response pathway. We next crossed CS165 cells with a strain carrying our original mutation, CS172 (isogenic to CS171, but *MATα*), generating diploid strain CS181. CS181 cells are also white on indicator plates and do not induce *KAR2* transcription upon tunicamycin treatment, as determined by S1 nuclease protection (data not shown). This indicates that the *ire1* disruption and the gene defective in our original isolate affect the same complementation group. Finally, CS181 cells were sporulated, the resulting tetrads were dissected, and the dissection products were analyzed on X-Gal–tunicamycin and X-Gal–2-deoxyglucose indicator plates. Of 48 tetrads analyzed, each of which gave rise to four viable spores, the white phenotype on indicator plates always segregated 4:0. This demonstrates that *IRE1* is allelic to the gene defective in CS171 cells, and we henceforth refer to the mutant allele in CS171 cells as *ire1-1*.

The *IRE1* gene was originally cloned by complementation of a yeast mutant auxotrophic for inositol (Nikawa and Yamashita, 1992). Although at present we can only speculate on how the two phenotypes (the requirement for inositol and the inability to induce the unfolded protein response) are related (see Discussion), the reported inositol auxotrophy of *ire1*$^-$ cells provided us with an additional phenotype to confirm that CS171 cells are defective in *IRE1*. As shown in Figure 4, both CS171 and CS165 cells, but not the parent JC103 cells, require inositol for growth. The inositol auxotrophy of CS165 cells is as severe as

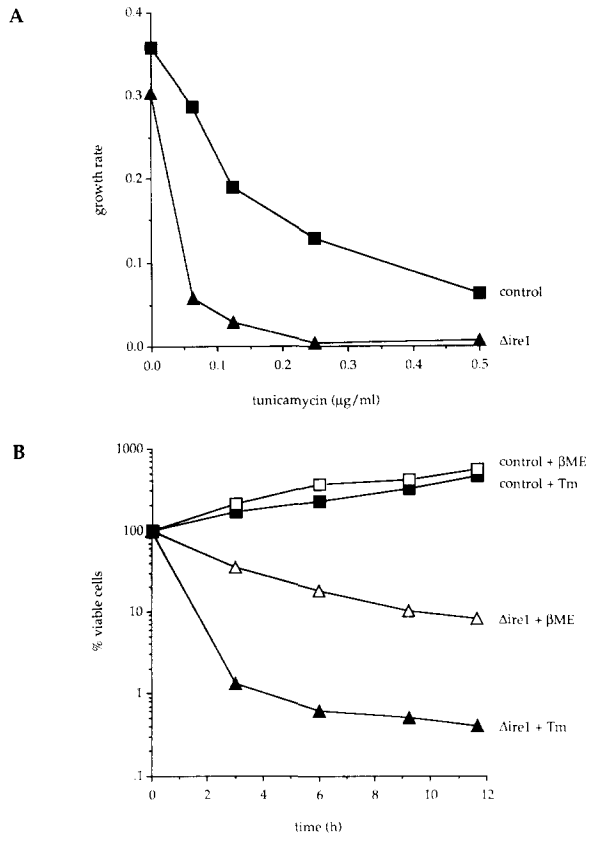

Figure 5. *Δire1* Cells Are Supersensitive to Tunicamycin and β-Mercaptoethanol

Tunicamycin and β-mercaptoethanol were added to JC103 and CS165 cultures growing in midlog phase.

(A) Growth rate of JC103 (closed squares) and CS165 (*Δire1*) (closed triangles) cells versus tunicamycin concentration. The growth rate is defined as the inverse of the doubling time for each strain at a given concentration of tunicamycin. The doubling time for cells in each culture was determined by the change in optical density at 600 nm that occurred between 7.5 hr and 10.75 hr of growth in the presence of tunicamycin.

(B) Cell viability was determined for strains JC103 and CS165 at various times after the addition of tunicamycin (to 0.125 μg/ml) or β-mercaptoethanol (to 15 mM). Aliquots from cultures were removed at different time points, and cells were plated on rich media. The fraction of viable cells was determined by counting colonies after 3 days of growth at 25°C. The viability of untreated cells at time 0 was set at 100%. The data were collected from cultures treated in parallel. Each data point represents the average of at least two determinations.

that of *ino1*$^-$ cells, which are defective in an enzyme essential for inositol biosynthesis (Donahue and Henry, 1981). CS171 cells grow slightly better than CS165 and *ino1*$^-$ cells on plates lacking inositol, indicating that, with regard to the inositol requirement, *ire1-1* is a slightly weaker allele than the *IRE1* disruption. Importantly, the addition of extra inositol to X-Gal–tunicamycin indicator plates does not cause CS165 cells to turn blue, indicating that the failure of these cells to induce transcription of the *lacZ* reporter gene is not due to the low concentration of inositol in X-Gal–tunicamycin plates.

IRE1 Is Required to Protect Cells from the Accumulation of Unfolded Proteins in the ER

With the exception of the requirement for inositol, *IRE1* seems to be dispensable for normal cell growth: cells deleted for *IRE1* grow at approximately the same rate in rich medium as wild-type cells (Figure 5A, point at Tm = 0) and are not sensitive to either low or elevated temperatures (data not shown). We wished to determine whether cells impaired in *IRE1* function are more sensitive to the specific stresses caused by the accumulation of unfolded proteins in the ER lumen. To this end, we tested the sensitivity of wild-type and CS165 cells to tunicamycin and β-mercaptoethanol, a compound that induces BiP in mammalian cells (Whelan and Hightower, 1985). β-Mercaptoethanol also induces UPRE-dependent transcription in yeast: JC103 cells are blue on X-Gal–β-mercaptoethanol plates. This response requires *IRE1* because CS171 and CS165 cells are white on X-Gal–β-mercaptoethanol plates.

The data in Figure 5 demonstrate that growth of CS165 cells was inhibited by low tunicamycin concentrations (Figure 5A, triangles) that only slightly impair the growth of JC103 control cells (Figure 5A, squares), indicating that *ire1*$^-$ cells are hypersensitive to tunicamycin. A qualitatively similar effect was observed when cells were grown in medium containing β-mercaptoethanol.

To distinguish whether these treatments lead to growth arrest or cell death, CS165 cells and wild-type JC103 cells were incubated in medium containing low concentrations of either tunicamycin or β-mercaptoethanol. After various lengths of time in the presence of the drugs, cells from each culture were plated on rich medium, and the number of viable cells was determined by colony-forming ability. The addition of either compound led to rapid cell death of CS165 cells (Figure 5B, triangles) but did not significantly impair the viability of the control JC103 cells (Figure 5B, squares). We conclude from this experiment that *IRE1* function is required to protect cells from the stress resulting from the accumulation of unfolded proteins in the ER and that this stress, in the absence of the protective unfolded protein response, is lethal to the cells. Hence, under conditions where unfolded proteins accumulate in the ER, *IRE1* is essential for cell viability.

Ire1p Is a Transmembrane Protein with a Cytosolic Kinase Domain

The data presented here suggest that the *IRE1* gene product, Ire1p, is an essential component in the unfolded protein response pathway. *IRE1* encodes a protein with a predicted molecular mass of 127 kd that bears a classic N-terminal signal sequence and has an internal stretch of 29 hydrophobic amino acids that is likely to be a single membrane-spanning region (Figure 6A). These features suggest that the N-terminal half of Ire1p is translocated into the ER lumen during its biogenesis and that the C-terminal half of the protein is exposed in the cytosol. Interestingly, the C-terminal portion of Ire1p contains a 300 amino acid domain that has the sequence characteris-

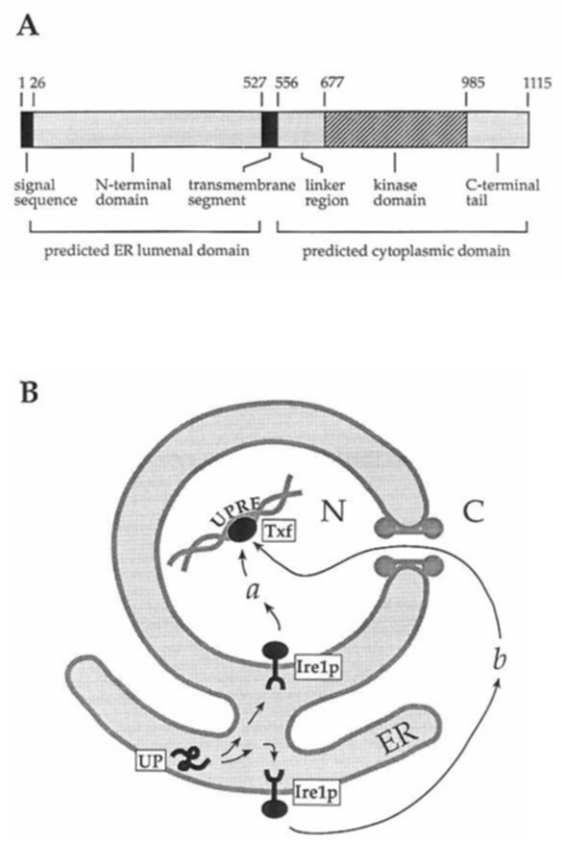

Figure 6. Predicted Domain Structure of Ire1p and Models for the Role of Ire1p in the Unfolded Protein Response Pathway

(A) A linear representation of Ire1p as deduced from the nucleotide sequence of the *IRE1* gene.

(B) Two possible routes (a and b) are depicted, along which the signal transmitted by the unfolded protein response pathway might travel. The routes are distinguished by the subcellular location of Ire1p. In route a, the ER membrane and the nuclear envelope are a continuous membrane system, and the kinase domain of Ire1p may face into the nucleus (N), where it transduces the signal generated by unfolded proteins (UP) across the inner nuclear membrane; activation of the kinase would lead to UPRE-dependent transcription, presumably by modulating the activity of a transcription factor (Txf). In route b, alternatively, the kinase domain of Ire1p may be in the cytoplasm (C), where its activation leads to a signal that traverses the nuclear envelope, most likely by traveling through nuclear pores.

tics of a serine/threonine protein kinase (Hanks et al., 1988; Nikawa and Yamashita, 1992). Other regions of Ire1p are not similar in sequence to any known protein. Thus, Ire1p appears to have the topology of a membrane protein kinase similar to that of many growth factor receptors in higher eukaryotic cells.

Discussion

We have identified a gene required for the unfolded protein response pathway in yeast and have shown that it is the previously identified gene *IRE1*. The sequence of Ire1p suggests that it is a serine/threonine protein kinase similar in structure to receptor kinases involved in transmembrane signaling. Ire1p thus belongs to a class of transmembrane kinases that, to date, includes only five other proteins: the activin and transforming growth factor β type II receptors from vertebrates (Mathews and Vale, 1991; Lin et al., 1992), the *daf-1* gene product from Caenorhabditis elegans (Georgi et al., 1990), the TMK1 kinase from Arabidopsis (Chang et al., 1992), and the Zea mays protein kinase 1 from maize (Walker and Zhang, 1990). Ire1p is the only known transmembrane kinase in yeast.

Because the accumulation of unfolded proteins in the ER lumen sends a signal to activate transcription in the nucleus and because of the similarity of Ire1p to plasma membrane receptors involved in other signaling events, we propose that Ire1p transmits the unfolded protein signal across the membrane surrounding the ER lumen. Ire1p could transmit the signal directly to the nucleus (Figure 6B, route a) or to the cytosol to components that then enter the nucleus through nuclear pores (Figure 6B, route b). Thus, we predict that Ire1p is localized either in the inner nuclear membrane or in the ER membrane. These possibilities can be readily distinguished by localization studies once antibodies that recognize Ire1p become available.

If Ire1p functions as proposed in Figure 6B, it must detect unfolded proteins in the ER lumen. It is likely that BiP plays a key role in the detection process. A growing body of evidence suggests that in both yeast and mammalian cells, the concentration of free BiP in the ER is monitored: when the concentration of free BiP falls, transcription of the genes encoding ER resident proteins is induced via the unfolded protein response pathway (Dorner et al., 1992; Hardwick et al., 1990; Kohno et al., 1993; Ng et al., 1992). It is possible that Ire1p senses the level of free BiP in the ER lumen directly. The activation of transmembrane receptor kinases is thought to involve their dimerization (Ullrich and Schlessinger, 1990; Hemmati-Brivanlou and Melton, 1992), and, by analogy, the Ire1p kinase may also be activated in this manner. One hypothesis is that when the level of free BiP in the ER is high, BiP binds the ER lumenal domain of Ire1p, thereby preventing dimerization and keeping Ire1p in an inactive state. In contrast, under conditions that cause the accumulation of unfolded proteins in the ER, preferential binding of BiP to unfolded proteins rather than to Ire1p may permit the dimerization of Ire1p. Dimerization would activate the kinase and transmit a signal via phosphorylation of nuclear or cytosolic proteins. In turn, the Ire1p-initiated phosphorylation events would activate UPRE-dependent transcription of genes in the nucleus. This simple model is consistent with the available data and with current ideas about transmembrane kinase function; however, we recognize the possibility that the pathway may be more complex.

The identification of *IRE1* as an essential gene in the unfolded protein response pathway has allowed us to address a number of issues regarding the physiological role of the pathway. For example, we have shown that *IRE1* is not essential for normal growth in rich medium. However, when cells are challenged with compounds that cause unfolded proteins to accumulate in the ER, we observe

that *ire1*$^-$ cells die quickly. By contrast, *IRE1*$^+$ cells remain viable under the same conditions. These observations demonstrate unambiguously that the unfolded protein response pathway is required for cell survival under conditions of stress in the ER.

We have also shown that *PDI1* transcription is induced in response to unfolded proteins in the ER. This is in contrast with arguments made by others that *PDI1* and *KAR2* are not coregulated (Tachibana and Stevens, 1992). Moreover, Ire1p is required for the tunicamycin-dependent induction of both *KAR2* and *PDI1* transcription, suggesting that the two genes are regulated by the same pathway or by pathways that are at least partially overlapping. While this work was in progress, another ER resident protein, Eug1p, was identified and shown to be related to PDI. Interestingly, transcription of *EUG1* is also induced by the accumulation of unfolded proteins in the ER and there is a UPRE-like sequence in the *EUG1* promoter (Tachibana and Stevens, 1992). Thus, as has been postulated for mammalian cells, it seems likely that the unfolded protein response pathway in S. cerevisiae operates on a set of genes that encode ER resident proteins.

Finally, with the identification of *IRE1*, we have discovered a surprising link between the unfolded protein response pathway and inositol metabolism. In yeast, phosphatidylinositol is a major constituent of membranes, and free inositol levels play a central role in the regulation of phospholipid synthesis (White et al., 1991). This suggests the possibility that the regulation of synthesis of ER resident proteins and the regulation of phospholipid biogenesis may be coupled. Such a link seems reasonable because as a cell synthesizes more ER proteins, it might also need to produce more ER membrane, and Ire1p may coordinate the two processes.

Experimental Procedures

Media and Genetic Methods

YPD (complete) and synthetic minimal media are described by Sherman (1991). X-Gal indicator plates are described by Chien et al. (1991) and contained 40 mg/ml of X-Gal (Biosynth International, Skokie, Illinois) and either 1 μg/ml tunicamycin (Boehringer Mannheim, Indianapolis, Indiana), 10 mM 2-deoxyglucose, 15 mM β-mercaptoethanol (Sigma Chemical Company, St. Louis, Missouri), or 1 mM deoxycorticosterone (gift of K. Yamamoto, University of California, San Francisco). Minimal medium lacking inositol was made as described by Culbertson and Henry (1975). When added, inositol (myo-inositol; Sigma, St. Louis, Missouri) was at a final concentration of 100 μg/ml. Yeast transformations were performed by lithium acetate procedures (Ito et al., 1983; Elble, 1992). Plasmids were isolated from yeast as described by Strathern and Higgins (1991).

Plasmid Construction

To make the UPRE–*lacZ* reporter construct, the following double-stranded oligonucleotide bearing the UPRE (Mori et al., 1992) was synthesized. Its protruding termini are complementary to those generated by the restriction endonucleases BglII and XhoI:

5′-GATCTGTCGACAGGAACTGGACAGCGTGTCGAAAAAGC-3′
3′-ACAGCTGTCCTTGACCTGTCGCACAGCTTTTTCGAGCT-5′

One copy of this oligonucleotide was inserted into the BglII and XhoI sites of plasmid pGA1696 to create plasmid pJC005. pGA1696 is a plasmid identical to pGA1695, described by Sorger and Pelham (1987), but it has a 5′ BglII site and a 3′ XhoI site. It is a derivative of plasmid pLG-Δ178 (Guarente and Mason, 1983) and bears the Escherichia coli *lacZ* gene under the control of a disabled *CYC1* promoter.

To create reporter plasmids that could integrate into the yeast genome, a 4 kb BglII–Tth111I fragment of pJC005 containing the UPRE, disabled *CYC1* promoter, and *lacZ* gene sequences was subcloned into the yeast-integrating vectors pRS303 (*HIS3*) and pRS305 (*LEU2*; Sikorski and Hieter, 1989) to replace the BamHI–NaeI fragments in both plasmids. The resulting plasmids were named pJC002 (*HIS3* UPRE–*lacZ*) and pJC003 (*LEU2* UPRE–*lacZ*).

pCS110 was constructed by inserting the XhoI–HindIII fragment bearing *IRE1* (see Figure 3) into the low copy *TRP1*$^+$ yeast vector pRS314 (Sikorski and Hieter, 1989) to replace the XhoI–EcoRI fragment in the polylinker.

Yeast Strains

Yeast strains used in this study are listed in Table 1. Reporter strains JC103 and JC104 were constructed by homologous recombination of both pJC002 (cut with NheI) and pJC003 (cut with BstEII) into the chromosomal copies of the *HIS3* and *LEU2* genes of strains W303-1A and W303-1B. Correct integration of the reporter genes was confirmed by Southern blot.

To disrupt the *IRE1* gene in strain CS165, a plasmid, pCS109, was constructed in which the 2.1 kb KpnI fragment of *IRE1* is replaced by the *URA3* gene. The haploid reporter strain JC103 was transformed with the XhoI–HindIII fragment from pCS109 that bears the disrupted *IRE1* gene, and cells were plated on *URA*$^-$ plates that had been supplemented with inositol. Inositol was added to a final concentration of 20 μg/ml in the selection plates to allow growth of the inositol auxotrophs that were created by disrupting the chromosomal copy of *IRE1*. The disruption of the *IRE1* gene with *URA3* in strain CS165 was confirmed by Southern blot.

RNA Analysis

RNA Preparation

Cultures were grown in log phase in the absence or presence of tunicamycin. Yeast cells from a 25 or 50 ml sample from each culture were collected by centrifugation and washed with water, and the cell pellets were stored at −80°C until processed for RNA. Whole-cell RNA was made according to a scaled-down version of the hot phenol method described by Kohrer and Domdey (1991).

Probes

The probes used for S1 analysis protect the following sequences: bases 375–561 of *KAR2* (Rose et al., 1989), bases 878–1016 of *ACT1* (Ng and Abelson, 1980), bases 207–306 of *PDI1* (LaMantia et al., 1991), and bases 2231–2330 of *lacZ* (Kalnins et al., 1983). In a three-fragment ligation, DNA fragments bearing these sequences were cloned into the polylinker of the phagemid pUC119 (Vieira and Messing, 1987) along with a DNA fragment from Staphylococcus aureus nuclease that is not homologous to yeast or *lacZ* DNA sequences. To make end-labeled probes, oligonucleotides specific for the mRNA sequences were labeled with [γ-^{32}P]ATP, annealed to single-stranded plasmid bearing the sense strand of the sequences to be analyzed, and extended with the Klenow fragment of DNA polymerase. The double-stranded product was cut with a restriction enzyme and probes of discrete lengths bearing *KAR2*, *ACT1*, *PDI1*, or *lacZ* sequences fused to 150 bp of S. aureus nuclease sequences were isolated by preparative denaturing gel electrophoresis (Sambrook et al., 1989).

S1 Nuclease Protection Assay

S1 nuclease protection assays were carried out as described by Favaloro et al. (1980). In brief, 15 μg of RNA was hybridized at 37°C overnight to 0.01 pmol of each probe. S1 digestion was carried out at 18°C for 2 hr using 500 U/ml of S1 nuclease (Sigma, St. Louis, Missouri). Samples were then fractionated on polyacrylamide (8%) denaturing gels and analyzed by autoradiography and by quantitation using the PhosphorImager (Molecular Dynamics, Sunnyvale, California).

Primary Screen

Strain JC103 was mutagenized with ethyl methanesulfonate (Sigma, St. Louis, Missouri) to 30% survival according to the protocol of Lawrence (1991). Cells were plated onto YPD plates and allowed to grow at room temperature for 3 days. The colonies were then replica-plated onto X-Gal–tunicamycin indicator plates and incubated overnight at 30°C. The desired mutants failed to turn blue on the indicator plates

and were patched from the master YPD plates onto fresh YPD plates. They were rescreened on X-Gal–tunicamycin indicator plates.

Secondary Screens

2-Deoxyglucose

Yeast patches were replica-plated from YPD onto X-Gal–2-deoxyglucose plates. Mutants that turned blue on these indicator plates were discarded.

Tunicamycin Resistance

Roughly equivalent numbers of yeast cells from each mutant strain were transferred to YPD plates containing 1 μg/ml tunicamycin by spotting diluted suspensions of cells onto the plates. The plates were then incubated at room temperature for 3 days in the dark. Wild-type JC103 failed to grow at this concentration of tunicamycin, and any mutants that grew equally well as the tunicamycin-resistant strain JRY318 (Barnes et al., 1984) were discarded.

Glucocorticoid Response

To identify those mutants that were able to activate transcription of a reporter construct regulated by the glucocorticoid response element, mutant cells were transformed with two plasmids. pRS314/F260SGR bears a mutant version of the GR driven by the glyceraldehyde-3-phosphate dehydrogenase promoter in a low copy *TRP1*$^+$ yeast vector. The F620S mutation in the GR seems to increase the affinity of the receptor for its ligand and is described by Garabedian and Yamamoto (1992). The reporter plasmid pΔS26X (Schena et al., 1989) has three copies of the glucocorticoid response element fused to a disabled *CYC1* promoter and the *lacZ* gene in a high copy *URA3*$^+$ yeast vector. Both plasmids were provided by S. Bohen. When replica-plated onto X-Gal indicator plates containing the GR ligand deoxycorticosterone, yeast that can carry out the GR response turn blue. We discarded mutants that failed to turn blue in this assay.

Measuring Tunicamycin-Dependent KAR2 *mRNA Induction*

Cultures (25 ml) of mutant cells were grown to midlog phase at room temperature in YPD. Tunicamycin was added to a final concentration of 1 μg/ml, and cells were grown for 1 hr in the presence of the drug before being harvested. Whole-cell RNA was isolated, and S1 nuclease protection experiments were carried out as described above. Mutants that induced expression of *KAR2* mRNA to similar levels as (control) JC103 cells were discarded.

Rescuing the CS171 Mutant Phenotype

Strain CS171 was transformed with a high copy yeast genomic library (Carlson and Botstein, 1982), and colonies were replica-plated to X-Gal–tunicamycin plates. One colony (out of 10,000 screened) turned blue, and the complementing plasmid, pJC012, was isolated. The 9 kb yeast genomic insert from pJC012 was subcloned into the low copy vector pRS314 (Sikorski and Hieter, 1989), and this new plasmid also complemented the CS171 defect. Tn10LUK mutagenesis of pJC012 was performed essentially as described in Huisman et al. (1987) except that pools of transposon-containing pJC012 were transformed into the *recA*–E. coli strain DH5α and amplified before being transformed into yeast. Plasmids containing integrated transposons were screened for their ability to complement the white phenotype of CS171 cells on X-Gal–tunicamycin plates. Ten plasmids that were unable to complement the defect were isolated from the yeast cells, transformed into DH5α cells, and reamplified. Restriction digest mapping of the pJC012::Tn10LUK plasmids revealed five different integration events that clustered within a 2.5 kb segment in the genomic insert. DNA sequencing across the junctions of the Tn10 integrations was carried out using a primer oligonucleotide specific to the 5′ end of the Tn10LUK *lacZ* gene. The identity of *IRE1* was determined by comparison of these sequences with the GenBank data base. The accession number of the *IRE1* sequence is Z11701.

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