Palindrome with Spacer of One Nucleotide Is Characteristic of the cis-Acting Unfolded Protein Response Element in *Saccharomyces cerevisiae*

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Eukaryotic cells possess multiple intracellular signaling pathways from the endoplasmic reticulum (ER) to the nucleus. One of these, the unfolded protein response (UPR), is activated to induce transcription of ER-localized molecular chaperones and folding enzymes in the nucleus. In *Saccharomyces cerevisiae*, at least six lumenal proteins including essential Kar2p and Pdi1p are known to be regulated by the UPR. We and others recently demonstrated that the basic-leucine zipper protein Hac1p/Ern4p functions as a trans-acting factor responsible for the UPR. Hac1p binds directly to the cis-acting unfolded protein response element (UPRE) necessary for induction of Kar2p. Moreover, we showed that the KAR2 UPRE contains an E box-like palindrome separated by one nucleotide (CAGGTG) that is essential for its function. We report here that the promoter regions of five target proteins (Kar2p, Pdi1p, Eug1p, Fkb2p, and Lhs1p) contain a single UPRE sequence that is necessary and sufficient for induction and that binds specifically to Hac1p in vitro. All of the five functional UPRE sequences identified contain a palindromic sequence that has, in four cases, a spacer of one C nucleotide. This unique characteristic of UPRE explains why only a specific set of proteins are induced in the UPR to cope with ER stress.

When unfolded proteins are accumulated in the endoplasmic reticulum (ER), an intracellular signaling pathway termed the unfolded protein response (UPR) is activated to induce transcription of ER-localized molecular chaperones and folding enzymes in the nucleus. In *Saccharomyces cerevisiae*, at least six lumenal proteins including essential Kar2p and Pdi1p are known to be regulated by the UPR. We and others recently demonstrated that the basic-leucine zipper protein Hac1p/Ern4p functions as a trans-acting factor responsible for Kar2p induction. Moreover, we showed that the KAR2 UPRE contains an E box-like palindrome separated by one nucleotide (CAGGTG) that is essential for its function. We report here that the promoter regions of five target proteins (Kar2p, Pdi1p, Eug1p, Fkb2p, and Lhs1p) contain a single UPRE sequence that is necessary and sufficient for induction and that binds specifically to Hac1p in vitro. All of the five functional UPRE sequences identified contain a palindromic sequence that has, in four cases, a spacer of one C nucleotide. This unique characteristic of UPRE explains why only a specific set of proteins are induced in the UPR to cope with ER stress.

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* The abbreviations used are: ER, endoplasmic reticulum; OFR, open reading frame; UPR, unfolded protein response; UPRE, unfolded protein response element; UPRF, unfolded protein response factor; kb, kilobase pair(s).

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trophoretic mobility shift assays (10, 11). More importantly, we demonstrated that Hac1p recognizes the palindromic separated by a one-nucleotide spacer in KAR2 UPRE both in vivo and in vitro (11). Furthermore, we showed that Hac1p is involved in induction of all known target proteins (11, 31). This indicated that, as in the case of KAR2 UPRE, the promoters of other target proteins might contain a palindromic sequence with a spacer of one nucleotide recognized directly by Hac1p.

EXPERIMENTAL PROCEDURES

Strains and Microbiological Techniques—The yeast strains used in this study were KMY1005 (MATa leu2–3, 112 ura3–52 his3–Δ200 trp1–Δ901 lys2–801), KMY1015 (MAT1/1015 enr1::TRP1), and KMY2005 (MATa leu2–3, 112 ura3–52 his3–Δ200 trp1–Δ901 lys2–801 sec53–6) (11). The SCJ deletion (pre1) strain SNT925 (MATa leu2–3, 112 ura3–52 his3–Δ200 trp1–Δ901 lys2–801 sec53–6) was a generous gift from D. S. Nishikawa and T. Endo (Nagoya University). The compositions of rich broth medium (YPD) and synthetic complete medium used for selection of transformants such as Sc (– Ura) have been described (33). Tunicamycin was obtained from Sigma (T-7765) and used at a concentration of 5 μg/ml throughout the experiments. Yeast cells were transformed by the lithium acetate method (34).

Construction of Reporter Plasmids—Recombinant DNA techniques were carried out as described (35). The reporter plasmids pSCZ2 and pMCZ2 were constructed by modifying the CYC1-laclZ fusion gene of the multicopy vector pLG178 (36), a derivative of pLG670-Z (37), which is often utilized to assess activities of cis-acting elements in S. cerevisiae (38). Because the laclZ reporter gene in pLG178 and pLG–C can be individually used (36), we transferred the CYC1 portion in pLG–C into another lacZ-containing vector in frame. Thus, inserted into pUC118 was the 0.25-kb XhoI-BamHI fragment of the CYC1-laclZ fusion gene in pLG–C, which contained the entire CYC1 promoter and 5′-terminal several nucleotides of the CYC1-coding region located upstream of the BamHI site (36, 37). From the result presented, one nucleotide C was deleted at the start codon of the BamHI site was deleted by site-directed mutagenesis (39) so that the ATG codon would be in frame with the lacZ-coding sequence embedded in pSEY102 (a CEN4-ARS1-based single-copy vector) or pSEY101 (a 2-μm-based multicopy vector), both containing the URA3 selectable marker (27). The 0.25-kb EcoRI (derived from the multicloning site of pUC118)-BamHI fragment of the mutagenized CYC1 gene was inserted between the EcoRI and BamHI sites of pSEY102 to create the single-copy vector pSCZ2 and multicopy vector pMCZ2, respectively. pMCZ2 possesses unique EcoRI and XhoI sites upstream of the CYC1 promoter for inserting oligonucleotides, whereas pLG178 contains only one XhoI site. Various double-stranded, synthetic oligonucleotides whose 5′- and 3′-termini are complementary to protruding termini generated by EcoRI and XhoI, respectively, were inserted between the EcoRI and XhoI sites of pMCZ2.

Comparison of β-galactosidase activity expressed from pLG178 and pMCZ2 suggested that the presence of the lacI portion between CYC1 and lacZ represses the expression of β-galactosidase. First, pMCZ2 produced considerably higher basal β-galactosidase produced from pMCZ2 containing the wild-type UPRE-Y in cells incubated in the presence of tunicamycin for 3 h was 3-fold higher than those from pLG–C containing UPRE-Y. Second, activity of UPRE-A, a point mutant of UPRE-Y described previously (36), was 3% of that of UPRE-Y when inserted into pMCZ2, whereas it was almost negligible (0.3% of UPRE-Y) when inserted into pLG–C. These results indicated that, although the extent of induction with pMCZ2 containing UPRE-Y is lower than that with pLG–C containing UPRE-Y (50–60-fold versus 200–300-fold induction by tunicamycin treatment for 3 h), β-galactosidase assay with pMCZ2 provides higher sensitivity for detecting weakly active elements than that with pLG–C.

Constructs to Determine Promoter Activity—The 2.5-kb Apal-PstI fragment containing the entire PD1 gene in the plasmid pMTY17 (24) was cloned into pUC118, and a BamHI site was created immediately downstream of the start ATG codon (ATGGATCC-- -- --) by site-directed mutagenesis. The 0.44-kb Apal-BamHI, 0.30-kb BstBI-BamHI, 0.21-kb SpeI-BamHI, or 0.09-kb MluI-BamHI fragment of the PD1 promoter was inserted between the Smal and BamHI sites of pSEY102. Two point mutations were introduced to PD1la UPRE by site-directed mutagenesis.

The plasmid pCT20 carrying both the EUG1 and FKB2 genes in tandem was kindly provided by D. T. H. Stevens (University of Oregon), and the 2.8-kb HindIII-SalI fragment was cloned into pUC119. The genomic LHS1 gene with framing regions was obtained by screening the yeast genomic library constructed on Yep15, a multicopy yeast vector (ATCC 37293, Ref. 40) and the 1.6-kb EcoRV fragment was cloned into pUC118. A BamHI site was created immediately downstream of the start ATG codon of EUG1, FKB2, or LHS1 (ATGATGCC-- -- --). The 0.19-kb HindIII–BamHI fragment of EUG1, a 0.29-kb Accl–BamHI fragment of FKB2, or a 0.27-kb BstXI–BamHI fragment of LHS1 was inserted between the Smal and BamHI sites of pSEY101. Two point mutations were introduced to EUG1 UPRE, FKB2 UPRE, or LHS1 UPRE (Fig. 1).

The plasmid pPS177 carrying the entire SCJ1 gene was kindly provided by Dr. P. A. Silver (Harvard Medical School). The 3.0-kb KpnI-HindIII fragment was transferred to pPS316 (a CEN4-ARS4-based single-copy vector containing the URA3 selectable marker, Ref. 41) to create pPS316-SCJ1. The 1.2-kb KpnI–SpI fragment of pPS177 was cloned into pUC118, and a BamHI site was created immediately downstream of the first of the two ATG codons (ATGATGCC-- -- --). The KpnI–BamHI, StyI–BamHI, Alfl–BamHI, or HgiI–BamHI fragment was inserted between the Smal and BamHI sites of pSEY101.

Assays—β-Galactosidase assays and Northern blot hybridization analysis were carried out as described previously (7, 11, 31). Electrophoretic mobility shift assays were performed as described (11). Cell extracts were prepared from the wild-type strain (KMY1005) that had been grown in YPD medium to a mid-log phase and incubated for 1 h with tunicamycin, and proteins were fractionated by ammonium sulfate precipitation according to our previous report (31). Hac1p of 238 amino acids was translated in vitro using TNT®-coupled wheat germ extract system (Promega) and a template HAC1 DNA according to the manufacturer’s instructions.

RESULTS

KAR2 UPRE Confers Inducibility on the CYC1-laclZ Gene Regardless of Its Orientation—We constructed the reporter plasmids pSCZ2 (a single-copy vector) and pMCZ2 (a multicopy vector) as described under “Experimental Procedures.” These plasmids contained a modified version of the CYC1-laclZ gene of pLG–C containing UPRE-Y (36) and provided a sensitive and convenient assay system for examining cis-acting elements in S. cerevisiae. Using pMCZ2, we showed that the KAR2 UPRE (previously referred to as UPRE-Y, Ref. 27) contained a partial palindromic sequence separated by one nucleotide (Fig. 1); nucleotides 10–12 (CAG)
Fig. 2. Binding of various UPRE-like sequences to Hac1p. Hac1p of 238 amino acids was translated in vitro, and cell extracts were prepared from the wild-type strain (KMY1005) that had been treated with tunicamycin as described under “Experimental Procedures.” A, 20 μg of proteins in cell extracts or 0.2 μl of in vitro translated Hac1p were mixed with 0.3 ng (approximately 12,000 cpm) of 32P-labeled synthetic oligonucleotides containing 22-base pair wild-type UPRE-Y or its point mutant (UPRE-Tv10), which is virtually inactive in mediating the UPR in vivo (11). Both 5′- and 3′-termini of double-stranded probes, cohesive to EcoRI and XhoI sites, respectively, were radiolabeled using the Klenow fragment of DNA polymerase I and α-32PdATP, and protein-bound probes were separated from free probes in a 5% nondenaturing gel as described previously (11). The specific binding of UPRE-Y to a protein(s) in cell extracts (termed UPRF) or Hac1p translated in vitro is marked by a black or white arrowhead, respectively. The band marked by an asterisk, migrating faster than UPRF, seems to represent a protein(s) recognizing nucleotides outside of the palindrome in UPRE. B, the specific binding between 0.3 ng of 32P-labeled UPRE-Y and UPRF in cell extracts (20 μg of proteins, upper panel) or 0.2 μl of in vitro translated Hac1p (lower panel) was competed by 50- or 250-fold molar excess of unlabeled UPRE-Y (KAR2 UPRE) or various UPRE-like sequences present in the promoters of ER stress-inducible proteins as indicated. Only specific binding is shown, and the positions of UPRF and Hac1p are marked as in A. Neither 5′- nor 3′-termini of competitor oligonucleotides were filled in, and possible formation of concatemers may explain the large molar excess of unlabeled KAR2 UPRE required for competition under these conditions.

Specific Binding of UPRE to UPRF in Cell Extracts and Hac1p Translated in Vitro—The cellular activity for specific binding to UPRE, namely UPRF activity, was detected only in ER-stressed cells (10, 31); binding to the wild-type UPRE-Y was obtained with extracts prepared from tunicamycin-treated cells (Fig. 2A) but not from untreated cells (data not shown). This binding was specific because UPFR did not bind to a point mutant of UPRE-Y designated Tv10, the activity of which was less than 1% of UPRE-Y due to a transversion (C to A) at critical nucleotide 10 (11). The faster migrating band marked by an asterisk may represent a protein(s) recognizing sequences outside of the palindrome. Similarly, binding to UPRE-Y but not to UPRE-Tv10 was obtained with in vitro translated Hac1p (Fig. 2A), which was demonstrated previously to be a transcription factor responsible for the UPR (10, 11). When this UPRE-Y (KAR2 UPRE) was inserted into pMCZ2, 56-fold induction of β-galactosidase was observed after treatment of the wild-type cells with tunicamycin at 30 °C for 3 h, whereas pMCZ2 alone caused marginal (2.7-fold) induction (Fig. 3). Thus, β-galactosidase assays with pMCZ2 as well as electrophoretic mobility shift assays using cell extracts and Hac1p translated in vitro allowed us to examine whether promoters of other ER stress-inducible proteins also included a palindromic sequence directly recognized by Hac1p.

FKB2 UPRE Contains a Partial Palindrome Recognized by Hac1p—The FKB2 promoter was shown previously to contain a cis-acting element that conferred inducibility by ER stress on the CYC1-lacZ gene (26). We confirmed the observation using the pMCZ2 system; β-galactosidase was induced 10-fold by tunicamycin treatment, although the extent of induction was much lower than that with KAR2 UPRE (Fig. 3). When aligned with KAR2 UPRE, FKB2 UPRE was found to contain a partial palindrome separated by one nucleotide (C), although the 3′ half was not well conserved. However, all three of the nucleotides shown to be important for KAR2 UPRE activity at 8, 13, and 18 (dotted in Fig. 3) were conserved. This FKB2 UPRE and 14–16 (GTG) were most critical (boxed), and nucleotides 8 (G), 13 (C), and 18 (C) were also important for UPRE activity (11). In previous experiments to dissect the UPRE, β-galactosidase activity was measured in cells incubated in the presence or absence of tunicamycin, which is known to elicit ER stress by inhibiting N-glycosylation of newly synthesized proteins in the ER (3, 42).

Since tunicamycin treatment may cause pleiotropic defects in cellular metabolism, the response of the lacZ gene in pMCZ2 to ER stress was confirmed here at the mRNA level using the temperature-sensitive sec53 strain (Fig. 1). At the nonpermissive temperature, sec53 cells accumulated full-length precursors of secretory proteins, which were abnormally glycosylated and malfolded in the ER due to the defect in phosphomannomutase activity (43), leading to activation of the UPR (16, 17). The sec53 strain was transformed with pMCZ2 alone or pMCZ2 containing UPRE-Y. Total RNAs were isolated from transformants that had been grown at the permissive temperature of 23 °C or after shifting to the semi-nonpermissive temperature of 30 °C for 90 min. KAR2 mRNA transcribed from the chromosomal gene was markedly induced at 30 °C in all transformants examined, because Kar2p expression was under the control of the KAR2 promoter containing a functional UPRE. In contrast, lacZ mRNA was not induced at 30 °C in sec53 cells carrying pMCZ2 alone. When UPRE-Y was inserted into pMCZ2, lacZ mRNA was markedly induced at 30 °C, indicating that transcription of the UPRE-CYC1-lacZ gene was induced only if unfolded proteins had accumulated in the ER regardless of the nature of the stress employed. Moreover, only slightly decreased response was observed when UPRE-Y was inserted in the opposite orientation, showing that UPRE functions as an upstream activator sequence regardless of its orientation. LHS1 UPRE is indeed in the opposite orientation to other functional UPREs (see Fig. 5).
were used for the presence or absence of tunicamycin (Ura) medium to a mid-log phase, and aliquots were incubated in SC (containing UPRE as indicated. Transformants were grown at 30 °C in KAR2

... otherwise known as CEN4-ARS1, was used for the generation of single-copy yeast vectors based on the pSEYc102 (a CEN4-ARS1-based single-copy vector). The wild-type UPRE (indicated by xx), which changed the central seven nucleotides from AGACGGT to CATCTTG, as described under “Experimental Procedures.” These PDII promoters were fused in-frame to the lacZ-coding sequence in pSEYc102 (a CEN4-ARS1-based single-copy vector). The wild-type (ERN+, KMY1005) or ernΔ (KMY1015) strain was transformed with each of these constructs. β-Galactosidase assays were carried out as in Fig. 3, and the activities are presented as means ± S.D. (bars), based on duplicate determinations with three independent transformants.

FIG. 3. **FKB2** UPRE contains a partially palindromic sequence. Nucleotide sequences of **KAR2**, **FKB2**, and **mutant** (FKB2*) UPREs are shown. Partially palindromic sequences between nucleotides 10 and 16 are indicated by arrows. Three nucleotides important for **KAR2** UPRE activity at 8, 13, and 18 are marked by dots. Another stretch of important nucleotides (positions 2–4) is double underlined. The wild-type strain (KMY1005) was transformed with pMCZ2 alone (none) or pMCZ2 containing UPRE as indicated. Transformants were grown at 30 °C in SC (–Ura) medium to a mid-log phase, and aliquots were incubated in the presence or absence of tunicamycin (TM). Samples taken after 3 h were used for β-galactosidase assays, and the activities presented are averages of duplicate determinations with three independent transformants. Standard deviation was less than 10% for all values shown. Δ(%) is expressed as a percentage of **KAR2** UPRE after subtracting β-galactosidase activities in unstressed cells (–TM) from those in tunicamycin-treated cells (+TM).

FIG. 4. **Analysis of the PDII promoter.** Schematic structure of the PDII promoter is presented at the top. The adenine of the first ATG codon is set as +1. The locations of two UPRE-like elements (designated PDIIa and PDIIb) and a putative TATA sequence are shown by boxes. 5′-Deletion mutants were constructed using appropriate restriction enzymes indicated, and two point mutations were introduced into PDIIa UPRE (indicated by xx), which changed the central seven nucleotides from AGACGGT to CATCTTG, as described under “Experimental Procedures.” These PDII promoters were fused in-frame to the lacZ-coding sequence in pSEYc102 (a CEN4-ARS1-based single-copy vector). The wild-type (ERN+, KMY1005) or ernΔ (KMY1015) strain was transformed with each of these constructs. β-Galactosidase assays were carried out as in Fig. 3, and the activities are presented as means ± S.D. (bars), based on duplicate determinations with three independent transformants.

The **PDII Promoter Contains a Functional UPRE**—Among the six target proteins of the UPR so far known, only Pdi1p and Kar2p are essential for vegetative growth of the cell. The presence of a functional UPRE responsible for the induction of Pdi1p by ER stress was proposed previously by sequence comparison, but its activity has not been determined (5). We thus fused the 0.44-kb **PDII** promoter region in frame to lacZ in the single-copy vector pSEYc102 as described under “Experimental Procedures.” The **PDII** promoter responded to ER stress by inducing a 6-fold increase in β-galactosidase expression when cells were treated with tunicamycin for 3 h (Fig. 4). The location of the previously proposed UPRE referred to here as **PDIIb** UPRE, Ref. 5) is indicated in Fig. 4. Deletion of 0.14 kb between the Apal and BstBI sites showed little effect on the promoter activity, whereas deletion of an additional 87 base-pairs containing PDIIb UPRE resulted in a decrease in basal activity to nearly half of that of the 0.44-kb promoter without affecting the extent of induction. In PDIIb UPRE, as shown in Fig. 5, nucleotides 10–12 and 14–16 were not palindromic, although all three of the nucleotides important for UPRE activity at 8, 13, and 18 were identical to those in **KAR2** and **FKB2** UPREs. PDIIb UPRE hardly competed for the binding of either UPRE in cell extracts or Hac1p translated in vitro to 32P-labeled **KAR2** UPRE (Fig. 2B). When inserted into pMCZ2, PDIIb UPRE increased basal activity by 4.3-fold but affected the induction only slightly; β-galactosidase was induced 3.5-fold by tunicamycin treatment, whereas 2.4-fold induction was observed with the vector alone (Fig. 5). These results raise doubts about the role of PDIIb UPRE in the induction of Pdi1p. The PDIIb UPRE with the surrounding sequence may provide a binding site for some factor(s) important for basal expression of Pdi1p.

In contrast, deletion between the SpeI and MluI sites containing a putative TATA sequence abolished tunicamycin-induced expression of β-galactosidase almost completely (Fig. 4), suggesting the presence of a functional UPRE in this region. By sequence homology search, we identified a potential UPRE designated PDI1a UPRE, in which nucleotides 10–12 and 14–16 were perfectly palindromic and the half-sites were separated by one nucleotide, C (Fig. 5). When inserted into pMCZ2, PDI1a UPRE conferred inducibility by ER stress on the CYC1-lacZ gene (15-fold induction by tunicamycin). Transversion of the important nucleotide 18 (C to A) is likely to explain the lower inducibility exhibited by PDI1a UPRE than by **KAR2** UPRE (see “Discussion”). PDI1a UPRE competed for the specific binding between 32P-labeled **KAR2** UPRE and...
concluded from these results that PDIIa UPRE is necessary and sufficient for the induction of PDI1p by ER stress.

A typical pattern of the induction of various endogenous mRNAs by tunicamycin treatment for 1 h is shown at the top of Fig. 5, and the correlation of the degree of induction with the corresponding UPRE activity will be discussed after characterization of all UPREs involved in the ER stress response (see "Discussion"). It should be noted, however, that the relative intensity of mRNA bands from different genes does not reflect the actual abundance in the cell; KAR2 and PDII mRNAs appeared to be much more abundant than other mRNAs, as suggested previously (18, 25).

Characterization of the EUG1 and FKB2 Promoters—When the sequence proposed to be responsible for the induction of Eug1p by ER stress in the previous study (25) was inserted into pMCZ2, this putative EUG1 UPRE increased basal activity by 4.8-fold but conferred only slight inducibility (3.4-fold increase by tunicamycin versus 2.4-fold increase with the vector alone) as in the case of PDI1b UPRE (Fig. 5). However, this EUG1 UPRE competed for the specific binding between $^{32}$P-labeled KAR2 UPRE and UPRE in cell extracts or Hac1p translated in vitro, albeit very weakly (Fig. 2B). Although the palindromic sequence in EUG1 UPRE is not typical of other functional UPREs, Hac1p seems to be capable of recognizing EUG1 UPRE (see "Discussion"). Sequences responsible for increased basal activity observed with EUG1 UPRE (Fig. 5) may be able to be separated from the palindromic sequence exerting weak UPRE activity. We found no other potential UPRE sequences in the 250 nucleotides located between the start ATG codon of Eug1p and the termination codon of the preceding open reading frame (ORF) of 372 amino acids (D9719.22; Ref. 44). To determine the EUG1 promoter activity accurately, we used the multicopy vector pSEY101, considering the previous finding that Eug1p is at least 10-fold less abundant than Pdi1p (25). The EUG1 promoter conferred marked induction (66-fold) of $\beta$-galactosidase by tunicamycin (Fig. 6). However, it should be noted that it showed very low basal activity (less than 1 units) and conferred only mild induction (4–5-fold) when inserted into the single-copy vector pSEYc102. Mutation of two nucleotides in the EUG1 UPRE (CAGCGTG was changed to CATCTTG) eliminated most of the response of the EUG1 promoter to ER stress. Similarly, introduction of two point mutations into the FKB2 UPRE mentioned above (CAGCGCA was mutated to CATCTCA) made the FKB2 promoter almost insensitive to ER stress (Fig. 6). Thus, the EUG1 and FKB2 promoters each contain a single UPRE which are primarily responsible for the induction of Eug1p and Fkb2p, respectively.

Identification of UPRE Responsible for the Induction of Lhs1p—The sequences proposed to be functional UPREs responsible for the induction of Lhs1p by ER stress in the previous reports (18, 19) did not confer inducibility on the CYC1-lacZ gene (data not shown). Instead, we found a sequence that contained the same partial palindromic spacer (CAGCGTG) as KAR2 UPRE. This LHS1 UPRE responded to ER stress by inducing a 29-fold increase in $\beta$-galactosidase expression when inserted into pMCZ2 (Fig. 5), although the orientation of the LHS1 UPRE used for in vivo assay was opposite to that present in the endogenous LHS1 promoter. In addition, this LHS1 UPRE competed for the specific binding between $^{32}$P-labeled KAR2 UPRE and UPRE in cell extracts or Hac1p translated in vitro, albeit less efficiently than KAR2 UPRE (Fig. 2B). As Lhs1p is thought to be expressed at a much lower level than Kar2p (18), we also used the multicopy vector pSEY101 to analyze the LHS1 promoter. Twelve-fold induction of $\beta$-galactosidase by tunicamycin treatment was observed when the LHS1 promoter was fused in

UGRF in cell extracts or Hac1p translated in vitro, albeit less efficiently than KAR2 UPRE (Fig. 2B). We found no other potential UPRE sequences in the region from −211 to −92.

When two point mutations were introduced into PDIIa UPRE present in the 0.44-kb promoter to inactivate both half-sites (CAGCGTG was mutated to CATCTTG), induction of $\beta$-galactosidase by tunicamycin was markedly reduced and the level of the remaining response was very close to that of the wild-type promoter in the ern1Δ strain (Fig. 4); the ern1Δ strain cannot transmit the signal from the ER to activate transcription in the nucleus due to the absence of Ern1p, a transmembrane protein kinase localized in the ER and essential for signal transduction across the ER membrane (6, 7). We
frame to lacZ (Fig. 6), and this induction was abolished almost completely by two point mutations introduced into the LHS1 UPRE redefined in this study (CAGCGTG was changed to CATCTTG). We concluded that Lhs1p is induced by ER stress through the interaction between Hac1p and this LHS1 UPRE, which functions as a cis-acting element that is necessary and sufficient for induction.

Apparent Absence of a Functional UPRE in the SCJ1 Promoter—The sequence proposed previously to be a functional UPRE responsible for the induction of Scj1p by ER stress (Ref. 22; indicated by the most downstream box f in Fig. 7) did not confer significant inducibility on the CYC1-lacZ gene when inserted into pMCZ2 (data not shown). Unlike the other promoters of ER stress-inducible proteins, an ORF of 590 amino acids (YM8261.07) was predicted immediately upstream of two putative TATA sequences for SCJ1 (see Fig. 7 and also compare the broken arrow in Fig. 9 with those in Fig. 6; see also Ref. 45). We found no other potential UPRE sequences between the termination codon of this ORF and the start ATG codon of Scj1p. By sequence homology search, two sequences (indicated by boxes a and d in Fig. 7) in which a partial palindrome was separated by one C nucleotide (CAGCGTA) and a sequence (box b) in which a perfect palindrome was separated by one C nucleotide (CAGCCTG) were found within the upstream ORF. When inserted into pMCZ2, however, these putative UPREs did not confer inducibility on the CYC1-lacZ gene, probably because two important nucleotides 8 and 18 were not conserved (see “Discussion”). Only the results with one putative UPRE (box d) are shown in Fig. 5. This putative SCJ1 UPRE increased basal activity by 2.9-fold without conferring significant inducibility. In addition, this sequence did not compete for the specific binding of 32P-labeled KAR2 UPRE to UPRF or Hac1p (Fig. 2 B).

Next, we transferred the 3.0-kb KpnI-HindIII fragment con-
contain a functional UPRE (26, 27). The KAR2 and FKB2 UPRE sequences were so divergent (see Fig. 3) that it was difficult to assess essential features of the UPRE. Indeed, although putative UPREs were proposed previously to be present in the promoters of Pdi1p (5), Scj1p (22), Lhs1p (18, 19), and Eug1p (25), most of these were hardly active as shown in this study.

Our recent extensive mutational analysis of KAR2 UPRE shed some light on this issue (11). The results revealed that KAR2 UPRE contains an E box (CANNTG)-like palindromic sequence that provides a binding site for Hac1p. Interestingly, Hac1p exhibits a strong preference for a spacer of one nucleotide between the half-sites, apparently a characteristic specific to Hac1p. The lack of spacing reduced induction to 13% of the normal level, whereas increased spacing abolished induction almost completely. Hac1p also shows a preference for a specific nucleotide in the order C > G > A > T. Furthermore, CAC is preferred to CAG as a half-site (11). Thus, the UPRE contains an E box (CANNTG)-like palindromic sequence that provides a binding site for Hac1p. Interestingly, Hac1p exhibits a strong preference for a spacer of one nucleotide between the half-sites, apparently a characteristic specific to Hac1p. The lack of spacing reduced induction to 13% of the normal level, whereas increased spacing abolished induction almost completely. Hac1p also shows a preference for a specific nucleotide in the order C > G > A > T. Furthermore, CAC is preferred to CAG as a half-site (11). Thus, the UPRE contains an E box (CANNTG)-like palindromic sequence that provides a binding site for Hac1p. Interestingly, Hac1p exhibits a strong preference for a spacer of one nucleotide between the half-sites, apparently a characteristic specific to Hac1p. The lack of spacing reduced induction to 13% of the normal level, whereas increased spacing abolished induction almost completely. Hac1p also shows a preference for a specific nucleotide in the order C > G > A > T. Furthermore, CAC is preferred to CAG as a half-site (11).

Fig. 9. The SCJ1 promoter region responds poorly to ER stress. Schematic structure of the SCJ1 gene is presented at the top with several restriction enzyme sites. The adenine of the first ATG codon is set as +1. Two closed boxes indicate putative TATA sequences, and the broken arrow denotes the location and direction of the preceding ORF. The full-length and various 5' deleted promoter regions were fused in frame to the lacZ-coding sequence in pSEY101 from the first (Met1) or second (Met28) methionine as described under "Experimental Procedures." The wild-type strain (KMY1005) was transformed with each of these constructs. Transfomants were grown at 30 °C in SC(-Ura) medium to a mid-log phase, and aliquots were incubated in the presence (+) or absence (−) of tunicamycin (EM) for 1 h. Total RNAs were extracted and analyzed by Northern blot hybridization.

Fig. 8. The 3.0-kb KpnI-HindIII fragment containing the SCJ1 gene fully responds to ER stress. Schematic structure of the plasmid pRS316-SCJ1 is shown at the bottom, which was constructed as described under "Experimental Procedures." The position and direction of the SCJ1-coding region is indicated. The wild-type (SCJ1, SEY6210) or scj1Δ (SNY1025) strain was transformed with pRS316 alone (pRS316-V) or pRS316-SCJ1. Transformants were grown at 30 °C in SC(-Ura) medium to a mid-log phase, and aliquots were incubated in the presence (+) or absence (−) of tunicamycin (EM) for 1 h. Total RNAs were extracted and analyzed by Northern blot hybridization.
tivities. The correlation would be even more significant if we consider the orientation of LHS1 UPRE. When inserted in the opposite orientation, KAR2 UPRE showed slightly decreased activity (Fig. 1). By analogy, the degree of LHS1 mRNA induction may be lower than that expected from the LHS1 UPRE activity obtained with in vivo assay, and therefore PDII, EUG1, and LHS1 mRNAs may show similar levels of induction (Fig. 5). In addition, the degree of KAR2 mRNA induction might be reduced by the heat shock element and the GC-rich region present in the KAR2 promoter (27) but lacking in the other five promoters.

Transcriptional regulation of Sq1p was exceptional; the promoter region apparently lacks any functional UPREs (Fig. 7) and is insufficient for induction (Fig. 9). Interestingly, β-galactosidase was expressed only when the lacZ-coding sequence was fused immediately downstream of Met28 but not of Met1, consistent with the previous report that Sq1p is translated from the second methionine (22). If Sq1p was translated from the first methionine, the first 25 amino acids would be predicted to function as a mitochondrial targeting signal (46), whereas if the second methionine was used to initiate translation, the next ~20 amino acids would target Sq1p into the lumen of the ER (22). Therefore, regardless of the mechanism, the preferential starting of Sq1p translation at Met28 would ensure its function as an ER-resident molecular chaperone. In fact, the absence of Sq1p caused slight activation of the UPR; the level of KAR2 mRNA was approximately 2-fold higher in tunicamycin-untreated cells than in untreated Scj1Δ cells presumably because of increased amounts of unfolded proteins accumulated in the ER (Fig. 8).

All of the five functional UPREs identified contained a palindromic sequence (Fig. 5) and competed for the specific binding between 32P-labeled KAR2 UPRE and Hac1p with various efficiencies (Fig. 2B). Based on the results of analysis of a number of active and inactive UPRE-like sequences, we can now deduce some important features required for the interaction between Hac1p and UPRE.

First, Hac1p recognizes not only the central seven nucleotides but also certain surrounding sequences. The central seven nucleotides, CAGGCGT or CAGCGTG, of PDIIa or LHS1 UPRE, respectively, may be expected to confer higher or equal activity, respectively, as compared with KAR2 UPRE containing CAGCGTG as discussed earlier. However, in vivo activities of PDIIa and LHS1 UPREs were much lower than that of KAR2 UPRE (Fig. 5). Since mutations of nucleotides 1–5 in PDIIa UPRE from CCAAT to GGAAC (KAR2 type) increased in vivo activity only slightly (data not shown), as was the case of FKB2 and FKB2′ UPREs (Fig. 3), a “natural” version of the important nucleotide 18 seemed to be responsible for the weak activity of PDIIa UPRE observed; in the case of KAR2 UPRE, the same transversion reduced the activity to less than 20% (11). On the other hand, nucleotides 8 and 18 in LHS1 UPRE are transitions rather than transversions of those in KAR2 UPRE, perhaps explaining why LHS1 UPRE is more active than PDIIa UPRE.

Second, at least one half-site sequence must be CAG or CAC since PDIIb UPRE is virtually inactive although all three important nucleotides 8, 13, and 18 are conserved. Third, when one of the half-site sequences diverges from CAC or CAG, the three important nucleotides 8, 13, and 18 must be conserved, because FKB2 UPRE is active, whereas SCJ1 UPRE shown in Fig. 5 as well as other UPRE-like sequences shown in Fig. 7 are virtually inactive.

Finally, the case of EUG1 UPRE is exceptional, because its half-sites are separated by two nucleotides (CAGCGTG), which is usually inactive as mentioned earlier. However, this EUG1 UPRE competed for the binding between KAR2 UPRE and Hac1p, although very weakly (Fig. 2B). The presence of two overlapping half-sites with no spacing (CACCGG and CGCGTG) flanked by two important nucleotides (at positions 8 and 18) might explain the recognition of EUG1 UPRE by Hac1p.

In addition to the one-nucleotide spacing between the E box-like half-sites, sequences outside of the central seven nucleotides are important for UPRE recognition by Hac1p. This unique characteristics may distinguish UPRE from among various cis-acting elements recognized by basic region-containing transcription factors, and are likely to explain why transcription of only molecular chaperones and folding enzymes in the ER is induced when unfolded proteins are accumulated in the ER. Without this induction system, yeast cells cannot survive under ER stress conditions (6, 7, 11). Furthermore, increased synthesis of the target proteins of the UPR in the absence of excess unfolded proteins in the ER is also toxic to the cell (10, 31). Therefore, the UPR must be tightly regulated to meet the requirements of this organelle. Recently, we and others showed that Hac1p itself is induced by ER stress and the induction is mediated by unconventional splicing of HAC1 precursor mRNA (10, 13, 31, 47, 48). The unique features of UPRE revealed in this study suggest a basis for the specificity of the UPR; Hac1p induced under ER stress conditions may activate transcription of a limited set of proteins only necessary to cope with deleterious effects of unfolded proteins accumulated in the ER. The UPR, an intracellular signaling from the ER to the nucleus, appears to possess multiple distinguished characteristics among biological signal transduction systems.

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