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Saccharomyces cerevisiae KAR2 (BiP) Gene Expression Is Induced by Loss of Cytosolic HSP70/Ssa1p through a Heat Shock Element-Mediated Pathway[1]

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A family of highly-conserved 70 kDa stress proteins is localized in various intracellular compartments of *Saccharomyces cerevisiae*. Their gene expression is specifically and/or sometimes cooperatively regulated at the transcriptional level by *cis*-acting elements found in their respective promoters. Here, we find that depletion of cytosolic Ssa1p induced BiP(Kar2p) in the endoplasmic reticulum at the transcriptional level. By analyzing internal deletion mutants of the *KAR2* promoter, we determined that the heat shock element (HSE) is necessary for *KAR2* gene induction in response to the depletion of Ssa1p. Furthermore, either the *KAR2*HSE or *SSA1*HSE is sufficient for gene activation, as assayed using HSE-*CYC1-lacZ* fusion reporter plasmids. Finally, temperature-sensitive *ssa1* mutants transformed with an HSE-*CYC1-lacZ* fusion vector exhibited strong induction of β-galactosidase activity when shifted to a restrictive temperature. These results show that loss of functional Ssa1p from the cytosol up-regulates *KAR2* gene expression through an HSE-mediated pathway and also support the idea that *SSA1* gene expression is auto-regulated.

Key words: BiP, heat shock element, heat shock protein, Ssa1, transcriptional regulation.

HSP70s comprise a major family of constitutive and stress-inducible 70 kDa ATP-binding proteins, conserved from all prokaryotes to eukaryotes (*1*). In eukaryotes, members of this family are localized in various cellular compartments, and exhibit chaperone activities that facilitate protein translocation across the membrane, protein folding, and assembly. BiP is a member of the HSP70 family that is localized in the lumen of the endoplasmic reticulum (ER), and plays an important role in the translocation of nascent proteins across the ER membrane, and their subsequent folding and assembly in the ER lumen (*2*). In mammalian cells, BiP has been identified as glucose-regulated protein 78 (GRP78) (*3, 4*) or as a protein that constitutively binds to the immunoglobulin heavy chains of myeloma light chain nonproducers in the ER (*5, 6*). BiP also transiently associates with a variety of secretory and transmembrane proteins, thereby promoting their folding and assembly by stabilizing the prefolded nascent polypeptide structure (*2, 7*).

Mammalian BiP is induced at the transcriptional level by various types of environmental stress such as glucose starvation, blocking of protein glycosylation, intracellular Ca^{2+} disturbance, and addition of reducing agents; treatments which lead to the accumulation of unfolded proteins in the ER (*8, 35*). In *Saccharomyces cerevisiae*, BiP is encoded by the *KAR2* gene, a gene that is required for karyogamy, *i.e.* nuclear fusion during mating (*9-11*). Similar to mammalian BiP, yeast BiP is induced at the transcriptional level on the accumulation of unfolded proteins in the ER and further induced by heat shock. Recently, we cloned and analyzed a 1.3 kb segment of DNA that lies upstream of the *KAR2* gene and found that a 236 bp fragment (nucleotides -245 to -9) contains all the information required for accurate transcription of the *KAR2* gene (*12*). Sequential deletion analysis has further identified a *cis*-acting regulatory sequence in the promoter region of the *KAR2* gene (*12, 13*). The upstream promoter region of *KAR2* contains three distinct transcriptional regulatory elements (Fig. 4A): (i) an unfolded protein response element (UPRE), (ii) a functional heat shock element (HSE), and (iii) a GC-rich region required for normal high basal expression. These elements function independently and their effects are additive (*12*). UPRE is responsible for gene activation in response to the accumulation of mutant or unfolded proteins within the ER (*12, 13, 30*). Some sensory system, involving at least *ERN1/IRE1*,

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Abbreviations: HSE, heat shock element; UPRE, unfolded protein response element; SRP, signal recognition particle; ER, endoplasmic reticulum.

detects these abnormal proteins in the ER and transduces a signal(s) across the ER membrane to the nucleus (*14, 15*). HSE, which is absent from the mammalian *BiP* gene promoter, is responsible for gene expression induced by heat shock mediated through heat shock factor (HSF) (*16*). Intracellular signals generated by heat shock or other types of stress lead to the activation of HSF (*16*). As a result, transcription of the *KAR2* gene is regulated in response to stimuli originating in both the ER lumen and the cytosol. On the other hand, *SSA*, another member of the HSP70 family, localized predominantly in the cytosol, plays an important role in the translocation of proteins into the ER (*1, 17, 18*). Some secretory or transmembrane proteins require both Ssap and BiP in order to be translocated across the ER membrane efficiently. These data lead to the idea that gene expression of *SSA* and *KAR2* needs to be regulated cooperatively.

Four members of the yeast *SSA* gene, *SSA1-4*, form an essential subfamily (*19*). Yeast strains carrying disruptions in either *SSA1* or *SSA2* are phenotypically indistinguishable from the wild-type, however, an *ssa1* and *ssa2* double disruptant showed temperature-sensitive cell growth, while an *ssa1 ssa2 ssa4* triple disruptant was inviable (*19*). Ssa1p appears to regulate its own gene expression by itself; overexpression of Ssa1p blocked the enhancemet of *SSA1* promoter activity by heat shock (*20*), and an *ssa1 ssa2* double mutant activated the *SSA3* and *SSA4* gene promoters (*21, 22*). Similar to Ssa1p, the level of BiP in the ER is also regulated through self-regulation; high level expression of BiP mitigates the unfolded protein response (*12*) and a yeast BiP mutant lacking the C-terminal ER retention signal has been shown to up-regulate the concentration of BiP in the ER (*25*).

In this study, we examined whether or not the concentration of wild-type Ssa1p in the cytosol is involved in the regulation of BiP expression localized in another compartment, *viz.* the ER. The depletion or inactivation of Ssa1p in the cytosol leads to higher expression of BiP in the ER *via* transcriptional enhancement of the *KAR2* gene, and not by altering the translocation of BiP across the ER membrane. Analyses of the upstream elements of the *KAR2* gene showed that this induction is mediated by HSE, and not by either the UPRE or GC-rich region.

MATERIALS AND METHODS

Yeast Strains, Media, and Microbiological Techniques—The MW141 (*MATα his3-11,-15 leu2-3,-112 trp1 ura3-52 ssa1::HIS3 ssa2::LEU2 ssa4::URA3* [pGAL1-SSA1 (*TRP1/ARS/CEN4*)]) and DS10 (*MATa his3-11,-15 leu2-3,-112 trp1 lys2 ura3-52*) strains were obtained from E. Craig (University of Wisconsin-Madison). The MO10 strain (*MATα his3-11,-15 leu2-3,-112 trp1 ura3-52 ssa1::HIS3 ssa2::LEU2 ssa4::ura3* [pGAL1-SSA1 (*TRP1/ARS/CEN4*)]) was obtained by isolation of spontaneous 5-fluoroorotic acid-resistant cells derived from the MW141 strain. The MO10 strain is isogenic as to the MW141 strain except for uracil auxotrophy. The HFSA/AW (*MATα his3-11,-15 leu2-3,-112 lys2 trp1 ura3 ssa1::HIS3 ssa2::LEU2 ssa4::LYS2* [pGAP-SSA1 (*TRP1/ARS/CEN3*)]), HFSA/A11 (*MATα his3-11,-15 leu2-3, -112 lys2 trp1 ura3 ssa1::HIS3 ssa2::LEU2 ssa4::LYS2* [pGAP-SSA1A11 (*TRP1/ARS/CEN3*)]), and HFSA/A22 (*MATα his3-11,-15 leu2-3,-112 lys2 trp1 ura3 ssa1::HIS3 ssa2::LEU2 ssa4::LYS2* [pGAP-SSA1A22 (*TRP1/ARS/CEN3*)]) strains were obtained from M. Nakai and T. Endo (Nagoya University) (manuscript in preparation). The MS137 strain (*MATa leu2-3,-112 ura3-52 ade2-101, kar2-159*) was obtained from M. Rose (Princeton University). Standard yeast genetic techniques were performed as described (*26*). The media used in this study were as follows: Glucose-based rich medium (YPD), comprising 1% yeast extract (Difco Laboratories), 2% Bacto-Peptone (Difco), and 2% glucose; galactose-based rich medium (YPG), comprising 1% yeast extract, 2% Bacto-Peptone, and 2% galactose; synthetic complete galactose medium (SC-Gal), comprising 0.7% yeast nitrogen base without amino acids, 2% galactose, and appropriate amounts of amino acids and nucleic acid bases; and synthetic minimal medium (SD), comprising 0.7% yeast nitrogen base without amino acids and 2% glucose. Recombinant DNA manipulations were performed according to standard methods (*27*).

Plasmid Construction—Plasmids pKAR2HSE-*lacZ* and pSSA1HSE2-*lacZ* were constructed by inserting a synthetic 22 bp double-stranded oligonucleotide encoding the *KAR2*HSE or a 26 bp *SSA1*HSE2 into the *Xho*I site of pLGΔ-178, a 2μ-based multicopy yeast vector containing the *CYC1-lacZ* fusion gene and the *URA3* selectable marker, kindly provided by L. Guarente (Massachusetts Institute of Technology). Plasmids pUPR-*lacZ*, pZ621 (wild-type *KAR2* promoter), pZ631(-HSE), pZ641(-GC), and pZ651(-UPRE) were described by Mori *et al.* (*13*).

Enzyme Assays—Assaying of β-galactosidase activity in yeast extracts was carried out as described previously (*12*). β-Galactosidase was measured in units defined as $(A_{420} \times 1{,}000)/(A_{600} \times tv)$, where A_{420} (absorbance at 420 nm) is the reading of the final color, A_{600} the turbidity of the culture at the time of harvest, t the number of minutes for which the reaction mixture was incubated, and v the volume of the sample in milliliters. The values are averages of single determinations for three independent yeast transformants.

Northern Hybridization—Northern hybridization was performed using 10 μg of total cellular RNA isolated from yeast cells. RNA was extracted by the heat-freeze extraction method of Schmitt *et al.* (*28*). RNA was electrophoresed in 1% agarose-formamide gels, transferred to nitrocellulose membranes (Schleicher and Schuell), and then probed with a ^{32}P-labeled SSA1 fragment (0.8 kb *Eco*RI fragment) or *KAR2* fragment (1.1 kb *Eco*RI fragment).

Gel Electrophoresis and Immunoblotting—Cell extracts were prepared by the glass-bead lysis method of Jazwinski (*29*). Protein concentrations in the extracts were determined using a Bio-Rad assay kit (Bio-Rad). Cell extracts (1 μg) were subjected to electrophoresis in 8% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes (Schleicher and Schuell). Following transfer, the membranes were incubated with anti-Kar2p rabbit antibodies (1:1,000 dilution) (*30*) or anti-Ssa1p rabbit antibodies (1:1,000 dilution), kindly provided by Dr. T. Endo. Rabbit antibody binding to the blots was detected with an ECL Western blotting detection kit (Amersham).

RESULTS

Depletion of Ssa1p Induces KAR2 Gene Expression—To

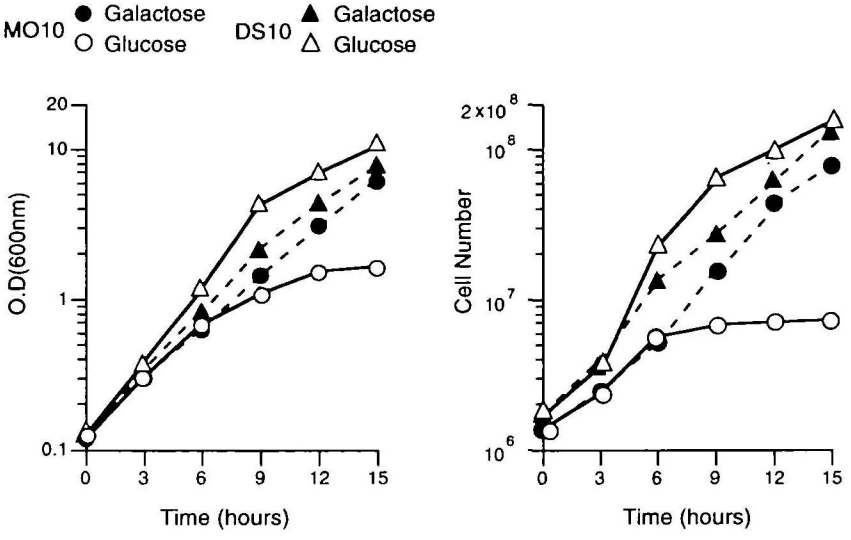

Fig. 1. **Growth of yeast strains MO10 and DS10.** Cells were grown to the log-phase in YPG, harvested, washed once with H_2O, and then transferred to fresh YPD or YPG medium. The optical density (600 nm) (left) and number of cells (right) were determined at the indicated times.

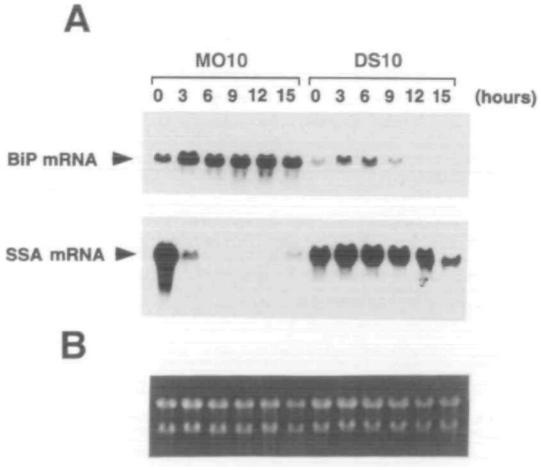

Fig. 2. **Effect of depletion of Ssa1p on the expression of BiP mRNA.** Cells were grown to the log-phase in YPG, harvested, washed once with H_2O, and then transferred to fresh YPD medium. RNA was obtained at the indicated times. Electrophoresis of denatured RNA (10 μg) was carried out in a 1% gel containing formaldehyde. (A) RNA was blotted on to a nitrocellulose membrane and then hybridized using a ^{32}P-labeled BiP or SSA1 probe. (B) Ethidium bromide staining of ribosomal RNA in the gel prior to transfer.

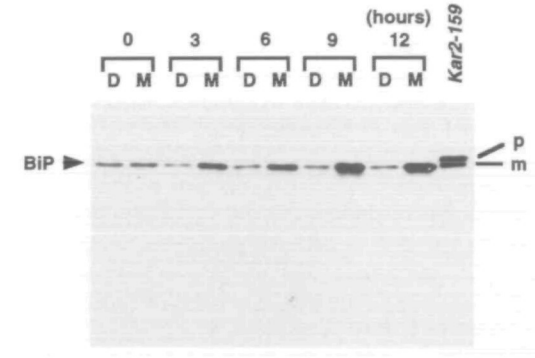

Fig. 3. **Induction of the mature form of BiP after depletion of Ssa1p.** Cells were harvested at the indicated times after shifting from YPG to YPD medium, and then whole extracts were prepared by the glass-bead lysis method (Jazwinski, 1990). Protein concentrations were measured, and an equal portion of each cell extract (1 μg) was loaded on an 8% SDS-polyacrylamide gel, electrophoresed, transferred to a nitrocellulose membrane, and then immunoblotted with rabbit anti-Kar2p or rabbit anti-Ssa1p antibodies. Bound IgG was visualized using a chemiluminescence detection kit as described under "MATERIALS AND METHODS." A cell lysate of *kar2-159* was prepared at 2 h after the temperature shift to 37°C. D: DS10 cells; M: MO10 cells; p: precursor form; m: mature form.

determine whether or not Ssa1p is involved in the regulation of *KAR2* gene expression, we manipulated the intracellular level of Ssa1p in the MO10 strain, which is isogenic as to the MW141 strain established by E.A. Craig (*17, 19*), except for uracil auxotrophy (see "MATERIALS AND METHODS"). The MO10 strain harbors chromosomal disruptions of the *SSA1*, *SSA2*, and *SSA4* genes, and is rescued by the presence of a centromeric plasmid carrying the *SSA1* gene under regulation by the *GAL1* promoter (*17, 19*). MO10 cells grown in galactose-based medium overproduced Ssa1p several fold, however, the *GAL1* promoter was extremely repressed after a shift to glucose-based medium (*17*). As shown in Fig. 1, when cells were transferred from galactose medium to glucose medium, the number of MO10 cells stopped increasing after 6 h. However, the absorbance (600 nm) continued to gradually increase for another 6 h since the cells became about 2- to 4-fold the size of logarithmically-growing cells. As expected, transcription of the *SSA1* mRNA was strongly repressed after MO10 cells had been transferred to glucose medium (Fig. 2). Consistent with this reduction in *SSA1* mRNA, the Ssa1p level in MO10 cells decreased after 3 h incubation in glucose medium and was further reduced to a half or third compared with that of the parental wild-type DS10 cells by 6 h (data not shown). In contrast, BiP mRNA was induced as soon as 3 h after the initiation of *SSA1* gene repression. Induction of the BiP mRNA appears to continue for at least 15 h, whereas BiP mRNA in wild-type DS10 cells was only slightly induced on growth in glucose medium, soon returning to a normal level (Fig. 2). Figure 3 shows that the BiP protein in MO10 cells was significantly induced with time. At 3 h following the shift to the repressive condition, the BiP protein level had increased by 2-fold, as measured by

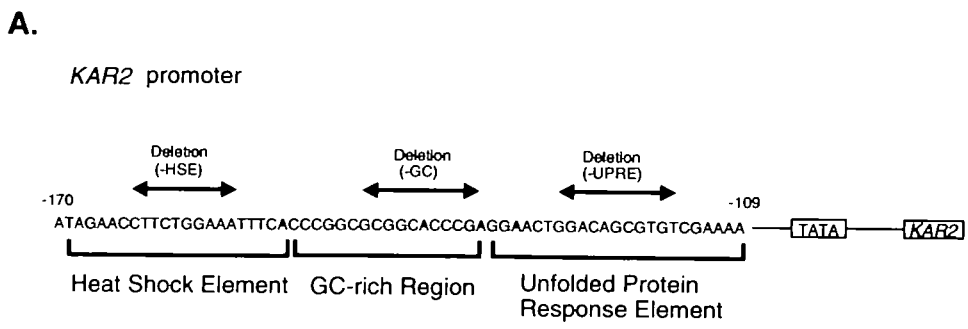

Fig. 4. **Transcriptional regulatory sequence of the *KAR2* gene.** (A) The *KAR2* promoter sequence. The arrows indicate sequences deleted in order to disrupt the HSE, GC-rich region, and UPRE, respectively. TATA and *KAR2* indicate the two TATA boxes and the *KAR2* coding region, respectively. (B) Comparison of the HSEs used in this study. GAA modules are indicated by closed squares.

Western blotting, and continued to increase up to about 4- or 5-fold after 9 h incubation. Since Ssa1p plays an important role in protein translocation across the ER membrane, it was necessary to determine whether or not the induced BiP is correctly processed into the mature form after translocation. A temperature-sensitive *kar2* mutant (strain *kar2-159*) was observed to accumulate the precursor form of BiP at the semi-permissive temperature of 30°C (Fig. 3) (*24*). In MO10 cells, however, BiP induced by switching the cells to glucose medium was processed into the mature form and no precursor form of BiP was detected. This indicates that induction of BiP in MO10 cells is not the result of a decrease of BiP in the ER caused by a defect in its translocation to the ER lumen.

Analysis of the KAR2 Promoter—To determine which region of the *KAR2* promoter is responsible for the induction caused by the depletion of Ssa1p, internal deletion analysis of the promoter was performed. The *KAR2* promoter can be divided into three functionally distinct regions, as shown in Fig. 4, the HSE, UPRE, and GC-rich regions (see "Introduction"). The plasmids (Z621, Z631, Z641, and Z651) in Fig. 5B harbor the wild-type and three different deleted versions of the *KAR2* promoter, respectively, with the *E. coli lacZ* gene fused to their downstream regions (Fig. 4A). These reporter plasmids were introduced individually into MO10 cells and then the β-galactosidase activity of each transfectant was measured 5 h after transfer to glucose medium. As shown in Fig. 5A, the induction of β-galactosidase activity was detected in cells transformed with Z621, which contains the intact *KAR2* promoter fused to the *lacZ* gene. However, no induction was observed in cells transformed with Z631(-HSE), in which only the HSE is deleted, despite the depletion of intracellular Ssa1p. The activity of Z631 in glucose medium was one-third of that in galactose medium (Fig. 5A). On the other hand, β-galactosidase activity in cells transformed with Z641(-GC) or Z651(-UPRE) was induced about 1.6- to 2-fold concomitant with the depletion of Ssa1p. This degree of activation was found to be similar to that in cells transformed with Z621. These results suggest that the promoter region containing the HSE plays a very important role in the induction of BiP when cytosolic Ssa1p is depleted.

To determine whether or not HSE is the sole element

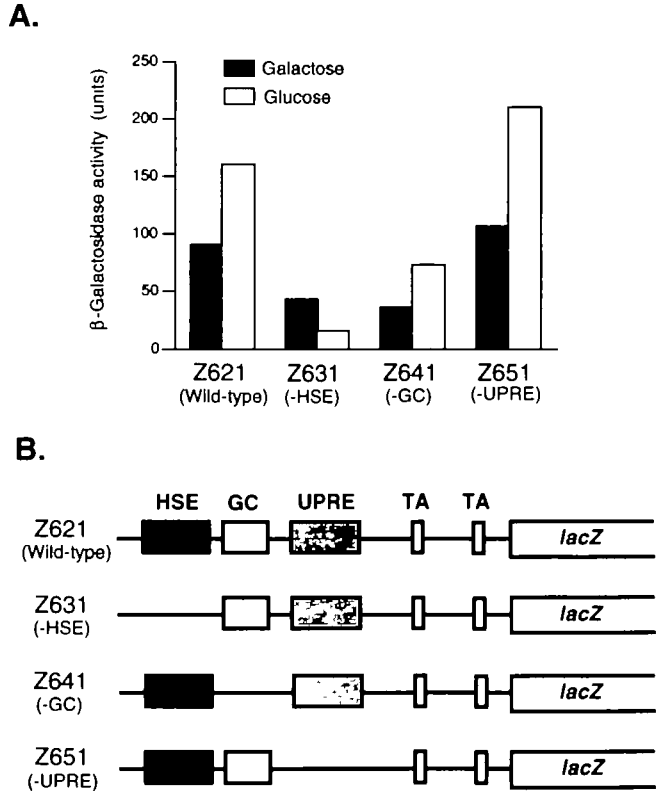

Fig. 5. **β-Galactosidase activities of cells transformed with internally-deleted promoter derivatives of *KAR2/lacZ* fusion plasmids.** MO10 cells were transformed with plasmids containing fusion genes in which the wild-type or mutant versions of the *KAR2* promoter were fused to the coding sequences of the *E. coli lacZ* gene. (A) β-Galactosidase activities of transformants growing exponentially in SC-Gal medium without uracil following 5 h culture in YPD or YPG are presented. (B) Diagrams of the promoters used in this study. Z621 contains the intact *KAR2* promoter fused to the upstream region of *lacZ*. Z631(-HSE), Z641(-GC), and Z651(-UPRE) contain an internally-deleted *KAR2* promoter. The sequences deleted in the constructs in order to disrupt the HSE, GC-rich region, and UPRE, respectively, are indicated in Fig. 4A by arrows.

responsible for the induction of *KAR2* gene expression, we inserted oligonucleotide sequences corresponding to the

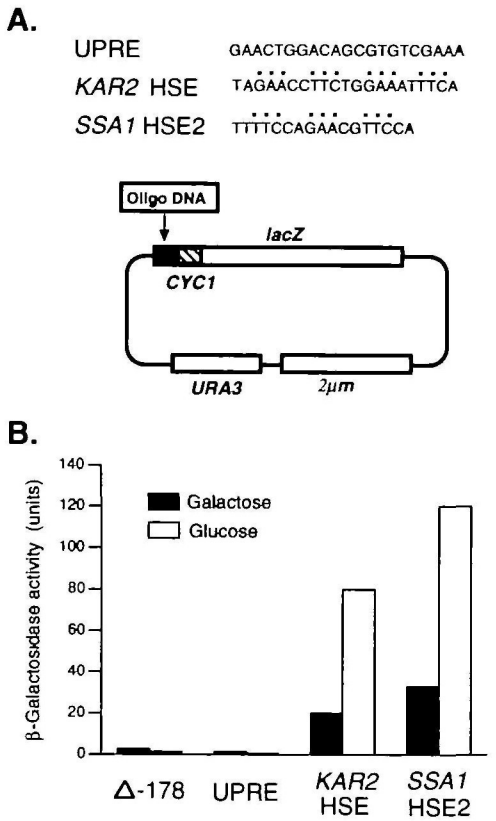

Fig. 6. **β-Galactosidase activities of cells transformed with UPRE/*lacZ* or HSE/*lacZ* fusion plasmids.** MO10 cells were transformed with plasmids containing fusion genes in which each promoter element sequence was inserted in the promoter of an *E. coli lacZ* reporter gene. (A) The DNA sequences of the UPRE, *KAR2*-HSE, and *SSA1*HSE2 oligonucleotides, and a diagram of the reporter plasmid are shown. The elements were cloned into the *Xho*I site of a *CYC1-lacZ* fusion vector (pLGΔ-178). (B) Cells were transformed with the pLGΔ-178 (control plasmid), UPRE-*lacZ*, *KAR2*HSE-*lacZ*, or *SSA1*HSE2-*lacZ* plasmid, respectively. β-Galactosidase activities of transformants growing exponentially in SC-Gal medium without uracil following 5 h culture in YPD or YPG are presented.

HSE or UPRE into the upstream region of the heterologous promoter, *CYC1*, fused to the *E. coli lacZ* gene (plasmid Δ-178) (Fig. 6). As expected, cells carrying the plasmid containing *KAR2*HSE exhibited about 4-fold induction of β-galactosidase activity in glucose medium compared with that in galactose medium, while cells transformed with control plasmid Δ-178 (null insertion) or a plasmid with UPRE sequences inserted upstream showed little β-galactosidase activity in any medium used. Since the sequences of HSEs are very conserved among different promoters, we further examined whether or not *SSA1*-HSE2 is as effective as the *KAR2*HSE in activating the promoter in response to the depletion of Ssa1p. Cells transformed with this plasmid also exhibited an about 4-fold increase in activity in glucose medium compared to in galactose medium (Fig. 6). These results support the idea that the intracellular level of Ssa1p regulates the gene expression of *KAR2* and *SSA1* itself through an HSE-mediated pathway.

Analysis of Temperature-Sensitive SSA1 Strains—It takes several hours for Ssa1p to be depleted in MO10 cells after a shift to glucose medium (Fig. 1, data not shown). To determine how depletion of Ssa1p caused the induction of the *SSA1* and *KAR2* genes more promptly and directly, we utilized temperature-sensitive *ssa1* mutant strains. Strain AW (wild-type *SSA1*) harboring chromosomal disruptions of the *SSA1*, *SSA2*, and *SSA4* genes is rescued by a centromeric plasmid carrying the wild-type *SSA1* gene under the control of the *GAP* (glyceraldehyde-3-phosphate dehydrogenase) promoter, and strains A11 and A22 have different temperature-sensitive *ssa1* genes constructed by plasmid shuffling of the wild-type *SSA1* (M. Nakai and T. Endoh, manuscript in preparation; M. Oka *et al.*, unpublished data). These cells were transformed with an *SSA1*-HSE2-*lacZ* reporter plasmid and then β-galactosidase activity was assayed (Fig. 7). After the temperature had been shifted to 30°C for 3 h, the β-galactosidase activities of wild-type AW cells and the two temperature-sensitive mutants were not very different. In contrast, the β-galactosidase activity of A22 cells increased about 4-fold after a shift to 37°C for 30 min, *i.e.*, a nonpermissive temperature for A22 cells (data not shown). When AW cells were similarly shifted to 37°C, to induce the expression of β-galactosidase by heat shock after 3 h, A22 cells still exhibited 6-fold stronger β-galactosidase activity than AW cells (Fig. 7). We obtained similar results for A11 cells (Fig. 7). These results clearly show that the concentration of functional Ssa1p in the cytosol affects the expression of the *SSA1* and *KAR2* genes.

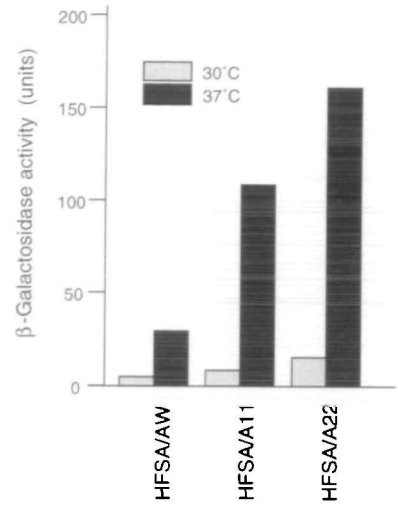

Fig. 7. **β-Galactosidase activities of temperature-sensitive *ssa1* cells.** *ssa1* mutant cells (HFSA/A22 and HFSA/A11 strains) and wild-type control cells (HFSA/AW strain) were transformed with an *SSA1*HSE2-*lacZ* fusion plasmid. β-Galactosidase activities were measured in transformants grown exponentially in SD at 23°C followed by 3 h incubation at 30 or 37°C.

DISCUSSION

Expression of yeast BiP is relatively high in the ER under normal cell growth conditions and is further induced under the following stress conditions: (i) treatment of cells with inhibitors of protein glycosylation, such as tunicamycin (*9, 10*); (ii) incubation of temperature-sensitive *sec* mutants at a nonpermissive temperature leading to the accumula-

tion of unfolded proteins or precursors of secretory proteins in the ER (*9, 10*); (iii) overexpression in wild-type cells of an aberrant heterologous protein constructed from mouse α-amylase fused to yeast killer prepro-signal peptide (*30*); and (iv) heat shock treatment (*9, 10, 12*). All the stimuli described above induce BiP at the transcriptional level *via* specific transcription elements. The former three types of stress cause the accumulation of unfolded or prefolded proteins in the ER, and this accumulation in turn triggers the induction of *KAR2* gene transcription *via* the UPRE, a 22 bp *cis*-acting element located in the upstream region of the *KAR2* promoter. The last is different from the former since induction is very rapid, transitional, and mediated through a canonical HSE.

Here we demonstrate another stimulus which induces BiP: (v) depletion or inactivation of the functional cytosolic HSP70/SSA subfamily. This induction was not merely due to cessation of cell growth, since BiP induction had been already detected at both the mRNA and protein levels (Figs. 2A and 3) when MO10 cells were still actively growing after 3 h incubation following a shift to glucose medium, as shown in Fig. 1. Induction of BiP in this case seems to be different from the usual heat shock response, because BiP mRNA induction occurred more slowly than in the case of the heat shock response and it continued to be expressed at high levels as long as Ssa1p was depleted (*12*). The BiP protein accumulated with time similar to in the case of the unfolded protein response (Figs. 2 and 3). There are two possible explanations for these phenomena. First, depletion or inactivation of Ssa1p in the cytosol may cause a defect in the translocation of BiP or other lumenal chaperones. Ssa1p is known to facilitate the posttranslational translocation of some secretory proteins in yeast, and its absence could result in a decrease in the amount of functional BiP or other chaperones in the ER, leading to the accumulation of unfolded proteins in the ER. This would trigger the unfolded protein response pathway whose induction is mediated by the UPRE. Second, there is an auto-regulatory system which adjusts the levels of Ssa1p and Kar2p to maintain the normal cellular function. Our data here support the second idea but not the first one. BiP induced by Ssa1 depletion was precisely processed to the mature form (Fig. 3). During preparation of this manuscript, Becker *et al.* reported that the translocation of BiP across the ER membrane was not affected in a temperature-sensitive *ssa1* mutant (*ssa1*^{ts} *ssa2 ssa3 ssa4*) growing at a nonpermissive temperature (*36*). This indicates that BiP could be translocated across the ER membrane even if all activities of Ssap were lost. Ng *et al.* reported that the mode of translocation of yeast secretory proteins across the ER membrane falls into three classes (*31*): those translocated through the SRP (signal recognition particle)-dependent pathway, the SRP-independent pathway, and both pathways. Since BiP can be translocated across the ER membrane *via* both pathways, it can use the SRP-dependent pathway even if the SRP-independent pathway was completely blocked (*31*). Further analysis of the *KAR2* promoter revealed that induction of BiP specifically depends on the HSE, but not the UPRE or any other element in the *KAR2* promoter (Figs. 5 and 6). Consequently, we conclude that Ssa1p is, directly or indirectly, involved in the activation of the *KAR2* gene and of itself through the HSE.

Craig and coworkers reported that in an *ssa1 ssa2* double mutant strain, Ssa3p and Ssa4p are highly induced, presumably to maintain the normal intracellular levels of Ssap subfamily proteins, and that this regulation is mediated by the HSE (*21, 22*). A similar induction phenotype has been observed in cells in which Srp54p, a component of SRP, is depleted resulting in the blocking of protein translocation *via* the SRP-dependent pathway. Depletion of Srp54p induces various heat shock proteins, including Ssap, Ydj1p, BiP, Hsp82p, and Hsp104p (*32*). Furthermore, we previously reported that SSA mRNA is greatly induced in a temperature-sensitive *sec62* strain incubated at a semipermissive temperature for 2 h (*9*), due to severe defects in the translocation of several secretory proteins, including alpha factor and carboxypeptidase Y (*33*). The common features of these various types of cytosolic stress, namely, (i) a decreased level of functional Ssap, (ii) depletion of Srp54, and (iii) inactivation of Sec62p, is the accumulation of untranslocated secretory proteins in the cytosol. We therefore speculate that this might be a primary signal for Ssap induction, and a mechanism by which the intracellular Ssa1p level can be critically monitored and regulated through the HSE-mediated pathway. In this study, both the *KAR2*HSE and *SSA1*HSE2 were activated similarly in response to the depletion of Ssa1p (Fig. 6). This may indicate that there is similarity in function between the *KAR2*HSE and *SSA1*HSE2 *in vivo*. It is not clear whether or not heat shock treatment and depletion of Ssa1p act *via* the same mechanism to activate HSE-mediated gene expression, but it is possible that the accumulation of aberrant proteins as a result of heat shock stress might sequester free Ssa1p in the cytosolic pool, causing the depletion of intracellular free Ssa1p, in analogy to the mammalian heat shock response (*23, 34*).

In conclusion, loss of functional Ssa1p alone, in the absence of heat shock, is sufficient for activation of the *SSA1* and *KAR2* genes through an HSE-mediated pathway. Thus, HSE may play an important role as the control element regulating the proper expression of functional cytosolic HSP70 and other heat shock proteins, including BiP. This pathway may function in parallel with one mediated by the UPRE, which controls the expression of BiP in response to the accumulation of unfolded proteins in the ER lumen.

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REFERENCES

1. Craig, E.A., Baxter, B.K., Becker, J., Halladay, J., and Ziegelhoffer, T. (1994) Cytosolic hsp70s of *Saccharomyces cerevisiae*: Roles in protein synthesis, protein translocation, proteolysis, and regulation in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R.I., Tissières, A., and Georgopoulos, C., eds.) pp. 31-52, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Gething, M.J. and Sambrook, J. (1992) Protein folding in the cell. *Nature* **355**, 33-45
3. Pouyssegur, J., Shiu, R.P.C., and Pastan, I. (1977) Induction of two transformation-sensitive membrane polypeptides in normal fibroblasts by a block in glycoprotein synthesis or glucose

deprivation. *Cell* **11**, 941-947
4. Shiu, R.P.C., Pouyssegur, J., and Pastan, I. (1977) Glucose deprivation accounts for the induction of two transformation-sensitive proteins in Rous sarcoma virus-transformed chick embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **74**, 3840-3844
5. Haas, I.G. and Wabl, M. (1983) Immunoglobulin heavy chain binding protein. *Nature* **306**, 387-389
6. Morrison, S.L. and Scharff, M.D. (1976) Heavy chain-producing variants of a mouse myeloma cell line. *J. Immunol.* **114**, 655-659
7. Gething, M.J., McCammon, K., and Sambrook, J. (1986) Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell* **46**, 939-950
8. Lee, A.S. (1987) Coordinated regulation of a set of genes by glucose and calcium ionophores in mammalian cells. *Trends Biochem. Sci.* **12**, 20-23
9. Normington, K., Kohno, K., Kozutsumi, Y., Gething, M.J., and Sambrook, J. (1989) *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell* **57**, 1223-1236
10. Rose, M.D., Misra, L.M., and Vogel, J.P. (1989) *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell* **57**, 1211-1221
11. Nicholson, R.C., Williams, D.B., and Moran, L.A. (1990) An essential member of the HSP70 gene family of *Saccharomyces cerevisiae* is homologous to immunoglobulin heavy chain binding protein. *Proc. Natl. Acad. Sci. USA* **87**, 1159-1163
12. Kohno, K., Normington, K., Sambrook, J., Gething, M.J., and Mori, K. (1993) The promoter region of the yeast *KAR2* (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. *Mol. Cell. Biol.* **13**, 877-890
13. 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
14. 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
15. 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
16. Fernandes, M., O'Brien, T., and Lis, J.T. (1994) Structure and regulation of heat shock gene promoters in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R.I., Tissières, A., and Georgopoulos, C., eds.) pp. 375-393, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
17. Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A., and Schekman, R. (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* **332**, 800-805
18. Chirico, W., Waters, M., and Blobel, G. (1988) 70K heat-shock related proteins stimulated protein translocation into microsomes. *Nature* **322**, 805-810
19. Werner-Washburne, M., Stone, D.E., and Craig, E.A. (1987) Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**, 2568-2577
20. Stone, D.E. and Craig, E.A. (1990) Self-regulation of 70-kilodalton heat shock proteins in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 1622-1632
21. Boorstein, W.R. and Craig, E.A. (1990) Transcriptional regulation of *SSA3*, an HSP70 gene from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 3262-3267
22. Boorstein, W.R. and Craig, E.A. (1990) Structure and regulation of the SSA4 HSP70 gene of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**, 18912-18921
23. Morimoto, R.I. (1993) Cells in stress: Transcriptional activation of heat shock genes. *Science* **259**, 1409-1410
24. Vogel, J.P., Misra, L.M., and Rose, M.D. (1990) Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. *J. Cell Biol.* **110**, 1885-1895
25. Hardwick, K.G., Lewis, M.J., Semenza, J., Dean, N., and Pelham, H.R. (1990) *ERD1*, a yeast gene required for the retention of luminal endoplasmic reticulum proteins, affects glycoprotein processing in the Golgi apparatus. *EMBO J.* **9**, 623-630
26. Rose, M.D., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28. Schmitt, M.E., Brown, T.A., and Trumpower, B.L. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **18**, 3091-3092
29. Jazwinski, S.M. (1990) Preparation of extracts from yeast. *Methods Enzymol.* **182**, 154-174
30. Tokunaga, M., Kawamura, A., and Kohno, K. (1992) Purification and characterization of BiP/Kar2 protein from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **267**, 17553-17559
31. Ng, D.T., Brown, D., and Walter, P. (1996) Signal sequences specify the targeting route to the endoplasmic reticulum membrane. *J. Cell Biol.* **134**, 269-278
32. Arnold, C.E. and Wittrup, K.D. (1994) The stress response to loss of signal recognition particle function in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **269**, 30412-30418
33. Rothblatt, J.A., Deshaies, R.J., Sanders, S.L., Daum, G., and Schekman, R. (1989) Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* **109**, 2641-2652
34. Baler, R., Welch, W.J., and Voellmy, R. (1992) Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *J. Cell Biol.* **117**, 1151-1159
35. Kozutsumi, Y., Segal, M., Normington, K., Gething, M.J., and Sambrook, J. (1988) The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature* **332**, 462-464
36. Becker, J., Walter, W., Yan, W., and Craig, E.A. (1996) Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation in vivo. *Mol. Cell. Biol.* **16**, 4378-4386