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 mRNA. Only spliced HAC1 mRNA (HAC1 protein factor Hac1p by controlling splicing of HAC1 inactivation of 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.

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-containing transcription factor Hac1p by controlling splicing of HAC1 mRNA. Only spliced HAC1 mRNA (HAC1) is translated, and Hac1p 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 ire1α 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.
in response to ER stress in ire1Δ and hac1Δ strains (Travers et al., 2000).

Here, we demonstrate the existence of additional, ire1Δ and hac1Δ-independent, ER stress response pathways in yeast. These pathways were revealed by elevation of basal transcription. Either sin4 mutations or recruitment of a LexA-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 ire1Δ, hac1Δ or the UPRE. We conclude that an ire1Δ- and hac1Δ-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 (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 ire1Δ- and hac1Δ-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 ire1Δ or hac1Δ function (Travers et al., 2000). Finally, about one-third of the activation of promoters containing an UPRE was independent of ire1Δ and hac1Δ 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 ire1Δ or hac1Δ.

Isolation of mutants with an ire1Δ/ern1Δ-independent ER to nucleus signalling phenotype

To identify genes involved in these ire1Δ-independent ER stress signalling pathways we isolated mutant yeast strains that efficiently signal ER stress in the absence of functional ire1Δ/ern1Δ. 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 ire1Δ) 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 eis1Δ 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 eis1Δ 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 ire1Δ.

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 eis1Δ 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
IRE1-independent transcripational activation by ER stress

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.

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In contrast, ER stress signalling was not observed when the \( \text{ire1}^{-D} \text{sin4}^{-D} \) strain was mated to a mutant strain that did not belong to the major complementation group (\( \text{ire1}^{-D} \text{mt no. 82} \), Fig. 2C). These data demonstrate that \( \text{SIN4} \) is allelic with \( \text{EIS1} \) and not a suppressor of \( \text{eis1} \) mutations. We conclude that \( \text{EIS1} \) is \( \text{SIN4} \). We designate the recessive \( \text{sin4}^{-} \) allele as \( \text{sin4}^{-68} \) (synonymous to \( \text{eis1}^{-68} \)).

**Fig. 2.** \( \text{EIS1} \) is \( \text{SIN4} \).

A. Schematic drawings of the insert in two complementing plasmids (Rose-1 and Rose-22), WT and mutant \( ytp1^{-D}::\text{kanMX2}, \text{sin4}^{-D}::\text{kanMX2}, \) and \( \text{sin4}^{-D}::\text{URA3} \) loci. The location of the mutation in allele \( \text{sin4}^{-68} \) (\( W705^* \), *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: \( \text{BamHI}; \) E: \( \text{EcoRI}; \) X: \( \text{XbaI}; \) Tm: tunicamycin.

B. Resistance of \( \text{ire1}^{-D} \text{sin4}^{-D} \) (RCY8) and \( \text{ire1}^{-D} ytp1^{-D} \) (RCY9) strains to tunicamycin. Serial 10-fold dilutions of fresh, overnight cultures were spotted onto YPD plates containing 0.5 \( \mu \text{g mL}^{-1} \) tunicamycin and control plates.

C. \( \text{SIN4} \) is allelic to \( \text{EIS1} \). An \( \text{ire1}^{-D} \text{sin4}^{-D} \) (RCY8), \( \text{ire1}^{-D} \text{eis1}^{-68} \) (derived from AWY19), and an \( \text{ire1}^{-D} \text{sin4}^{-D} \) (RCY8) strain were mated to an \( \text{ire1}^{-D} \) (MSY9-1), and two mutant strains (\( \text{ire1}^{-D} \text{eis1}^{-13} \), 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.
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 transition 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\(^{YC1-178}\)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\(^{YC1-178}\)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

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**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).
IRE1-independent transcriptional activation by ER stress

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_{CYC1-172}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 Hac1p 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

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.

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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). Similar results were obtained with three independent clones for each LexA fusion protein.

D. 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.
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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
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 sin4Δ 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. 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.

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 ECGs through the core promoter. A dashed line is used to indicate that the IRE1- and HAC1-independent signal transduction mechanism(s) are unknown.
scripational 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 RAS2Val192000). Further, genetic interactions of the hyperactive promoter a classical 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 manner. 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 manner. In this case we expect this mechanism to be also present in higher eukaryotes.

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. Lastly, 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>DTT</th>
<th>Tm</th>
<th>DTT</th>
<th>Tm</th>
<th>Function/description</th>
<th>Relation to ER stress</th>
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</thead>
<tbody>
<tr>
<td>SSA4</td>
<td>3.5</td>
<td>5.8</td>
<td>22.5</td>
<td>11.6</td>
<td>11.4</td>
<td>Heat shock protein</td>
<td>Y</td>
</tr>
<tr>
<td>UBC5</td>
<td>1.7</td>
<td>1.7</td>
<td>2.3</td>
<td>7.5</td>
<td>4.4</td>
<td>Ubiquitin conjugating enzyme</td>
<td>Y</td>
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<tr>
<td>HSP26</td>
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<td>1.9</td>
<td>6.3</td>
<td>5.7</td>
<td>6.0</td>
<td>Heat shock protein</td>
<td>Y</td>
</tr>
<tr>
<td>KTR2</td>
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<td>4.5</td>
<td>8.4</td>
<td>4.8</td>
<td>2.9</td>
<td>Mannosyltransferase</td>
<td>Y</td>
</tr>
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<td>GFA1</td>
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<td>4.3</td>
<td>7.1</td>
<td>4.6</td>
<td>1.3</td>
<td>Glucosamine-fructose-6-phosphate aminotransferase (isomerizing)</td>
<td>Y</td>
</tr>
<tr>
<td>NDI1</td>
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<td>2.9</td>
<td>2.0</td>
<td>4.5</td>
<td>1.3</td>
<td>NADH dehydrogenase (ubiquinone)</td>
<td>Y</td>
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<td>6.9</td>
<td>5.2</td>
<td>9.1</td>
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<td>3.6</td>
<td>Nucleotide sugar transporter</td>
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<td>2.5</td>
<td>3.9</td>
<td>4.1</td>
<td>3.8</td>
<td>Pkb-activating kinase homologue</td>
<td>?</td>
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<td>4.8</td>
<td>3.5</td>
<td>4.9</td>
<td>1,3-β-glucan synthase</td>
<td>?</td>
</tr>
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<td>3.5</td>
<td>2.5</td>
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<td>SUR1</td>
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<td>3.4</td>
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<td>Involved in maintenance of phospholipid levels</td>
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<td>3.5</td>
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<td>1.6</td>
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<td>4.8</td>
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<td>Structural component of chitin synthase 3 complex</td>
<td>?</td>
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<td>1.6</td>
<td>3.0</td>
<td>3.1</td>
<td>2.0</td>
<td>Aldehyde dehydrogenase, induced in response to heat shock</td>
<td>Y</td>
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<tr>
<td>DFR48</td>
<td>7.1</td>
<td>3.7</td>
<td>5.4</td>
<td>3.0</td>
<td>5.5</td>
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<td>N</td>
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<td>1.8</td>
<td>4.2</td>
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<td>Protein degradation tagging</td>
<td>Y</td>
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<td>3.1</td>
<td>2.9</td>
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<td>GPI-anchored aspartic-type endopeptidase</td>
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<td>AUT7</td>
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<td>3.5</td>
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<td>Autophagy</td>
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<td>G1-cyclin that associates with PHO85</td>
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<td>SKM1</td>
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<td>3.1</td>
<td>2.9</td>
<td>3.1</td>
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<td>2.9</td>
<td>2.6</td>
<td>3-methyladenine DNA glycosylase</td>
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<td>OCH1</td>
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<td>3.8</td>
<td>2.7</td>
<td>5.1</td>
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<tr>
<td>AFR1</td>
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<td>2.8</td>
<td>2.9</td>
<td>2.3</td>
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<td>Mating response</td>
<td>Y</td>
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<td>TPS2</td>
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<td>2.8</td>
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<td>Heat shock protein</td>
<td>Y</td>
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<td>Heat shock protein</td>
<td>Y</td>
</tr>
<tr>
<td>YPS1</td>
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<td>4.3</td>
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<td>2.1</td>
<td>5.9</td>
<td>GPI-anchored aspartic protease</td>
<td>Y</td>
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<td>APG7</td>
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<td>1.8</td>
<td>3.2</td>
<td>2.1</td>
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<td>Ubiquitin-like conjugating enzyme, autophagy</td>
<td>Y</td>
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<td>SDS22</td>
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<td>1.8</td>
<td>2.8</td>
<td>2.1</td>
<td>2.5</td>
<td>Glic7p regulatory subunit</td>
<td>Y</td>
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</tbody>
</table>

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 [lexAop-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 NcoI-linearized pRS306 (Sikorski and Hieter, 1989). To delete *HAC1*, the 1.2 kb *KpnI*-Xbal piece of pHAKO1 (Cox and Walter, 1996) was cloned into the *KpnI*-Xbal sites of YPlac204 (Gietz and Sugino, 1988) to yield pλ.hac1::TRP1. Correct integration of *BamHl*-linearized pλ.hac1::TRP1 into the *HAC1* locus was verified by PCR with primers 5413G 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 *BamHl*-linearized plasmid pλ.sin4::URA3 in the case of *SIN4*. To construct pλ.sin4::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 SpeI–XhoI 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 *BamHl*-linearized pλ.sin4::URA3 into the *SIN4* locus was verified with primers 2742K and 2743K. HAC1 was deleted by PCR-mediated gene disruption using the nourseothricin resistance gene in pAG25 (Goldstein and Walter, 1996).
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 BglII-XhoI sites of the core CYC1 promoter up to basepair –178. We designate this reporter construct as UPRE-PCYC1(-178)lacZ. To construct a reporter plasmid lacking the UPRE, pJC002 was digested with XhoI and religated to remove the UPRE sequence to yield pJC002-UPRE (P_CYC1(-178)lacZ). Both, pJC002 and pJC002-UPRE were linearized with Nhel and integrated into the HIS3 locus. Integration into the HIS3 locus was verified by PCR on the 5’ end of the recombinant 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. 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. Hypotonic stress was induced by shifting cells from YPD to YPD + 2 M sorbitol + 1 M NaCl for 2 h, and hypertonic 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

The 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-PCYC1(-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 DH110B. 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 respective mutation.

Table 2. Yeast strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303 1A</td>
<td>MATa</td>
<td>A. Welihinda</td>
</tr>
<tr>
<td>W303 1B</td>
<td>MATca</td>
<td>A. Welihinda</td>
</tr>
<tr>
<td>AWY 14</td>
<td>MATa UPRE-P_CYC1(-178)lacZ::HIS3 UPRE-P_CYC1(-178)lacZ::TRP1</td>
<td>Liu et al. (2000)</td>
</tr>
<tr>
<td>AWY 19</td>
<td>MATa UPRE-P_CYC1(-178)lacZ::HIS3 UPRE-P_CYC1(-178)lacZ::TRP1 ire1Δ</td>
<td>Liu et al. (2000)</td>
</tr>
<tr>
<td>RCY 7</td>
<td>MATa UPRE-P_CYC1(-178)lacZ::HIS3 UPRE-P_CYC1(-178)lacZ::TRP1 sin4Δ::kanMX2</td>
<td>This study</td>
</tr>
<tr>
<td>RCY 8</td>
<td>MATa UPRE-P_CYC1(-178)lacZ::HIS3 UPRE-P_CYC1(-178)lacZ::TRP1 ire1Δ sin4Δ::kanMX2</td>
<td>This study</td>
</tr>
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</tr>
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<tr>
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<tr>
<td>MSY 8</td>
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<td>this study</td>
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<td>this study</td>
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<tr>
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<td>MATa ire1Δ::kanMX2</td>
<td>this study</td>
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</tr>
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</tr>
<tr>
<td>RCY 3</td>
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<tr>
<td>RCY 6</td>
<td>MATa P_CYC1(-178)lacZ::HIS3 ire1Δ::kanMX2 hac1Δ::TRP1 sin4Δ::URA3</td>
<td>this study</td>
</tr>
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</table>

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.
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 EcoRI–XbaI 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

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