Molecular Microbiology (2003) **49**(3), 591–606 doi:10.1046/j.1365-2958.2003.03585.x

IRE1- and *HAC1*-independent transcriptional regulation in the unfolded protein response of yeast

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Summary

The unfolded protein response (UPR) is a signalling pathway leading to transcriptional activation of genes that protect cells from accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER). In yeast, the only known ER stress signalling pathway originates at the type I transmembrane protein kinase/endoribonuclease Ire1p. Ire1p regulates synthesis of the basic leucine-zipper (bZIP)-containing transcription factor Hac1p by controlling splicing of HAC1 mRNA. Only spliced HAC1 mRNA (HAC1^{i}) is translated, and Hac1^{i}p activates transcription of genes that contain a conserved UPR element (UPRE) in their promoters. Here, we demonstrate that in addition to this well-understood ER stress signalling pathway, a second, IRE1, HAC1 and UPRE-independent mechanism for transcriptional activation upon ER stress, exists in yeast. A genetic screen identified recessive SIN4 alleles as suppressors of a defective UPR in ire1Δ strains. Elevation of basal transcription in sin4 strains or by tethering the RNA polymerase II holoenzyme with LexAp-holoenzyme component fusion proteins to a promoter allowed for activation of the promoter by ER stress in an IRE1, HAC1 and UPRE-independent manner. We propose that this novel second ER-to-nucleus signal transduction pathway culminates in core promoter activation (CPA) through stimulation of RNA polymerase II holoenzyme activity. Core promoter activation was observed upon diverse cellular stresses, suggesting it represents a primordial stress-induced gene activation mechanism.

Introduction

All eukaryotic cells deal with the accumulation of unfolded proteins in the ER by mounting protective responses, including the induction of ER-resident molecular chaperones and upregulation of ER-associated protein degradation. In higher eukaryotes, at least three signalling pathways contribute to the UPR (Shen *et al*., 2001). First, the type I transmembrane kinase/endoribonuclease Ire1p is activated by release of *BiP/GRP78/KAR2* from its lumenal domain upon accumulation of unfolded proteins in the ER lumen (Bertolotti *et al*., 2000). Activated Ire1p initiates spliceosome-independent RNA splicing of mRNAs encoding the bZIP transcription factors *HAC1* in yeast (Sidrauski and Walter, 1997) and *XBP-1* in higher eukaryotes (Shen *et al*., 2001; Yoshida *et al*., 2001; Calfon *et al*., 2002). Second, the eukaryotic initiation factor 2α (eIF2α) kinase PERK, that shares a functional ER lumenal domain with Ire1p (Liu *et al*., 2000), attenuates translation by phosphorylation and inactivation of eIF2α. This translational control event contributes to transcriptional activation of ER chaperone genes (Scheuner *et al*., 2001). Third, the transmembrane protein *ATF6*, residing in the ER membrane, is transported to the Golgi complex and its cytoplasmic domain released by proteolysis by S1P and S2P (Ye *et al*., 2000). The cytoplasmic portion of cleaved *ATF6* translocates to the nucleus and activates transcription of target genes (Haze *et al*., 1999; Wang *et al*., 2000). Common to these three pathways is the transcriptional induction of a set of genes.

Yeast lacks both ATF6 and PERK. Thus, the only known UPR pathway in this organism is the one originating at Ire1p. Activated Ire1p cleaves the mRNA for the bZIP transcription factor *HAC1* at the 5′- and 3′-exon/intron junctions (Sidrauski and Walter, 1997) and both exons are then joined by tRNA ligase (Sidrauski *et al*., 1996). This unconventional splicing reaction results in the removal of a translational attenuator residing in the intron of *HAC1* mRNA and allows for regulated synthesis of Hac1p only during periods of ER stress (Chapman and Walter, 1997). Hac1p derived from spliced *HAC1* mRNA is a more potent transcriptional activator than Hac1p derived from unspliced *HAC1* mRNA (Mori *et al*., 2000). Hac1p activates transcription of genes containing a conserved UPR element (UPRE) in their promoters as a homodimer (Mori *et al*., 1998). However, about one-third of the UPRE promoter activation is independent of *IRE1* and *HAC1* (Shamu and Walter, 1996) and a genome-wide analysis identified 87 open reading frames (ORF) that are induced

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in response to ER stress in *ire1Δ* and *hac1Δ* strains (Travers *et al*., 2000).

Here, we demonstrate the existence of additional, *IRE1*- and *HAC1*-independent, ER stress response pathways in yeast. These pathways were revealed by elevation of basal transcription. Either *sin4* mutations or recruitment of a LexAp-RNA polymerase II holoenzyme fusion protein to a *lacZ* reporter with LexA-binding sites, but no UPRE, increased basal transcription. Induction of transcription in response to ER stress in these two systems was two- to threefold and independent of any component of the classic UPR, that is *IRE1*, *HAC1* or the UPRE. We conclude that an *IRE1*- and *HAC1*-independent pathway for transcriptional activation exists in yeast and propose that this pathway culminates in stimulation of the activity of a core promoter, a process we term core promoter activation (CPA). Our data are consistent with stimulation of RNA polymerase II holoenzyme activity by ER stress as a possible mechanism for CPA. In addition, CPA was also observed when other physiological stresses were induced. These findings significantly contribute to our understanding of *IRE1*- and *HAC1*-independent promoter activation in response to ER stress.

Results

Additional ER stress response pathways exist in yeast

In yeast, the UPR is thought to be exclusively dependent on Ire1p-mediated splicing of *HAC1* mRNA. However, colonies of *ire1Δ* and *hac1Δ* strains that carry a UPRE-*lacZ* reporter developed a light blue colour in the presence of tunicamycin on X-Gal plates after prolonged incubation, but not in the absence of tunicamycin (data not shown). In addition, using DNA microarrays, a twofold or stronger induction of 87 ORFs by ER stress did not require *IRE1* or *HAC1* function (Travers *et al*., 2000). Finally, about one-third of the activation of promoters containing an UPRE was independent of *IRE1* and *HAC1* function (Shamu and Walter, 1996). These observations suggest that additional signal transduction pathways from the ER to the nucleus exist in yeast that do not require either *IRE1* or *HAC1*.

Isolation of mutants with an IRE1/ERN1*-independent ER to nucleus signalling phenotype*

To identify genes involved in these *IRE1*-independent ER stress signalling pathways we isolated mutant yeast strains that efficiently signal ER stress in the absence of functional *IRE1/ERN1*. *ire1Δ* strains were subjected to random mutagenesis with ethyl methanesulfonate. About 1×10^7 mutants were selected for increased resistance to tunicamycin (0.1–0.25 μg ml^{-1}). Colonies with increased resistance to tunicamycin were then screened for activation of an integrated UPRE-*lacZ* reporter [UPRE-$P_{CYC1(-178)}$-*lacZ*, see *Experimental procedures* for details] in response to ER stress (Fig. 1A). A total of 91 mutant strains activated the reporter in response to ER stress. All strains carried recessive mutations and 84 mutant strains belonged to the same complementation group. This strongly suggested allelism of these mutations. We designate this gene as *EIS1* for *ERN1* (synonymous to *IRE1*) independent signalling. However, seven mutants did not fall into this complementation group. These mutants can constitute up to seven additional alleles.

Next we determined the extent of the ER stress response in an *ire1Δ eis1-68* strain. We also included one mutant (*ire1Δ* mt no. 82) that did not fall into the major complementation group. Resistance to tunicamycin and 2-deoxy-D-glucose was increased in the *ire1Δ eis1-68* strain and mutant no. 82 compared with the parental *ire1Δ* strain (Fig. 1B). Because tunicamycin and 2-deoxy-D-glucose induce accumulation of unfolded proteins in the lumen of the ER by different mechanisms (Schwarz *et al*., 1979; Hubbard and Ivatt, 1981), resistance to both drugs indicates an improved handling of unfolded proteins in the ER lumen in both strains. In addition, both strains showed a rapid two to threefold increase in β-galactosidase production upon tunicamycin treatment (Fig. 1C). As early as 30 min after the addition of tunicamycin, an increase in β-galactosidase activity was detectable. This indicated that these strains responded to the presence of unfolded proteins in the ER lumen itself, and not to secondary perturbations of cellular metabolism caused by the accumulation of unfolded proteins. However, we also observed elevated levels of β-galactosidase expression prior to induction of ER stress (Fig. 1C), which reflects increased basal transcription of the reporter in these strains. In summary, several mutants were isolated that allow for efficient ER-to-nucleus signalling in the absence of functional *IRE1*.

EIS1 *is* SIN4

Based on the large number of mutants that fell into a single complementation group, we reasoned that a single gene mutation is responsible for restoration of ER stress signalling in our *eis1* mutants. The gene encoding the recessive mutation was isolated by complementation with a yeast genomic DNA library. Twenty-eight colonies containing plasmids that complemented induction of the *lacZ* reporter in response to ER stress were isolated. Resistance to tunicamycin was also reduced by these plasmids. Plasmid DNA was rescued from these strains and five different complementing plasmids were identified by DNA sequencing. One plasmid (Rose-1) was isolated 24 times, whereas the other four were isolated only once. Introduction of

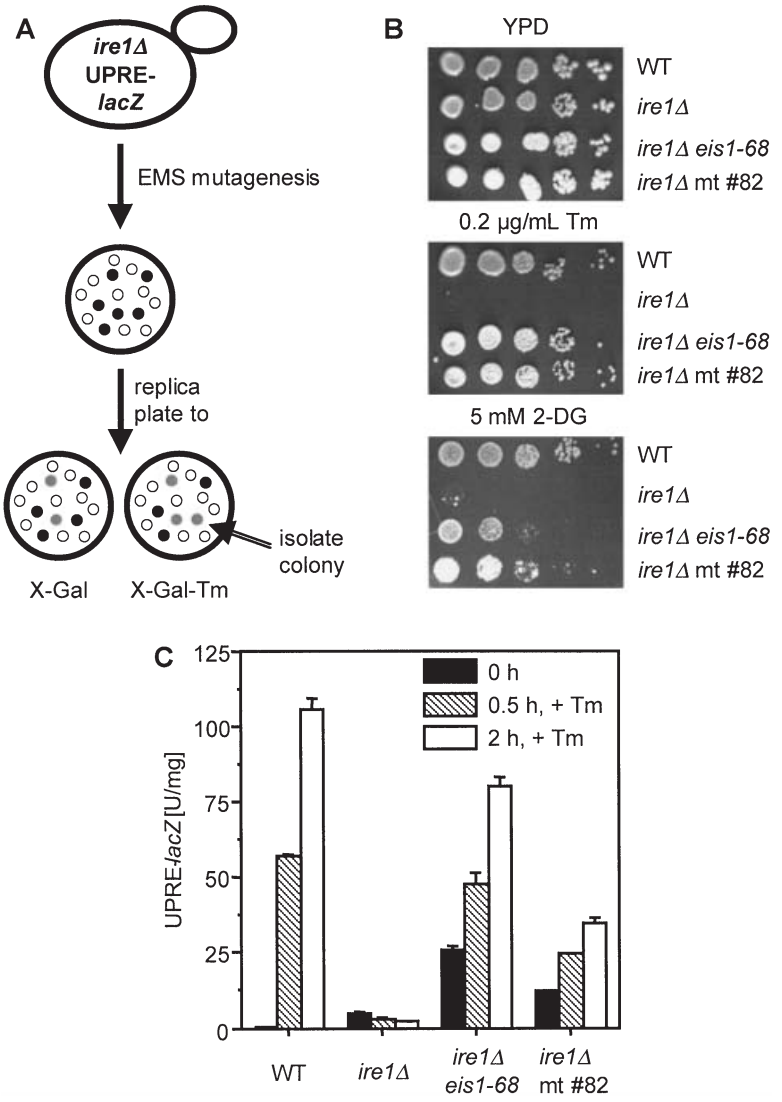

Fig. 1. Characterization of *eis* (ERN1 independent signalling) mutations.
A. Schematic outline of the genetic screen to isolate *eis* mutations. An *ire1Δ* strain (AWY19 or MSY9-1) was mutagenized with ethyl methanesulfonate (EMS) as described in *Experimental procedures*. Colonies resistant to 0.25 μg ml^{-1} tunicamycin (Tm; black circles) were screened for induction of an UPRE-*lacZ* reporter in response to ER stress (grey circles) by replica plating to X-Gal– and X-Gal–Tm-plates. Open circles represent colonies sensitive to 0.25 μg ml^{-1} tunicamycin.
B. *eis* mutants *ire1Δ eis1-68* and *ire1Δ* mt no. 82 are resistant to tunicamycin and 2-deoxy-D-glucose. Serial 10-fold dilutions of fresh overnight cultures were spotted onto yeast extract-peptone-dextrose (YPD) plates containing 0.2 μg ml^{-1} tunicamycin, 5 mM 2-deoxy-D-glucose (2-DG) and control plates.
C. Induction of an UPRE-$P_{CYC1(-178)}$-*lacZ* reporter by ER stress in *eis* mutants *ire1Δ eis1-68* and a second mutant (*ire1Δ* mt no. 82) that does not belong to the major complementation group. Cells were grown to mid-log phase and tunicamycin added to 2 μg ml^{-1}. Samples were taken before (solid bars), 30 (crossed bars) and 120 min (open bars) after addition of tunicamycin. The average and standard error from four independent protein and β-galactosidase determinations are shown. In both panels the strains used were: WT (AWY14), *ire1Δ* (AWY19).

Rose-1 and Rose-2 (data not shown) into an *ire1Δ eis1-68* strain reduced both tunicamycin resistance and tunicamycin-induced expression from the UPRE-*lacZ* reporter construct (Fig. 2A). The DNA inserts common to both plasmids encode two known genes, *SIN4* and *YTP1,* and one putative ORF, YNL235c (Fig. 2A). Thus, it is possible that *SIN4*, *YTP1* or YNL235c are allelic with *EIS1*. Because the isolated mutations in *EIS1* were recessive and therefore most likely loss-of-function alleles, we introduced *sin4* and *ytp1* null alleles into an *ire1Δ* background. The *ire1Δ sin4Δ* strain displayed tunicamycin resistance and tunicamycin-induced UPRE-*lacZ* reporter gene expression identical to an *ire1Δ eis1-68* strain (Fig. 2A, B). In contrast, the *ire1Δ ytp1Δ* strain was neither resistant to tunicamycin nor displayed induction of the UPRE-*lacZ* reporter. The *sin4* deletion leaves about 500 bp of 3′-sequence for ORF YNL235c intact. Therefore we do not expect that expression of YNL235c was affected by this deletion.

Null mutations in *SIN4* cause broad alterations in transcription of a large variety of genes. Loss-of-function mutations in *SIN4* are associated with a decrease in nucleosome density in the chromatin (Jiang and Stillman, 1992) and changes in transcription of many, but not all, genes (Mizuno and Harashima, 2000). Thus, it is possible that the additional plasmid-borne copy of *SIN4* is only a suppressor of induction of UPRE-$P_{CYC1(-178)}$-*lacZ* transcription in response to ER stress, due to altered expression of *SIN4* and subsequent Sin4p dosage-dependent effects on chromatin structure. To confirm that *SIN4* is indeed the complementing gene and not only a suppressor of the *eis1-68* allele, a *sin4* null mutation was introduced into an *ire1Δ* strain, and the resulting *ire1Δ sin4Δ* strain crossed to a representative mutant of the major complementation group (*ire1Δ eis1-13*). The resulting diploid strain (*ire1Δ/ire1Δ sin4Δ/eis1-13*) induced the UPRE-$P_{CYC1(-178)}$-*lacZ* reporter in response to ER stress

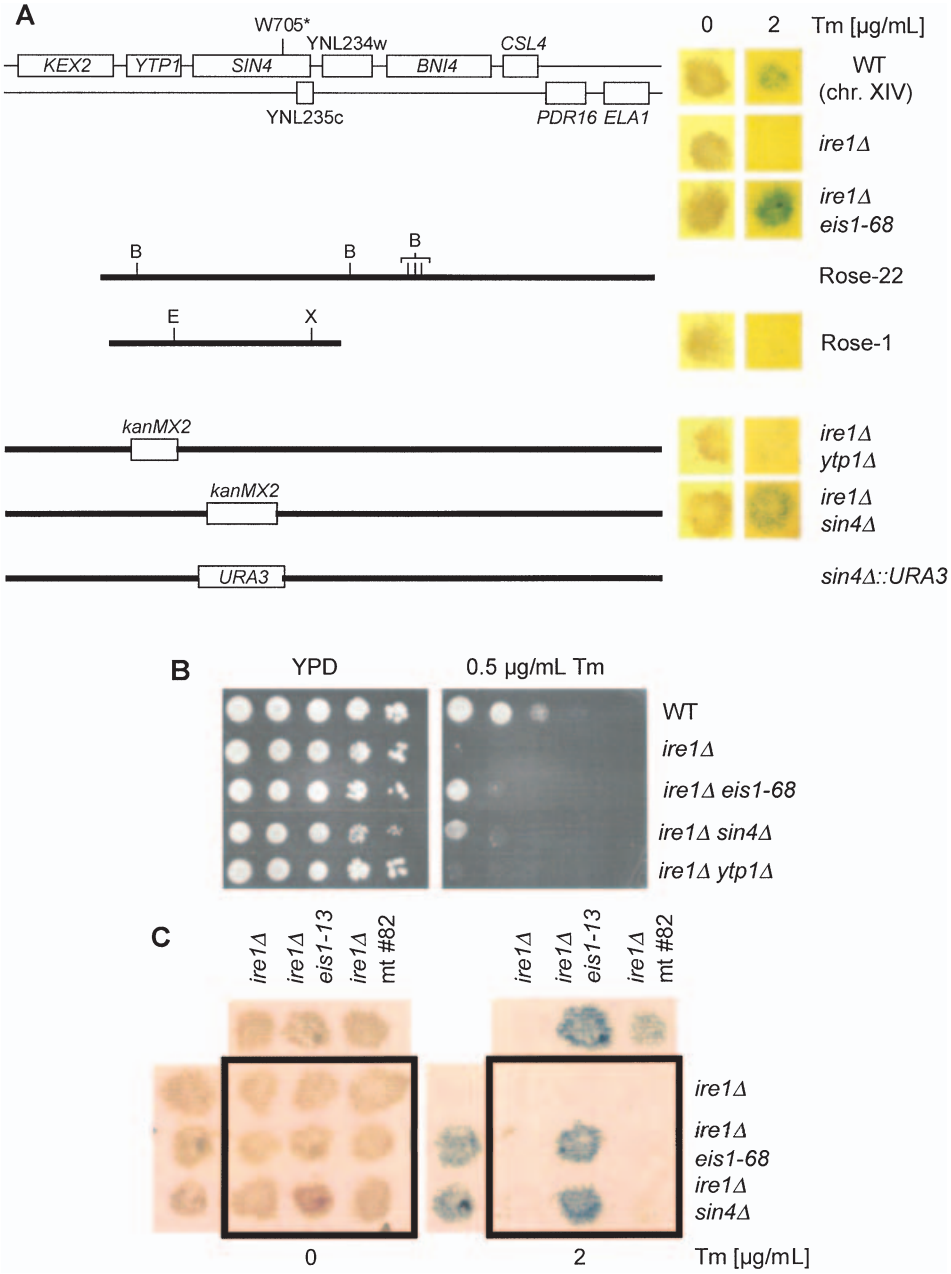

Fig. 2. *EIS1* is *SIN4*.
A. Schematic drawings of the insert in two complementing plasmids (Rose-1 and Rose-22), WT and mutant *ytp1Δ*::*kanMX2*, *sin4Δ*::*kanMX2*, and *sin4Δ::URA3* loci. The location of the mutation in allele *sin4-68* (W705*, *indicates a stop codon) is also indicated. Shown to the right is the growth phenotype and induction of an UPRE-*lacZ* reporter in response to ER stress. B: *Bam*HI; E: *Eco*RI; X: *Xba*I; Tm: tunicamycin.
B. Resistance of *ire1Δ sin4Δ* (RCY8) and *ire1Δ ytp1Δ* (RCY9) strains to tunicamycin. Serial 10-fold dilutions of fresh, overnight cultures were spotted onto YPD plates containing 0.5 μg ml^{-1} tunicamycin and control plates.
C. *SIN4* is allelic to *EIS1*. An *ire1Δ* (AWY19), *ire1Δ eis1-68* (derived from AWY19), and an *ire1Δ sin4Δ* (RCY8) strain were mated to an *ire1Δ* (MSY9-1), and two mutant strains (*ire1Δ eis1-13*, and mt. no. 82) isolated after ethyl methanesulfonate mutagenesis of MSY9-1. Diploid and parental haploid cells were tested for growth and induction of the UPRE-*lacZ* reporter by replica plating to X-Gal (left) and X-Gal–tunicamycin (right) plates. The phenotype of diploids derived from mating the indicated haploid strains in the corresponding rows and columns is shown inside the box. The phenotype of the parental haploid strain is shown outside the box. The WT strain used in all panels was AWY14.

(Fig. 2C). In contrast, ER stress signalling was not observed when the *ire1Δ sin4Δ* strain was mated to a mutant strain that did not belong to the major complementation group (*ire1Δ* mt no. 82, Fig. 2C). These data demonstrate that *SIN4* is allelic with *EIS1* and not a suppressor of *eis1* mutations. We conclude that *EIS1* is *SIN4*. We designate the recessive *sin4* allele as *sin4-68* (synonymous to *eis1-68*).

To identify the mutation in the *sin4-68* allele, the *sin4-68* allele was cloned into pRS306. The *SIN4* ORF was sequenced in both directions, and compared with the published sequence (Chen *et al*., 1993). Two point mutations were identified, at position +1836 a G→T transversion (Glu612Asp), and at position +2114 a G→A transition; resulting in a stop codon at amino acid position 705. Because mutagenesis with ethyl methanesulfonate resulted exclusively in transitions (Kohalmi and Kunz, 1988), we reasoned that the transversion at position +1836 is present in the parental strain. To verify this hypothesis, the *SIN4* gene from the parental *ire1Δ* strain (AWY19) was cloned, and the relevant region sequenced. Indeed, the G→T transversion at position +1836, but not the transition, was present in the parental strain (data not shown). The Glu612Asp–Sin4p is functional, as no phenotypes related to *sin4* mutations were observed in the parental strain (data not shown). Thus, *sin4-68* encodes a C-terminal truncation of Sin4p in which about 28% of the protein is missing.

Mutations in SIN4 *activate transcription in response to ER stress in* ire1Δ *strains*

Next, we compared the phenotype of the *ire1Δ sin4Δ* and the *ire1Δ sin4-68* strain. Both strains showed a similar elevation of basal transcription from the UPRE-$P_{CYC1(-178)}$-*lacZ* reporter, and showed a similar induction of β-galactosidase after addition of tunicamycin (Fig. 3A) or DTT (Fig. 3B). DTT unfolds proteins in the ER lumen by reducing intra- and intermolecular disulphide bonds. Resistance to tunicamycin of the *ire1Δ sin4Δ* strain was also indistinguishable from the *ire1Δ sin4-68* strain (Fig. 2B). Taken together, these data show that *ire1Δ sin4-68* and *ire1Δ sin4Δ* strains have a very similar phenotype and that transcriptional activation of the UPRE-*lacZ* reporter can be detected reproducibly in *ire1Δ sin4Δ* strains. Further, basal transcription and induction of the UPRE-$P_{CYC1(-178)}$-*lacZ* reporter in both the *ire1Δ sin4Δ* and the *ire1Δ sin4-68* strain were indistinguishable from a *sin4Δ* strain. This suggests that *SIN4, IRE1* and *HAC1* act in the same genetic pathway to activate transcription by the *IRE1-HAC1*-pathway.

Sin4p is part of the mediator complex that regulates activity of the RNA polymerase II holoenzyme and the level of transcription of target promoters. Sin4p forms a subcomplex with Gal11p and Rgr1p within the mediator that can be dissociated from the other mediator components. *RGR1* is an essential gene (Carlson, 1997). *gal11* and *sin4* mutants often have similar transcriptional defects (Fassler *et al*., 1991; Jiang and Stillman, 1992; Carlson, 1997). However, both proteins do not play identical roles in transcriptional regulation (Kim *et al*., 2000). To determine whether restoration of ER stress signalling in *ire1Δ sin4Δ* strains results from a defect in mediator function, an *ire1Δ gal11Δ* double null mutant strain was constructed and analysed. Deletion of *GAL11* did not increase basal transcription from the reporter, or induction by ER stress (Fig. 3). This demonstrates that increased transcription by ER stress seen in *sin4Δ* strains is specific to selective components of the mediator. Further, the *gal11* deletion abolished the two to threefold ER-stress induction seen in *ire1Δ* and *ire1Δ sin4Δ* strains (Fig. 3). This observation shows that the increase in β-galactosidase activity by ER stress in the *ire1Δ* strains has a transcriptional basis and is not an artefact that is associated with the *IRE1* deletion.

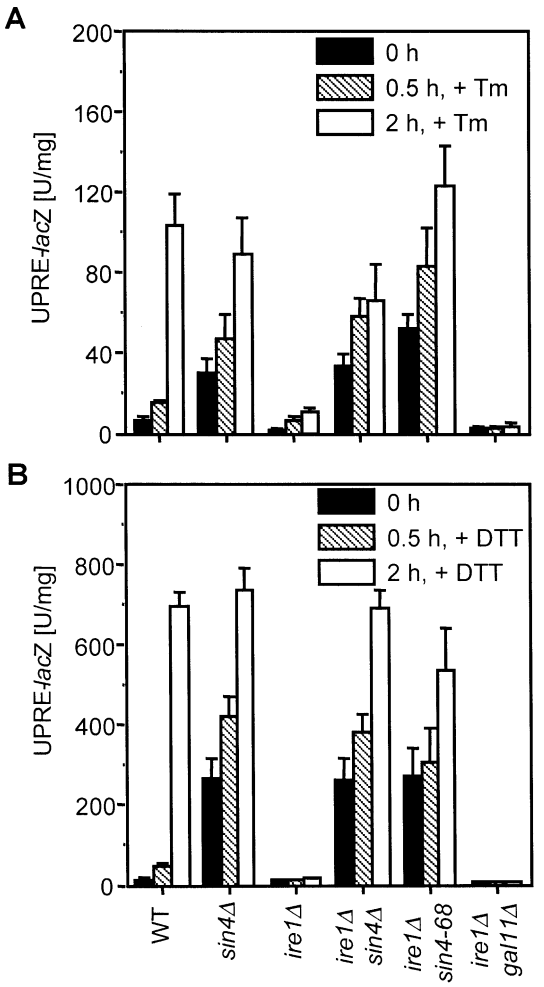

Fig. 3. Basal and stimulation of transcription by ER stress in *ire1Δ sin4Δ* and *ire1Δ gal11Δ* mutants. Induction of a *lacZ* reporter by ER stress in a WT (AWY14), *sin4Δ* (RCY7), *ire1Δ* (AWY19), *ire1Δ sin4Δ* (RCY8), *ire1Δ sin4-68* (AWY19 mt. no. 68) and *ire1Δ gal11Δ* (MSY293-01) strain by treatment of mid-log phase cells with (A) 0.4 μg ml^{-1} tunicamycin (Tm) or (B) 3 mM DTT, for 0.5 (crossed bars) or 2 h (open bars). Black bars represent untreated samples. The average and standard error from four independent protein and β-galactosidase determinations are shown.

IRE1 *and* HAC1 *are dispensable for transcriptional induction in response to ER stress in* sin4 *mutants*

Next we asked whether induction of the UPRE-$P_{CYC1(-178)}$-*lacZ* reporter in response to ER stress requires *HAC1* function. Hac1p is a bZIP-transcription factor (Nojima *et al*., 1994) that binds to the conserved UPRE and promotes transcription of target genes, e.g. *KAR2* (Mori *et al*., 1998). Thus, *hac1Δ* strains are defective in activation of the UPR and display a phenotype similar to *ire1Δ* strains (Cox and Walter, 1996). On the other hand, deletion of the UPRE from the *lacZ* reporter construct should also eliminate Hac1p-dependent transcription of the reporter construct. Therefore, we constructed a series of *ire1Δ*, *ire1Δ sin4Δ* and *ire1Δ hac1Δ sin4Δ* strains with the UPRE-$P_{CYC1(-178)}$-*lacZ*::*HIS3* reporter and a *lacZ* reporter construct without a UPRE (called $P_{CYC1(-178)}$-*lacZ*::*HIS*). All strains displayed a markedly similar behaviour (Fig. 4A). Basal expression of the reporter construct was elevated in all strains. This demonstrates that *SIN4* negatively regulates expression of the $P_{CYC1(-178)}$-*lacZ*::*HIS3* reporter in non-induced cells. More importantly, all strains induced the *lacZ* reporter after addition of tunicamycin to a similar degree (Fig. 4A) and showed a similar level of resistance to 1 mM 2-deoxy-D-glucose, a concentration that killed the *ire1Δ* strain (Fig. 4B). Furthermore, resistance to ER stress is not increased by a *sin4* mutation in an otherwise WT background (data not shown). Taken together, these data show that ER stress signalling in *sin4* strains is completely independent of the classic UPR, as it does not require *IRE1*, *HAC1* or the UPRE.

A core promoter is sufficient for activation of transcription by ER stress

Mutations in *SIN4* are associated with a less dense nucleosome packaging of DNA and therefore an increased accessibility of promoters for the transcriptional machinery (Jiang and Stillman, 1992). Increased accessibility of the promoter for RNA polymerase II in *sin4Δ* strains may account for the increase in basal transcription of the $P_{CYC1(-178)}$-*lacZ* reporter in unstressed cells before induction of ER stress by treating the cells with tunicamycin (Figs 1A, 3 and 4A). However, we also observed an increase in transcription in *sin4Δ* strains after induction of ER stress (see for example Fig. 3 and compare the bars before addition of the drug, which correspond to the 0 h values with those after addition of tunicamycin or DTT). This increase was independent of *IRE1*, *HAC1* or the

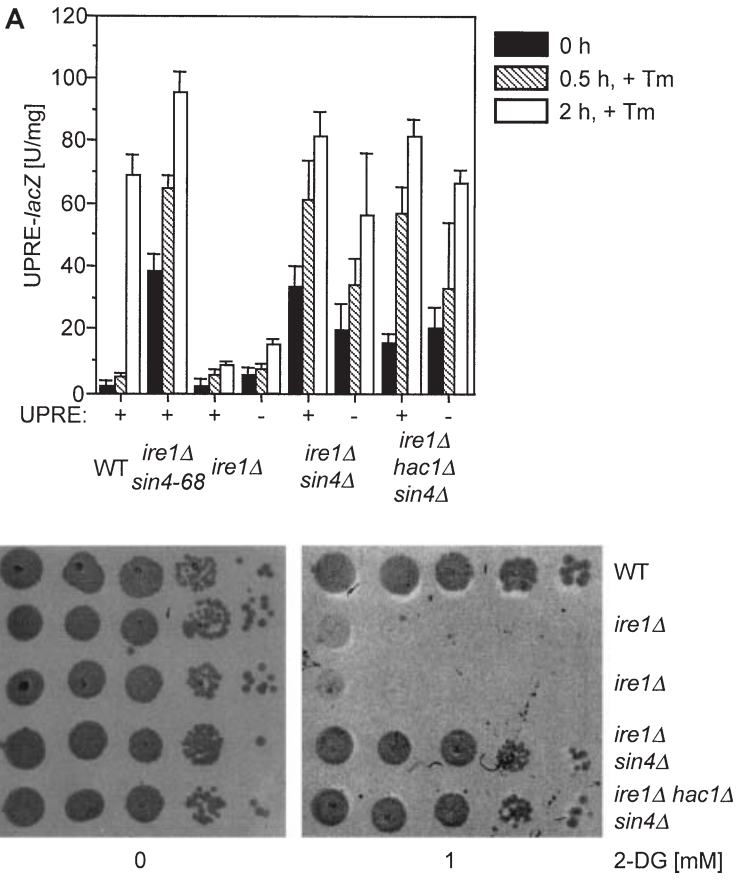

Fig. 4. Components of the UPR are not required for stimulation of transcription by ER stress in *sin4* mutants.
A. Induction of a *lacZ* reporter by ER stress in WT (AWY14), *ire1Δ* (RCY1), *ire1Δ sin4Δ* (RCY3) and *ire1Δ hac1Δ sin4Δ* (RCY5) strains with the UPRE-$P_{CYC1(-178)}$-*lacZ*::*HIS3* reporter and *ire1Δ* (RCY2), *ire1Δ sin4Δ* (RCY4) and *ire1Δ hac1Δ sin4Δ* (RCY6) strains with the $P_{CYC1(-178)}$-*lacZ*::*HIS3* reporter. Cells were treated with 0.4 μg ml^{-1} tunicamycin (Tm), for 0.5 (crossed bars) or 2 h (open bars); black bars represent untreated samples. The average and standard error from four independent protein and β-galactosidase determinations are shown.
B. Resistance of some of the strains used in (A) to 1 mM 2-deoxy-D-glucose (2-DG).

UPRE (Fig. 4). Therefore, we speculated if a core promoter is sufficient to mediate this stimulating effect of ER stress on transcription. To rule out changes in accessibility of the promoter for the holoenzyme by ER stress, the holoenzyme was artificially recruited to a $lexA_{op}$-*lacZ* reporter using fusions of the DNA binding protein LexA to the mediator proteins Sin4p or Srb11p (Kuchin *et al.*, 2000) (Fig. 5A). To eliminate effects of the classic UPR pathway, the experiment was performed in an *ire1Δ* and *SIN4* WT background. Expression of either LexA-Sin4p or LexA-Srb11p increased basal transcription of the reporter several hundred fold (Fig. 5B). Addition of tunicamycin (Fig. 5B) or 2-deoxy-D-glucose (10 mM, data not shown) further increased β-galactosidase activity by 1.5- to twofold (Fig. 5B). Northern blot analysis (Fig. 5C) confirmed that the increase in β-galactosidase activity correlated with a 1.5-fold increase in *lacZ* mRNA (Fig. 5D). Western blot analysis demonstrated that the increase in expression of the $lexA_{op}$-*lacZ* reporter after induction of ER stress was not due to changes in the level of LexA-Sin4p or LexA-Srb11p (Fig. 5E). ER stress-induced transcription mediated by LexA-Sin4p or LexA-Srb11p fusion proteins in the absence of an intact classic UPR pathway was of the same magnitude as observed in *sin4Δ* strains (Figs 3 and 4A). These results demonstrate that a core promoter is sufficient for induction of transcription by ER stress and that activation of a promoter in response to ER stress can be seen in a *SIN4* WT background provided that transcription is artificially elevated by another means, e.g. recruitment of RNA polymerase II to a promoter. These data are also consistent with the idea that RNA polymerase II holoenzyme activity is stimulated in response to ER stress.

Physiological targets for IRE1- *and* HAC1-*independent ER stress signalling*

To analyse if *IRE1*- and *HAC1*-independent ER stress signalling targets ER chaperone genes we performed a Northern analysis. This analysis revealed that moderate activation of the ER chaperone genes *KAR2*, *LHS1* and *PDI1* is retained in *ire1Δ* and *ire1Δ sin4Δ* strains (Fig. 6). Further, activation of the UPRE-*lacZ* reporter is to a large degree retained, which is consistent with our reporter assays (Fig. 3A, B), and *CYC1* shows an approximately twofold activation by ER stress in all strains. Because the UPRE-*lacZ* reporter is driven from the core *CYC1* promoter, this finding is also consistent with our reporter assays (Fig. 3A, B). In contrast, activation of the ER chaperone genes *EUG1* and *SCJ1* is completely abolished in *ire1Δ* strains (Fig. 6). We conclude that some ER chaperone genes are targeted by this new pathway and that a mechanism to selectively target only these promoters must exist.

CPA is a general phenomenon of stress responses

To elucidate whether ER-stress induction of CPA is a more general response to cell stress, we measured CPA induced by diverse cellular stress responses. Therefore, we assayed the expression level of the $P_{CYC1(-178)}$-*lacZ* reporter construct before and after induction of various stresses. WT, *ire1Δ*, and *ire1Δ sin4Δ* strains were either heat shocked, or exposed to hyper- or hypotonic conditions to induce osmotic stress. Under all conditions the *ire1Δ sin4Δ* strain displayed a 1.5- to twofold induction of β-galactosidase activity (Fig. 7). We conclude that activation of the core promoter is not limited to ER stress and a phenomenon generally associated with stress responses.

Discussion

In yeast, stress signalling from the ER to the nucleus is dependent on the type I transmembrane protein kinase/endoribonuclease Ire1p. Ire1p regulates the synthesis of the bZIP transcription factor Hac1ip through cleavage of *HAC1* mRNA. However, one-third of UPRE-*lacZ* reporter gene induction by ER stress was independent of *IRE1* (Shamu and Walter, 1996). In this study, we show that besides the *IRE1-HAC1* pathway, additional ER stress response pathway(s) culminating in transcriptional activation from a core promoter exist in *Saccharomyces cerevisiae*, which may be responsible for the residual, *IRE1*- and *HAC1*-independent transcriptional activation (Figs 8 and 9). Our data are consistent with ER stress stimulating RNA polymerase II holoenzyme activity in an *IRE1*- and *HAC1*-independent manner. We used random mutagenesis of an *ire1Δ* strain to uncover these new pathways. Several mutations were isolated that activated a *lacZ* reporter in response to ER stress in an *IRE1*-independent manner. All mutations were recessive and most comprised one complementation group. Characterization of the recessive allele of the major complementation group demonstrated that loss-of-function mutations in *SIN4* allow for transcriptional activation of the *lacZ* reporter by ER stress independent of the classic UPR. In addition to these findings, the identical response of *sin4Δ* and *ire1Δ sin4Δ* strains to ER stress (Fig. 3) suggests that *HAC1*, *IRE1* and *SIN4* act in the same genetic pathway that is responsible for transcriptional activation by the classic UPR. This also explains the otherwise surprising specificity of our genetic screen for loss-of-function mutations in *SIN4*.

In addition to revealing transcriptional activation upon induction of ER stress, *sin4* mutations elevated basal transcription of the *lacZ* reporter construct, a phenomenon well established in previous studies (Mizuno and Harashima, 2000). Two models can explain how *sin4* mutations

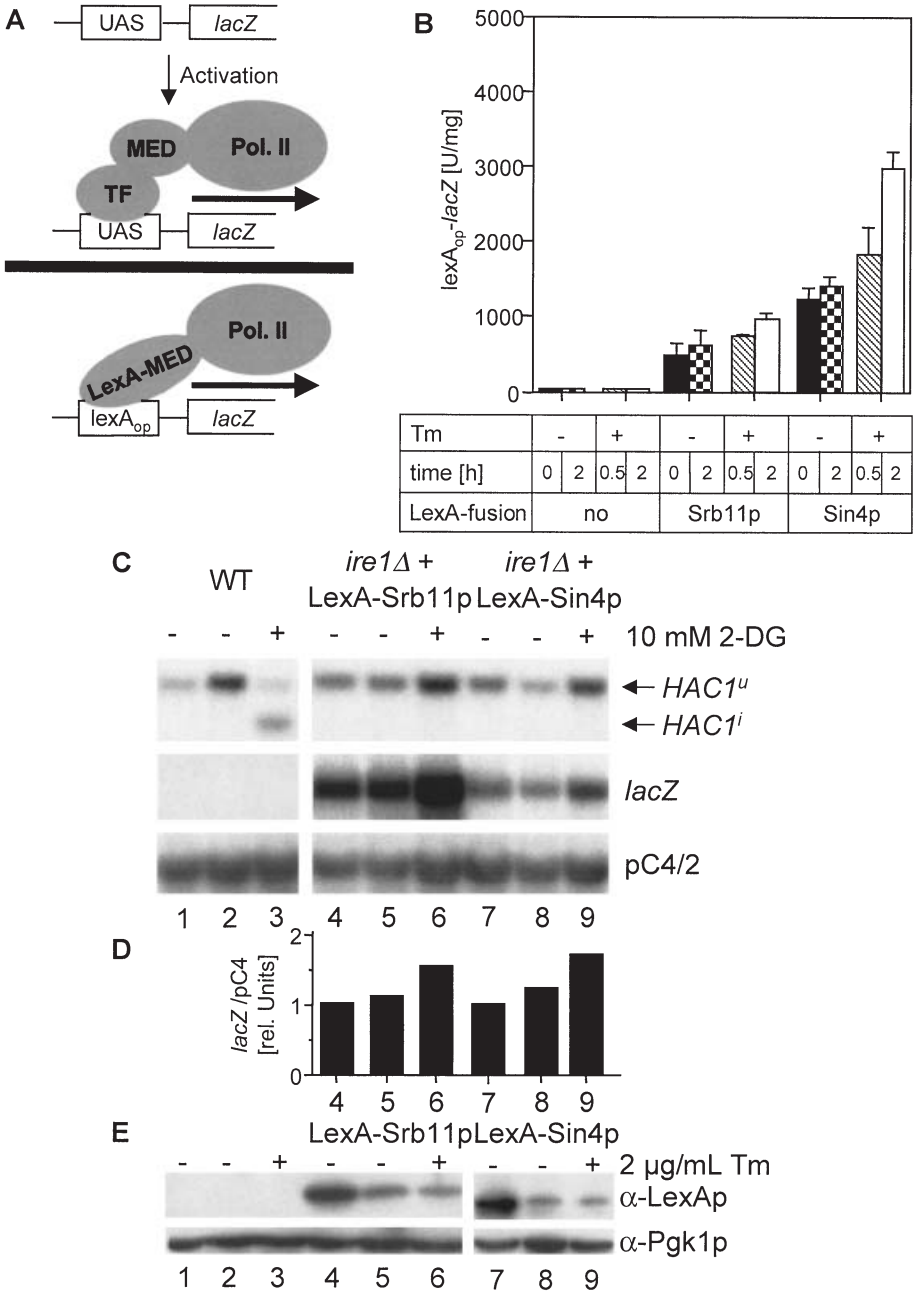

Fig. 5. ER stress increases transcription from a core promoter to which the RNA polymerase II holoenzyme is constitutively tethered in *ire1Δ* cells.
A. Recruitment of the RNA polymerase II holoenzyme to the promoter of a lexA$_{op}$-*lacZ* reporter by LexAp-mediator component fusion proteins obviates the need for a transcription factor (TF). Top part: Upon an environmental stimulus a transcription factor binds to an upstream activating sequence (UAS) and recruits the RNA polymerase II holoenzyme (Pol. II) to the promoter by interaction with mediator components (MED), resulting in activation of transcription. Bottom part: The LexAp-fusions constitutively recruit the RNA polymerase II holoenzyme to the promoter, resulting in 'constitutive' activated transcription.
B. Induction of ER stress with tunicamycin (0.4 µg ml^{-1}) stimulates transcription of a lexA$_{op}$-*lacZ* reporter in an *ire1Δ* strain (MSY24-3). Similar results were obtained with three independent clones for each LexA fusion protein.
C. Northern blot and (D) quantification by PhosphorImaging demonstrate an increase in *lacZ* mRNA relative to the loading control pC4/2 (Schröder *et al.*, 2000) in an *ire1Δ* strain (MSY24-3).
E. Western blot for LexA-Sin4p and LexA-Srb11p. Mid-log phase cells were treated for 2 h with 10 mM 2-deoxy-D-glucose in (C) and 2 µg ml^{-1} tunicamycin in (E) to induce ER stress.

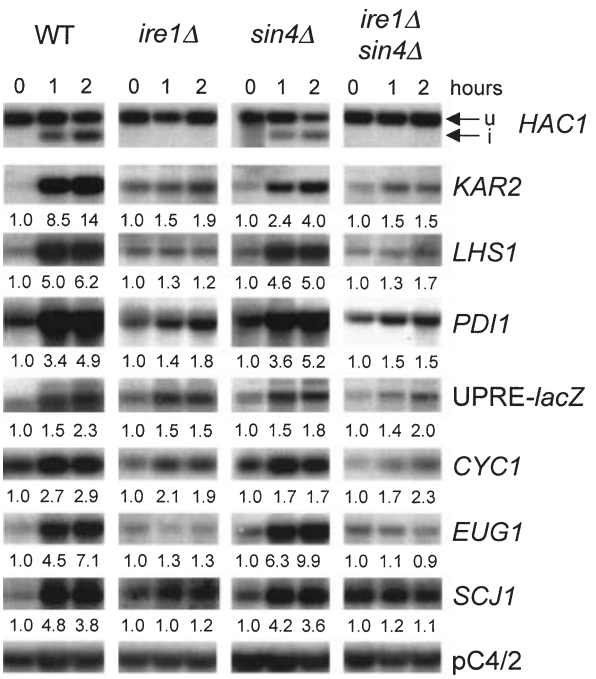

Fig. 6. Northern analysis of ER chaperone induction after treatment of WT (AWY14), *ire1Δ* (AWY19), *sin4Δ* (RCY7) and *ire1Δ sin4Δ* (RCY8) strains with 0.2 μg ml^{-1} tunicamycin for 1 or 2 h. Fold inductions are shown below each blot and were calculated as the ratio of the relative mRNA level at 1 or 2 h to the relative mRNA at 0 h.

affect basal transcription (Fig. 8). In the first model (Fig. 8A), mutations in *SIN4* result in less dense packing of nucleosomes on DNA (Jiang and Stillman, 1992), a chromatin structure correlating in many cases with increased transcriptional activity (Han and Grunstein, 1988; Han *et al*., 1988). Because *sin4* mutations increase basal transcription of only a subset of genes, additional specificity factors must exist (Mizuno and Harashima, 2000). In the second model (Fig. 8B), abrogation of an interaction of Sin4p with a transcriptional repressor is responsible for increased basal transcription in *sin4* mutants. Sin4p is part of a subcomplex that is dissociable from the mediator, indicating that Sin4p is distally localized in the mediator. In addition to propagating the stimulatory effect of transcriptional activators, mediator also propagates the repressing effect of Sfl1p and Tup1p to the RNA polymerase II holoenzyme (Song and Carlson, 1998; Zaman *et al*., 2001). Consistent with this idea are the observations that *sin4* mutations relieve repression by Rme1p (Covitz *et al*., 1994; Shimizu *et al*., 1997), by α2-Mcm1p (Chen *et al*., 1993; Wahi and Johnson, 1995) and partially by Mig1p (Kuchin and Carlson, 1998). In addition, mutations in *SRB10,* encoding another component of the mediator complex, relieve repression by Tup1p (Zaman *et al*., 2001). The repressor can restrain the RNA polymerase II holoenzyme on the promoter through preventing access to the TATA element or by preventing release of the holoenzyme once initiation is completed.

However, both models (Fig. 8A, B) can only explain elevated basal transcription in the *sin4* strains and not stimulation of transcription in response to ER stress independent of *IRE1* and *HAC1* function. In the first model (Fig. 8A), activated transcription in response to ER stress requires loss of Sin4p or at least inactivation of Sin4p, prior to induction and a subsequent opening of the chromatin. *lacZ* reporters without UPRE [Fig. 4A, see also Kohno *et al*. (1993) and Mori *et al*. (1992)] showed only a

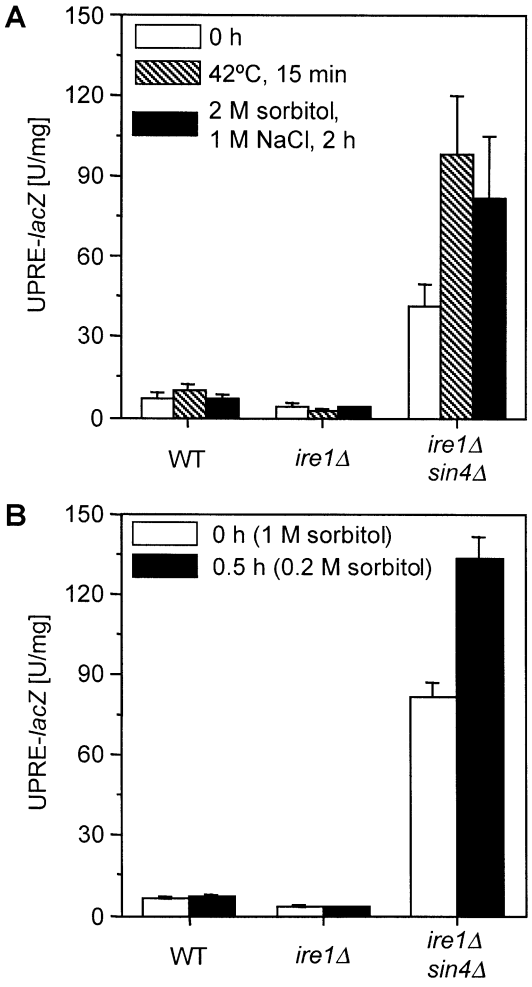

Fig. 7. Core promoter activation is a general phenomenon of stress responses.
A. Induction of a *lacZ* reporter containing only a core promoter by heat shock and hypertonic stress. A WT (AWY14), *ire1Δ* (AWY19) and *ire1Δ sin4Δ* (RCY8) were shifted from 30°C to 42°C for 15 min (crossed bars), or to YPD containing 2 M sorbitol and 1 M NaCl to induce hypertonic stress (black bars).
B. Induction of a *lacZ* reporter containing only a core promoter by hypotonic stress. The same strains as in (A) were shifted from YPD + 1 M sorbitol to YPD + 0.2 M sorbitol for 2 h (black bars). Open bars in (A) and (B) represent the expression level of the *lacZ* reporter before induction of stress. The average and standard error from two independent protein and β-galactosidase determinations are shown.

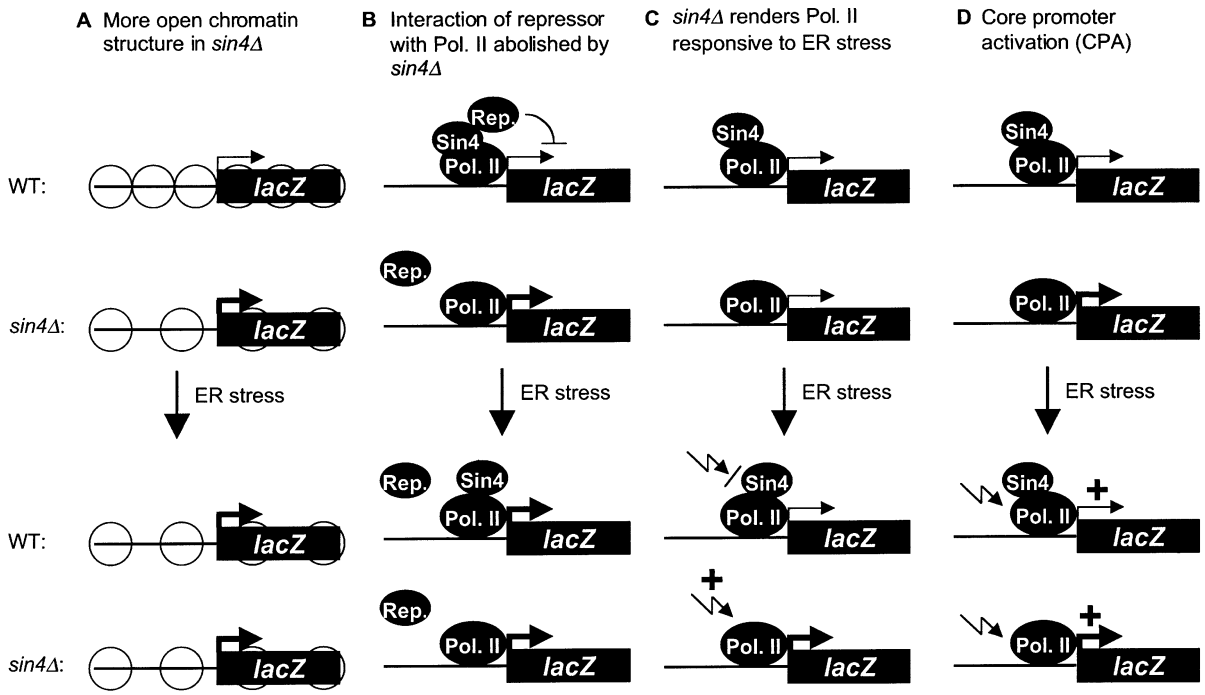

Fig. 8. Summary of models discussed in the text. Please refer to the text for details. Open circles represent nucleosomes. Pol. II: RNA polymerase II transcriptional machinery; Rep: repressor.

small, but detectable, response to ER stress in WT strains. Because β-galactosidase levels in *ire1Δ* strains were significantly lower, even after induction of ER stress, than in *sin4Δ* strains, we believe that Sin4p is intact and functional during periods of ER stress. Further, Western blot analysis demonstrated that the level of a LexA-Sin4p fusion protein was unaffected by ER stress (Fig. 5E). Thus, loss of Sin4p function as a mechanism for activated transcription upon ER stress can be ruled out. In the second model (Fig. 8B), ER stress interferes with the interaction between the repressor and Sin4p. This interaction is already abolished in a *sin4Δ* strain. We cannot rule out that the repressor contacts other components of mediator in addition to Sin4p, but at least deletion of *SIN4* and *GAL11* yielded opposite results (Fig. 3), indicating that the phenotype is specifically related to *SIN4* and not *GAL11*. It is also possible that *sin4* mutations render some parts of the transcriptional machinery responsive to ER stress (Fig. 8C). However, this third model is not consistent with the observation of stimulation of transcription by ER stress when the RNA polymerase II holoenzyme was tethered to a promoter in a *SIN4* WT background (Fig. 5B, D). Taken together, these findings support that *SIN4* does not have a direct role in the *IRE1*- and *HAC1*-independent tran-

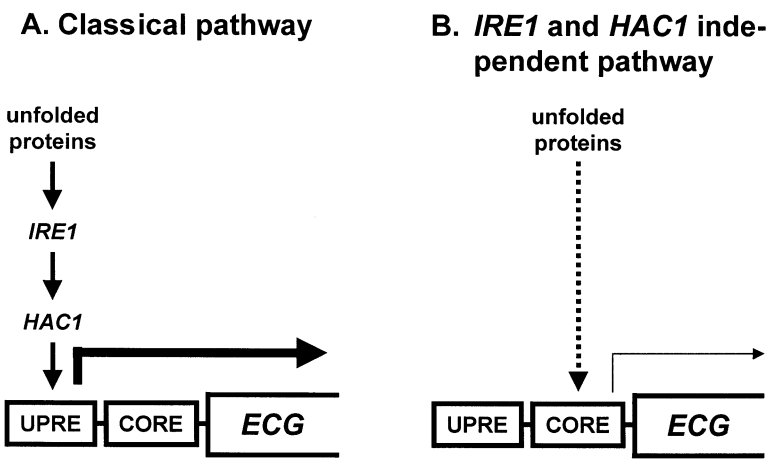

Fig. 9. Summary of unfolded protein response signalling pathways in yeast.
A. The classic pathway activates transcription of ER chaperone genes (*ECG*) through *IRE1*, *HAC1* and the UPRE.
B. *IRE1*- and *HAC1*-independent pathways activate transcription of some *ECG*s through the core promoter. A dashed line is used to indicate that the *IRE1*- and *HAC1*-independent signal transduction mechanism(s) are unknown.

scriptional activation after induction of ER stress. We conclude that stimulation of transcription in *sin4Δ* strains (Figs 3 and 4) or in the recruitment experiment (Fig. 5) is revealed due to the high level of basal expression prior to induction of ER stress in these systems and therefore present, but hard to detect, in WT cells.

To explain stimulation of transcription in response to ER stress in *ire1Δ sin4Δ* strains (Figs 3 and 4) or the recruitment experiment (Fig. 5) a new signal transduction pathway from the ER to the nucleus needs to be invoked. Because this response to ER stress is fast, that is, is detectable as early as 30 min after induction of ER stress (Figs 1A, 3A and 4A), we think that this pathway responds to unfolded proteins in the ER lumen, and not to secondary perturbations of cellular metabolism. Two models how transcription can be stimulated in the *ire1Δ sin4Δ* strains are at hand: (i) an increase in the accessibility of the promoter for the RNA polymerase II holoenzyme (Fig. 8A), or (ii) an increase in the specific activity of the holoenzyme (Fig. 8D). Examples for the first model (Fig. 8A) are changes in chromatin structure, such as less dense packaging of nucleosomes or increased histone acetylation, or binding of another, not yet identified transcriptional activator, to the promoter and subsequent recruitment of the holoenzyme to the promoter. Examples for the second model (Fig. 8D) include an intrinsic structural change in the holoenzyme, such as a post-translational modification, e.g. phosphorylation, or the addition or elimination of regulatory factors resulting in increased specific activity of the holoenzyme. This idea is not unprecedented. Direct stimulation of the holoenzyme by glucose starvation through the Snf1p kinase was recently reported (Kuchin *et al.*, 2000). Further, genetic interactions of the hyperactive *RAS2*^{Val19} allele with *sin4*, *srb8*, *srb9*, and *srb11* mutations, as well as truncations of the C-terminal domain of *RBP1*, the largest subunit of RNA polymerase II, also suggest a direct modification of the holoenzyme, which is important for entry into stationary phase (Howard *et al.* 2001; Howard *et al.*, 2002). Alternatively, these changes can affect the activity of repressors, which could, for example, result in a more rapid release of the holoenzyme once initiation is completed. To discriminate between these two possibilities, we recruited the holoenzyme to a lexA$_{op}$-*lacZ* reporter with LexA-holoenzyme component fusions, which should completely saturate the promoter with holoenzyme. Expression of LexA-Sin4p or LexA-Srb11p fusion proteins resulted in very strong elevation of basal transcription (Fig. 5B). Further, Western blot analysis demonstrated that the levels of both LexA-fusion proteins dropped after another 2 h of growth (Fig. 5E), without affecting expression of the reporter significantly (Fig. 5B). These observations are consistent with saturation of the promoter with holoenzyme. We also saw stimulation of transcription after induction of ER stress in this system (Figs 5B, C). These findings are consistent with the idea that RNA polymerase II holoenzyme activity is stimulated by ER stress, and that accessibility of the promoter for the holoenzyme is not affected. However, our data cannot rule out that ER stress leads to a more elongation proficient state of the chromatin.

We term this stress-induced transcriptional activation of a core promoter core promoter activation or CPA. CPA was also observed during heat or osmotic shock and is therefore not limited to ER stress (Fig. 7). CPA may be physiologically important to further amplify a stress response by direct stimulation of holoenzyme activity, as suggested by Kuchin *et al.* (2000). For example, CPA contributes about 10% of the total activation of the *PDI1* gene by ER stress (Fig. 6), and contributes to an even higher degree to the activation of the UPRE-*lacZ* reporter (Fig. 6). CPA may mediate the *IRE1*- and *HAC1*-independent transcriptional activation from a UPRE (Shamu and Walter, 1996). Modest activation of a subset of ER chaperone genes was observed in *ire1Δ* and *ire1Δ sin4Δ* strains (Fig. 6). This demonstrates that CPA indeed targets some, but not all, ER chaperone genes. Alternatively, CPA can be responsible for a genome-wide modulation of transcription (Kuchin *et al.*, 2000), or constitute a pathway separate from the classic UPR and then be responsible for induction of a separate set of genes for which a moderate induction level is sufficient to contribute to cell survival during stress conditions. These functions are supported by three observations: First, survival of ER stress by *ire1Δ sin4Δ* strains is improved compared with *ire1Δ* strains (Figs 2B and 4B). Second, *CYC1* is about twofold induced upon ER stress in an *IRE1*-independent manner (Fig. 6). Third, most of the 87 genes that were identified as ER stress responsive in an *IRE1*- and *HAC1*-independent manner encode functions that could protect from ER stress (Table 1; Travers *et al.*, 2000). In this case, CPA may play a role in activation of many genes in response to stress conditions. Last, direct stimulation of holoenzyme activity may be the most archaic stress response that evolved long before nature invented more specialized transcriptional regulators. In this case we expect this mechanism to be also present in higher eukaryotes.

In conclusion, the data provided in this study demonstrate that moderate transcriptional activation of a core promoter occurs after induction of ER stress, and that the classic UPR, that is, *IRE1*, *HAC1* and an UPRE, are dispensable for this response (Fig. 9). These findings provide a plausible explanation for the residual activation of transcription in an *IRE1*- and *HAC1*-independent fashion reported earlier (Shamu and Walter, 1996). Further, our data indicate that increased transcription after induction of ER stress in *ire1Δ* cells is not due to changes in promoter accessibility, but rather an intrinsic structural change that increases RNA polymerase II holoenzyme activity. Future work will address the question how the

Table 1. Open reading frames (ORF) induced in an *IRE1*- and *HAC1*-independent manner by ER stress with a likely function in alleviating ER stress.

	WT		*ire1Δ*		*hac1Δ*			
Gene	DTT	Tm	DTT	Tm	DTT	Tm	Function/description	Relation to ER stress
SSA4	3.5	5.8	22.5	11.6	11.4	8.6	Heat shock protein	Y
UBC5	1.7	1.7	2.3	7.5	4.4	4.6	Ubiquitin conjugating enzyme	Y
HSP26	2.6	1.9	6.3	5.7	6.0	5.5	Heat shock protein	Y
KTR2	5.7	4.5	8.4	4.8	2.9	2.9	Mannosyltransferase	Y
GFA1	5.3	4.3	7.1	4.6	1.3	1.3	Glucosamine-fructose-6-phosphate aminotransferase (isomerizing)	Y
NDI1	2.3	2.9	2.0	4.5	1.3	1.9	NADH dehydrogenase (ubiquinone)	Y
HVG1	6.9	5.2	9.1	4.2	3.6	3.2	Nucleotide sugar transporter	Y
PKH2	2.6	2.5	3.9	4.1	3.8	3.0	Pkb-activating kinase homologue	?
GSC2	9.6	4.8	4.8	3.5	4.9	2.0	1,3-β-glucan synthase	Y
SLT2	3.1	3.6	4.5	3.5	2.5	2.4	MAP kinase, cell wall defects	?
HSP42	2.3	2.4	4.6	3.4	8.2	7.0	Heat shock protein	Y
SUR1	1.2	1.9	2.8	3.4	2.6	2.6	Involved in maintenance of phospholipid levels	Y
SSE2	3.3	2.9	3.5	3.1	3.6	4.3	Heat shock protein	Y
BAG7	2.3	1.4	2.8	3.1	1.6	2.5	Rho GTPase activator	?
CSI2	3.9	5.2	4.8	3.1	2.7	1.9	Structural component of chitin synthase 3 complex	Y
ALD3	2.6	1.6	3.0	3.1	2.0	1.3	Aldehyde dehydrogenase, induced in response to heat shock	Y
DDR48	7.1	3.7	5.4	3.0	5.5	2.2	DNA repair	N
UBI4	2.4	1.8	4.2	2.9	3.0	2.4	Protein degradation tagging	Y
YPS3	3.4	2.3	3.1	2.9	3.4	6.6	GPI-anchored aspartic-type endopeptidase	Y
AUT7	1.8	1.9	3.5	2.9	3.2	4.5	Autophagy	Y
PCL1	5.1	5.3	3.9	2.9	4.3	3.0	G_1-cyclin that associates with PHO85	N
SKM1	1.8	1.9	3.1	2.9	3.1	2.6	Protein kinase, cell cycle control	N
MAG1	1.6	1.8	4.1	2.9	2.6	2.3	3-methyladenine DNA glycosylase	N
OCH1	3.0	2.3	3.8	2.7	5.1	2.7	α-1,6-mannosyltransferase	Y
AFR1	2.1	2.8	2.9	2.3	2.2	2.8	Mating response	?
TPS2	2.1	1.7	2.3	2.3	4.6	2.7	Trehalose phosphatase, stress response	Y
HSP82	1.1	2.1	2.9	2.2	7.7	9.0	Heat shock protein	Y
HSP104	1.3	1.5	3.0	2.2	5.5	5.3	Heat shock protein	Y
YPS1	8.0	4.3	2.7	2.1	5.9	4.4	GPI-anchored aspartic protease	Y
APG7	2.0	1.8	3.2	2.1	2.3	2.8	Ubiquitin-like conjugating enzyme, autophagy	Y
SDS22	2.1	1.8	2.8	2.1	2.5	2.1	Glc7p regulatory subunit	Y

The fold inductions after treatment with 2 mM DTT or 1 μg ml^{-1} tunicamycin (Tm) for 1 h are reproduced from Travers *et al*. (2000). The genes were sorted on the basis of their extent of induction with tunicamycin in the *ire1Δ*, and then *hac1Δ* strain. Only genes with a well-characterized function and more than a twofold induction in at least two of the three strains (WT, *ire1Δ*, and *hac1Δ*) are shown. A function or description of each gene is given, as provided by the *Saccharomyces* genome database (SGD). A putative relation to perturbation of secretory pathway function is denoted by a 'Y' for yes and 'N' for no. A question mark (?) is used in cases where, due to limited information, no decision could be made.

activity of the holoenzyme is regulated, and what signalling pathways are involved.

Experimental procedures

Strains and plasmids

Escherichia coli DH10B (Invitrogen) was used for all cloning purposes. Plasmids pSH18-18 [lexA$_{op}$-*lacZ* reporter (Hanes and Brent, 1989)], pSK34 [LexA-Srb11p (Kuchin *et al*., 2000)] and pSK151 [LexA-Sin4p (Kuchin *et al*., 2000)] were a gift of M. Carlson (Columbia University, New York, NY). *Saccharomyces cerevisiae* strains are summarized in Table 2.

Genomic manipulations

IRE1 was deleted as described before (Schröder *et al*., 2000). *ura3-1* strains were transformed to uracil prototrophy using *Nco*I-linearized pRS306 (Sikorski and Hieter, 1989). To delete *HAC1*, the 1.2 kb *Kpn*I-*Xba*I piece of pHAKO1 (Cox and Walter, 1996) was cloned into the *Kpn*I–*Xba*I sites of YIplac204 (Gietz and Sugino, 1988) to yield pΔhac1::TRP1. Correct integration of *BamH*I-linearized pΔhac1::TRP1 into the *HAC1* locus was verified by PCR with primers 5413G (Table 3) and 4149K, and by Southern analysis. *SIN4* and *YTP1* were deleted either by PCR-mediated gene disruption using the kanMX2-cassette of pFA6a (Wach *et al*., 1994) and oligonucleotide pairs 8701G, 8702G and 8699G, 8700G or with *BamH*I-linearized plasmid pΔsin4::URA3 in the case of *SIN4*. To construct pΔsin4::URA3, the 5′- and 3′-piece of the *SIN4* locus were amplified from genomic DNA with oligonucleotides 1684K, 1685K, 1686K and 1687K respectively. The PCR products were fused by overlapping PCR and cloned into the *Spe*I–*Xho*I sites of pRS306. Integration of the PCR generated deletion constructs into the *SIN4* and *YTP1* loci was verified with primers 7256G, 9059J, and 9060J. Correct integration of *BamH*I-linearized pΔsin4::URA3 into the *SIN4* locus was verified with primers 2742K and 2743K. *GAL11* was deleted by PCR-mediated gene disruption using the nourseothricin resistance gene in pAG25 (Goldstein and

Table 2. Yeast strains.

Strain	Genotype	Source
W303 1A	MAT**a**	A. Welihinda
W303 1B	MATα	A. Welihinda
AWY 14	MAT**a** UPRE-$P_{CYC1(-178)}$-*LEU2*::*HIS3* UPRE-$P_{CYC1(-178)}$-*lacZ*::*TRP1*	Liu *et al.* (2000)
AWY 19	MAT**a** UPRE-$P_{CYC1(-178)}$-*LEU2*::*HIS3* UPRE-$P_{CYC1(-178)}$-*lacZ*::*TRP1 ire1Δ*	Liu *et al.* (2000)
RCY 7	MAT**a** UPRE-$P_{CYC1(-178)}$-*LEU2*::*HIS3* UPRE-$P_{CYC1(-178)}$-*lacZ*::*TRP1 sin4Δ*::*kanMX2*	This study
RCY 8	MAT**a** UPRE-$P_{CYC1(-178)}$-*LEU2*::*HIS3* UPRE-$P_{CYC1(-178)}$-*lacZ*::*TRP1 ire1Δ sin4Δ*::*kanMX2*	This study
RCY 9	MAT**a** UPRE-$P_{CYC1(-178)}$-*LEU2*::*HIS3* UPRE-$P_{CYC1(-178)}$-*lacZ*::*TRP1 ire1Δ ytp1Δ*::*kanMX2*	This study
MSY 293-01	MAT**a** UPRE-$P_{CYC1(-178)}$-*LEU2*::*HIS3* UPRE-$P_{CYC1(-178)}$-*lacZ*::*TRP1 ire1Δ gal11Δ*::*natMX4*	This study
AWY 500	MATα UPRE-$P_{CYC1(-178)}$-*lacZ*::*LEU2*	Welihinda *et al.* (1998)
AWY 503	MATα UPRE-$P_{CYC1(-178)}$-*lacZ*::*HIS3*	A. Welihinda
MSY 6	MATα UPRE-$P_{CYC1(-178)}$-*lacZ*::*LEU2 ire1Δ*::*kanMX2*	this study
MSY 7	MATα UPRE-$P_{CYC1(-178)}$-*lacZ*::*HIS3 ire1Δ*::*kanMX2*	this study
MSY 8	MATα UPRE-$P_{CYC1(-178)}$-*lacZ*::*LEU2 ire1Δ*::*kanMX2 ura3-1*::*URA3*	this study
MSY 9-1	MATα UPRE-$P_{CYC1(-178)}$-*lacZ*::*HIS3 ire1Δ*::*kanMX2 ura3-1*::*URA3*	This study
MSY 24-3	MAT**a** *ire1Δ*::*kanMX2*	This study
RCY 1	MAT**a** UPRE-$P_{CYC1(-178)}$-*lacZ*::*HIS3 ire1Δ*::*kanMX2*	This study
RCY 2	MAT**a** $P_{CYC1(-178)}$-*lacZ*::*HIS3 ire1Δ*::*kanMX2*	This study
RCY 3	MAT**a** UPRE-$P_{CYC1(-178)}$-*lacZ*::*HIS3 ire1Δ*::*kanMX2 sin4Δ*::*URA3*	This study
RCY 4	MAT**a** $P_{CYC1(-178)}$-*lacZ*::*HIS3 ire1Δ*::*kanMX2 sin4Δ*::*URA3*	This study
RCY 5	MAT**a** UPRE-$P_{CYC1(-178)}$-*lacZ*::*HIS3 ire1Δ*::*kanMX2 hac1Δ*::*TRP1 sin4Δ*::*URA3*	This study
RCY 6	MAT**a** $P_{CYC1(-178)}$-*lacZ*::*HIS3 ire1Δ*::*kanMX2 hac1Δ*::*TRP1 sin4Δ*::*URA3*	This study

All strains are in the W303-1 genetic background and carry the mutations *ade2-1*, *can1-100*, *his3-11,-15*, *leu2-3,-112*, *trp1-1*, *ura3-1*.

McCusker, 1999) and oligonucleotides 5578K and 5579K. Correct integration of the deletion construct into the *GAL11* locus was verified with primers 6016K, 7305K and 3169l. Plasmid pJC002 (Cox *et al*., 1993) contains the *lacZ* gene under control of an UPRE inserted into the *Bgl*II–*Xho*I sites of the core *CYC1* promoter up to basepair –178. We designate this reporter construct as UPRE-$P_{CYC1(-178)}$-*lacZ*. To construct a reporter plasmid lacking the UPRE, pJC002 was digested with *Xho*I and religated to remove the UPRE sequence to yield pJC002-UPRE ($P_{CYC1(-178)}$-*lacZ*). Both, pJC002 and pJC002-UPRE were linearized with *Nhe*I and integrated into the *HIS3* locus. Integration into the *HIS3* locus was verified by PCR on the 5′ end of the recombined locus with primers 798K and 799K. All transformations were done using the LiOAc-method (Chen *et al*., 1992).

Media, growth conditions and stress induction

Yeast extract-peptone-dextrose (YPD) and synthetic dextrose (SD) media were described previously (Sherman, 1991). For plates, 2% (w/v) bacto-agar (Difco) was added. G418 (Invitrogen) and nourseothricin (Werner Bioagents) were used at 400 mg l^{-1} and 25 mg l^{-1} respectively. To monitor induction of *lacZ* reporters on plates, 0.1 M sodium phosphate (pH 7.0), 50 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal; Diagnostic Chemicals) and 2 mg ml^{-1} tunicamycin (Calbiochem) were added. To induce unfolded proteins in the ER lumen in liquid culture, 0.4 or 2 mg ml^{-1} tunicamycin were added to mid-log phase cultures. Alternatively, 2-deoxy-D-glucose or dithiothreitol (DTT) were used at 10 or 3 mM respectively. Resistance to tunicamycin and 2-deoxy-D-glucose was assayed by spotting 10-fold serial dilutions of freshly grown overnight cultures onto YPD or SD plates containing increasing concentrations of the drugs. The plates were then incubated at 30°C for 2–3 days. Heat shock was induced by shifting cells from 30°C to 42°C for 15 min. Hypertonic stress was induced by shifting cells from YPD to YPD + 2 M sorbitol + 1 M NaCl for 2 h, and hypotonic stress by shifting cells grown in YPD + 1 M sorbitol to YPD + 0.2 M sorbitol for 2 h.

Isolation of eis *(ERN1-independent signalling) mutations*

ire1Δ strains (AWY19 and MSY9-1) were mutagenized with 3% (v/v) ethyl methanesulfonate (Sigma) as described by Lawrence (1991). Cells were first selected for resistance to tunicamycin (0.1–0.25 μg ml^{-1} tunicamycin) and resistant colonies then screened for induction of the UPRE-$P_{CYC1(-178)}$-*lacZ* reporter by replica plating to YPD–X-Gal and YPD–X-Gal–tunicamycin plates. Colonies that developed a blue colour in the presence of tunicamycin were isolated. Dominance, recessiveness and complementation groups were established using classic yeast genetic methods.

Cloning of EIS1

Recessive mutations were cloned by complementation (Rose and Broach, 1991) using a yeast genomic DNA library in YCp50 [ATCC 37415 (Rose *et al*., 1987)]. Approximately 31 000 transformants were analysed for induction of the *lacZ* reporter in response to ER stress. Colonies showing complementation by the plasmid were further tested for sensitivity to 0.2 μg ml^{-1} tunicamycin. A total of 28 complementing clones were obtained. Plasmid DNA was isolated from these clones (Strathern and Higgins, 1991) and electroporated (Seidman *et al*., 2000) into *E. coli* DH10B. Sequencing of the 5′ and 3′ ends identified the inserts in YCp50. All plasmids were reintroduced into yeast to verify their ability to complement the recessive mutation.

Table 3. Oligonucleotides.

Name	Sequence (5′ to 3′)
5413G	CTATTGAGCTCCTGTCTTCCTCTACTGGGC
7256G	CGCGGCCTCGAAACGTGA
8699G	GGAGCAAGGTATTGGAATGAATGTATTTTGTCC AGCGTTCTTTTATCACGAGGCCACTAGTGGA TCTG
8700G	CCTTACCTGCCTAGAAGGAATGACAGCAGCTAA TAAGAATATTGTCTTCGAAGCTTCGTACGCTG CAG
8701G	AATAACGTTACAGCTGCTGGACACTTCAAATCA AGATTCAAGGATCTTCAAGCTTCGTACGCTG CAG
8702G	CATTTCTTCAATATTTTCTTGCGAAAAACAACTG CCAAGTTCCAGCTGCAAGGCCACTAGTGGA TCTG
9059J	GCAGCCGCATTCGCATGG
9060J	ATGGTCGCCTCCCAACCG
798K	CTGCCAGGTATCGTTTGAAC
799K	ACGCATCTGTGCGGTATTTC
1674K	CAGGCGCATTTTTGGTCTAC
1675K	GAGAGAAATCGCTACCCTTG
1684K	GCTAGATAACATATGGATCCAAGATGTGGATGAA GATACC
1685K	CTACTTTCTCGAGATTGAACTTCGTGCAATTGC
1686K	CTCATTCACTAGTTGATTCCTGTTGCTAAATGG
1687K	GGATCCATATGTTATCTAGCTCCCTCTTCTAATGG
2742K	TGTGGTCTCTACAGGATCTG
2743K	CACAGGTTACATCGTCGTAC
3490K	CCTACACCCTCTTTGTTAGA
3491K	AACTAGCAGACCTGACCTTC
3492K	TGGAAGCACTCCAAACTTCG
3493K	CTTGGAATTTCGCTGGCTCA
3494K	CACCTATGAATGATGATCAG
3495K	GCTCCAACACAACTTTCAGC
3496K	TATTGCATCCCCACTAAGCG
3497K	CATTTCTCCTGATGCGCCTA
3498K	CACAAACAAGGAATACACAC
3499K	GAATATGCCAAAGTGGGAGA
3500K	TAAGAGATGCAGTAGGTGCG
3501K	ACAAAGGAGACTAACGAAGG
3502K	TGGCATTTGAGGACTGTGAG
3503K	GATCAGGACGTTGTCCAGTA
3504K	TTCCTTATTGCCAAGCACGC
3505K	TAGACACAACATGCAGGTTC
3506K	ACGATATCACTGTGGCGACG
3507K	CACGTATCCAAATGCCAAAG
3508K	CCACACCATTGAACGCAAAC
3509K	TTCGCCACTATTTGGGTGAC
3510K	TCAAGCCACTTGTATCTGCA
4149K	TGTAAGCGGAGGTGTGGAG
3511K	ACCTGAACATATGCACAGTC
5578K	TCATGGACATTAACACTCTGAACGGAGGGAGC TCCGACACTGCTGATAAGAAGCTTCGTACGC TGCAGG
5579K	ACCCATAGGAGACTGTACAGTCTTCATATTATTG GGGTTTGGTGTGCCATAGGCCACTAGTGGAT CTG
6016K	CGTATCGTTTCGTATAGTGC
6932K	GATGGTAGTGGTCAAATGGC
6933K	CAACTGGTAATGGTAGCGAC
7305K	AGGTGCCACTTTCATCTGG
3169L	CCGTGTCGTCAAGAGTGG

Sequencing of sin4 *alleles*

The complete wild type (WT) and mutant *sin4-68* ORF were amplified with *Pwo* DNA Polymerase (Roche Molecular Biochemicals) using primers 1674K and 1675K, and cloned into the *Eco*RI–*Xba*I sites of pRS306. Both strands were sequenced with primers 3490K to 3511K. Two independent bacterial clones were analysed to eliminate mutations introduced during PCR.

β-Galactosidase assays

To assay induction of the *lacZ* reporter in response to ER stress in liquid culture, cells were grown to mid-log phase, drugs added at the concentration stated in the text, and samples taken before, 30 and 120 min after addition of the drug. The preparation of protein extracts, the determination of protein concentrations and β-galactosidase activity were previously described (Schröder *et al*., 2000). β-Galactosidase was standardized in all experiments to the amount of intracellular protein.

RNA preparation and analysis

Isolation and Northern analysis of RNA, the probes for *HAC1* and the loading control pC4/2 were described previously (Schröder *et al*., 2000). The *lacZ* probe was amplified by PCR with primers 6932K and 6933K from plasmid Z691 (Mori *et al*., 1993) as template. Probes for *CYC1*, *EUG1*, *LHS1*, *KAR2*, *PDI1* and *SCJ1* were amplified from genomic yeast DNA. All signals were quantified by PhosphorImaging (Molecular Dynamics) and standardized to the loading control pC4/2.

Western blots

To determine protein concentrations, protein was extracted as previously described (Kuchin et al., 2000), diluted 1:30 into 0.1 M 2-iodoacetamide in 0.1 M Tris-HCl (pH 8.0), and then incubated 15 min at 37°C to destroy β-mercaptoethanol. Protein was then determined using the Bio-Rad DC kit (Bio-Rad). Immunoblotting with goat-α-LexA-antibody (Invitrogen) and mouse-α-Pgk1p-antibody (Molecular Probes) as primary antibodies and rabbit-α-goat-IgG-horseradish peroxidase (1:5000, Invitrogen) or goat-α-mouse-IgG-POD (1:400, Roche Molecular Biochemicals) as secondary antibodies was done according to each manufacturer's instructions. Blots were stripped for 10 min at room temperature with 0.1 M glycine·HCl (pH 2.5). Chemiluminescence detection was performed as described previously (Schröder and Friedl, 1997).

Acknowledgements

We thank Dr Ajith A. Welihinda (Galileo Laboratories, Santa Clara, CA) for strains and plasmids, Marian Carlson for kindly providing plasmids, Jason S. Chang for technical assistance, and Dennis Thiele (University of Michigan, Ann Arbor, MI) for use of the Fotodyne electronic imaging system (Fotodyne, Hartland, WI). We also thank Jan Mitchell for critically reading the manuscript.

References

Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P.,

and Ron, D. (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature Cell Biol* **2:** 326–332.

Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., *et al.* (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415:** 92–96.

Carlson, M. (1997) Genetics of transcriptional regulation in yeast: connections to the RNA polymerase II CTD. *Annu Rev Cell Dev Biol* **13:** 1–23.

Chapman, R.E., and Walter, P. (1997) Translational attenuation mediated by an mRNA intron. *Curr Biol* **7:** 850–859.

Chen, D.-C., Yang, B.-C., and Kuo, T.-T. (1992) One step-transformation of yeast in stationary phase. *Curr Genet* **21:** 83–84.

Chen, S., West, R.W., Johnson, S.L., Gans, H., Kruger, B., and Ma, J. (1993) TSF3, a global regulatory protein that silences transcription of yeast *GAL* genes, also mediates repression by α2 repressor and is identical to SIN4. *Mol Cell Biol* **13:** 831–840.

Covitz, P.A., Song, W., and Mitchell, A.P. (1994) Requirement for *RGR1* and *SIN4*. *RME1*-dependent repression in *Saccharomyces cerevisiae*. *Genetics* **138:** 577–586.

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

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.

Fassler, J.S., Gray, W., and Lee, J.P., Yu, G.Y., and Gingerich, G. (1991) The Saccharomyces cerevisiae SPT14 gene is essential for normal expression of the yeast transposon, Ty, as well as for expression of the HIS4 gene and several genes in the mating pathway. *Mol Gen Genet* **230:** 310–320.

Gietz, R.D., and Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:** 527–534.

Goldstein, A.L., and McCusker, J.H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15:** 1541–1553.

Han, M., and Grunstein, M. (1988) Nucleosome loss activates yeast downstream promoters in vivo. *Cell* **55:** 1137–1145.

Han, M., Kim, U.J., Kayne, P., and Grunstein, M. (1988) Depletion of histone H4 and nucleosomes activates the PHO5 gene in Saccharomyces cerevisiae. *EMBO J* **7:** 2221–2228.

Hanes, S.D., and Brent, R. (1989) DNA specificity of the Bicoid activator protein is determined by homeodomain recognition helix residue 9. *Cell* **57:** 1275–1283.

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* **10:** 3787–3799.

Howard, S.C., Chang, Y.-W., Budovskaya, Y.V., and Herman, P.K. (2001) The Ras/PKA signaling pathway of Saccharomyces cerevisiae exhibits a functional interaction with the Sin4p complex of the RNA polymerase II holoenzyme. *Genetics* **159:** 77–89.

Howard, S.C., Budoskaya, Y.V., Chang, Y.-W., and Herman, P.K. (2002) The C-terminal domain of the largest subunit of RNA polymerase II is required for stationary phase entry and functionally interacts with the Ras/PKA signaling pathway. *J Biol Chem* **277:** 19488–19497.

Hubbard, S.C., and Ivatt, R.J. (1981) Synthesis and processing of asparagine-linked oligosaccharides. *Annu Rev Biochem* **50:** 555–583.

Jiang, Y.W., and Stillman, D.J. (1992) Involvement of the *SIN4* global transcriptional regulator in the chromatin structure of *Saccharomyces cerevisiae*. *Mol Cell Biol* **12:** 4503–4514.

Kim, S., Cabane, K., Hampsey, M., and Reinberg, D. (2000) Genetic analysis of the Ydr1-Bur6 repressor complex reveals an intricate balance among transcriptional regulatory proteins in yeast. *Mol Cell Biol* **20:** 2455–2465.

Kohalmi, S.E., and Kunz, R.A. (1988) Role of neighbouring bases and assessment of strand specificity in ethylmethanesulphonate and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutagenesis in the *SUP4-o* gene of *Saccharomyces cerevisiae*. *J Mol Biol* **204:** 561–568.

Kohno, K., Normington, K., Sambrook, J., Gething, M.-J., and Mori, K. (1993) The promoter 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.

Kuchin, S., and Carlson, M. (1998) Functional relationships of Srb10-Srb11 kinase, carboxy-terminal domain kinase CTDK-I and transcriptional corepressor Ssn6-Tup1. *Mol Cell Biol* **18:** 1163–1171.

Kuchin, S., Treich, I., and Carlson, M. (2000) A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. *Proc Natl Acad Sci USA* **97:** 7916–7920.

Lawrence, C.W. (1991) Classical mutagenesis techniques. In *Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology*, Vol. 194. Guthrie, C., and Fink, G.R. (eds). San Diego: Academic Press, pp. 273–281.

Liu, C.Y., Schröder, M., and Kaufman, R.J. (2000) Ligand-independent dimerization activates the stress response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. *J Biol Chem* **275:** 24881–24885.

Mizuno, T., and Harashima, S. (2000) Activation of basal transcription by a mutation in *SIN4*, a yeast global repressor, occurs through a mechanism different from activator-mediated transcriptional enhancement. *Mol Gen Genet* **263:** 48–59.

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.

Mori, K., Ogawa, N., Kawahara, T., Yanagi, H., and Yura, T. (1998) Palindrome with spacer of one nucleotide is characteristic of the *cis*-acting unfolded protein response ele-

ment in Saccharomyces cerevisiae. *J Biol Chem* **273:** 9912–9920.

Mori, K., Ogawa, N., Kawahara, T., Yanagi, H., and Yura, T. (2000) mRNA splicing-mediated C-terminal replacement of transcription factor Hac1p is required for efficient activation of the unfolded protein response. *Proc Natl Acad Sci USA* **97:** 4660–4665.

Nojima, H., Leem, S.-H., 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*. *Nucleic Acids Res* **22:** 5279–5288.

Ye, J., Rawson, R.B., Komuro, R., Chen, X., Dave, U.P., Prywes, R., *et al.* (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBP2. *Mol Cell* **6:** 1355–1364.

Rose, M.D., and Broach, J.R. (1991) Cloning genes by complementation in yeast. In *Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology*, Vol. 194. Guthrie, C., and Fink, G.R. (eds). San Diego: Academic Press, pp. 195–230.

Rose, M.D., Novick, P., Thomas, J.H., Botstein, D., and Fink, G.R. (1987) *A Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* **60:** 237–243.

Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., *et al.* (2001) Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell* **7:** 1165–1176.

Schröder, M., and Friedl, P. (1997) Overexpression of recombinant human antithrombin III in Chinese hamster ovary cells results in malformation and decreased secretion of the recombinant protein. *Biotechnol Bioeng* **53:** 547–559.

Schröder, M., Chang, J.S., and Kaufman, R.J. (2000) The unfolded protein response represses nitrogen-starvation induced developmental differentiation in yeast. *Genes Dev* **14:** 2962–2975.

Schwarz, R.T., Schmidt, M.F.G., and Datema, R. (1979) Inhibition of glycosylation of viral glycoproteins. *Biochem Soc Trans* **7:** 322–326.

Seidman, C.E., Struhl, K., Sheen, J., and Jessen, T. (2000) Introduction of plasmid DNA into cells. In *Current Protocols in Molecular Biology.* Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds). New York: John Wiley & Sons, pp. 1.8.1–1.8.10.

Sherman, F. (1991) Getting started with yeast. In *Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology,* vol. 194. Guthrie, C., and Fink, G.R. (eds). San Diego: Academic Press, pp. 3–21.

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.

Shen, X., Ellis, R.E., Lee, K., Liu, C.-Y., Yang, K., Solomon, A., *et al.* (2001) Complementary signaling pathways regulate the unfolded protein response and are required for C. elegans development. *Cell* **107:** 893–903.

Shimizu, M., Li, W., Shindo, H., and Mitchell, A.P. (1997) Transcriptional repression at a distance through exclusion of activator binding *in vivo*. *Proc Natl Acad Sci USA* **94:** 790–795.

Sidrauski, C., and Walter, P. (1997) The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. *Cell* **90:** 1031–1039.

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

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.

Song, W., and Carlson, M. (1998) Srb/mediator proteins interact functionally and physically with transcriptional repressor Sfl1. *EMBO J* **17:** 5757–5765.

Strathern, J.N., and Higgins, D.R. (1991) Recovery of plasmids from yeast into *Escherichia coli*: Shuttle vectors. In *Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology*, Vol. 194. Guthrie, C., and Fink, G.R. (eds). San Diego: Academic Press, pp. 319–329.

Travers, K.J., Patil, C.K., Wodicka, L., Lockhart, D.J., Weissman, J.S., and Walter, P. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101:** 249–258.

Wach, A., Brachat, A., Pöhlmann, R., and Philippsen, P. (1994) New heterologous modules for classical of PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10:** 1793–1808.

Wahi, M., and Johnson, A.D. (1995) Identification of genes required for $\alpha 2$ repression in *Saccharomyces cerevisiae*. *Genetics* **140:** 821–831.

Wang, Y., Shen, J., Arenzana, N., Tirasophon, W., Kaufman, R.J., and Prywes, R. (2000) Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J Biol Chem* **275:** 27013–27020.

Welihinda, A.A., Tirasophon, W., Green, S.R., and Kaufman, R.J. (1998) Protein serine/threonine phosphatase Ptc2p negatively regulates the unfolded-protein response by dephosphorylating Ire1p kinase. *Mol Cell Biol* **18:** 1967–1977.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107:** 881–891.

Zaman, Z., Ansari, A.Z., Koh, S.S., Young, R., and Ptashne, M. (2001) Interaction of a transcriptional repressor with the RNA polymerase II holoenzyme plays a crucial role in repression. *Proc Natl Acad Sci USA* **98:** 2550–2554.