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A 22 bp *cis*-acting element is necessary and sufficient for the induction of the yeast *KAR2* (BiP) gene by unfolded proteins

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The *KAR2* gene of *Saccharomyces cerevisiae* codes for an essential chaperone protein (BiP) that is localized in the lumen of the endoplasmic reticulum (ER). The high basal rate of transcription of *KAR2* is increased transiently by heat shock: prolonged induction occurs when unfolded proteins accumulate in the ER. Three *cis*-acting elements in the *KAR2* promoter control expression of *KAR2*: (i) a GC-rich region that contributes to the high level of constitutive expression, (ii) a functional heat shock element (HSE) and (iii) an element (UPR) that is involved in the induction of BiP mRNA by unfolded proteins. By analyzing internal deletion mutants of the *KAR2* promoter, we demonstrate here that these three elements regulate transcription of *KAR2* independently. Furthermore, the 22 bp UPR element causes a heterologous (*CYC1*) promoter to respond to the presence of unfolded proteins in the ER. Extracts of both stressed and unstressed yeast cells contain proteins that bind specifically to synthetic HSE and UPR elements and retard their migration through gels. Binding proteins specific for the UPR element can be fractionated by ammonium sulfate precipitation. Two of the proteins UPRF-1 and UPRF-2 (which is apparently a proteolytic degradation product of UPRF-1) bind inefficiently to mutant versions of the UPR that are unable to confer responsiveness to unfolded proteins to the (*CYC1*) promoter. UPRF-1 therefore displays the properties expected of a transcription factor that is involved in the sustained response of the *KAR2* promoter to unfolded proteins in the ER. These experiments show that yeast cells can activate a transcription factor that stimulates expression of a nuclear gene in response to the accumulation of unfolded proteins in another cellular compartment.

Key words: BiP/heat shock/HSE/transcription factors/UPR

Introduction

Proteins of the stress-70 family are abundant components of virtually all eukaryotic cells (for a review see Gething and Sambrook, 1992). Typically, an organism contains several stress-70 proteins, which are highly homologous in sequence but which are localized in different cellular compartments. The genes encoding these proteins are regulated at the transcriptional level and are induced by many forms of environmental stress—for example, heat shock, anoxia, exposure to cold, glucose starvation, infection with viruses and treatment with amino acid analogs for example (for reviews see Nover 1984; Craig, 1985; Neidhardt *et al.*, 1985; Lindquist and Craig, 1988; Morimoto *et al.*, 1990). The denominator common to all of these diverse stimuli is believed to be the accumulation of unfolded proteins in particular intracellular compartments (Kozutsumi *et al.*, 1988; Nakaki *et al.*, 1989). However, the mechanism(s) that senses the presence of unfolded proteins and stimulates transcription of the appropriate stress-70 gene(s) has not yet been defined.

BiP, which comprises 5-10% of the luminal protein of the ER of eukaryotic cells, is a member of the stress-70 family and, at least in mammalian cells, is believed to play a role in the folding and assembly of proteins that are translocated across the membrane of the ER. These include proteins that are resident in the secretory pathway as well as newly synthesized secretory and cell-surface proteins (reviewed by Gething and Sambrook, 1992). Mammalian BiP, which is also named GRP78, was first identified together with GRP94, another abundant protein of the ER, as a protein whose synthesis is stimulated by glucose starvation (Shiu *et al.*, 1977). Subsequently, both BiP and GRP94 proteins were shown to be expressed constitutively under normal conditions of growth and induced by a wide variety of stress conditions (reviewed by Lee, 1987) that cause the accumulation of unfolded proteins in the ER (Kozutsumi *et al.*, 1988).

Lee and co-workers have cloned and sequenced the promoters of BiP and GRP94 genes from a number of different mammalian species. By extensive deletion analysis and DNA footprinting, they have identified highly conserved upstream regulatory domains that are required for stress-induced stimulation of transcription. The mammalian GRP94 and BiP genes appear to be co-ordinately regulated by the binding of *trans*-acting factors to these conserved regulatory domains (Resendez *et al.*, 1988; Chang *et al.* 1989).

The BiP protein of the yeast *Saccharomyces cerevisiae* is encoded by the essential *KAR2* gene (Normington *et al.*, 1989; Rose *et al.*, 1989; Nicholson *et al.*, 1990). The protein is located in the lumen of the ER, where one of its functions is to maintain the translocational machinery in a functional state (Vogel *et al.*, 1990; Nguyen *et al.*, 1991). Unlike its mammalian homolog, yeast BiP has not yet been shown to interact directly with unfolded, newly synthesized secretory proteins. However, its synthesis, like that of mammalian BiP, is stimulated by the accumulation of unfolded proteins in the ER (Normington *et al.*, 1989; Rose *et al.*, 1989). Transcription of *KAR2* is also stimulated, albeit

transiently, by heat shock (Normington *et al.*, 1989; Rose *et al.*, 1989). This is the only significant difference between the regulation of the yeast gene and its mammalian counterparts, which are insensitive to heat shock.

To analyze the elements in the *KAR2* promoter that respond to unfolded proteins and heat shock, we have recently cloned and sequenced a 1.3 kb segment of DNA that lies immediately upstream of the BiP coding region (K.Kohno, K.Normington, J.F.Sambrook, M.J.Gething and K.Mori, submitted). A set of unidirectional deletion mutants was then generated lacking progressively longer segments of the upstream sequences. By fusing these truncated promoters to a segment of DNA encoding *Escherichia coli* β-galactosidase, we showed that a 236 bp fragment contains all of the information required for accurate transcription of the yeast *KAR2* gene. Analysis of a series of 5′-deletions of this fragment revealed three separate elements involved in the control of transcription of *KAR2*. A 20 bp consensus heat shock element (HSE) located between positions −168 to −149 is required for the transient increase in transcrip-

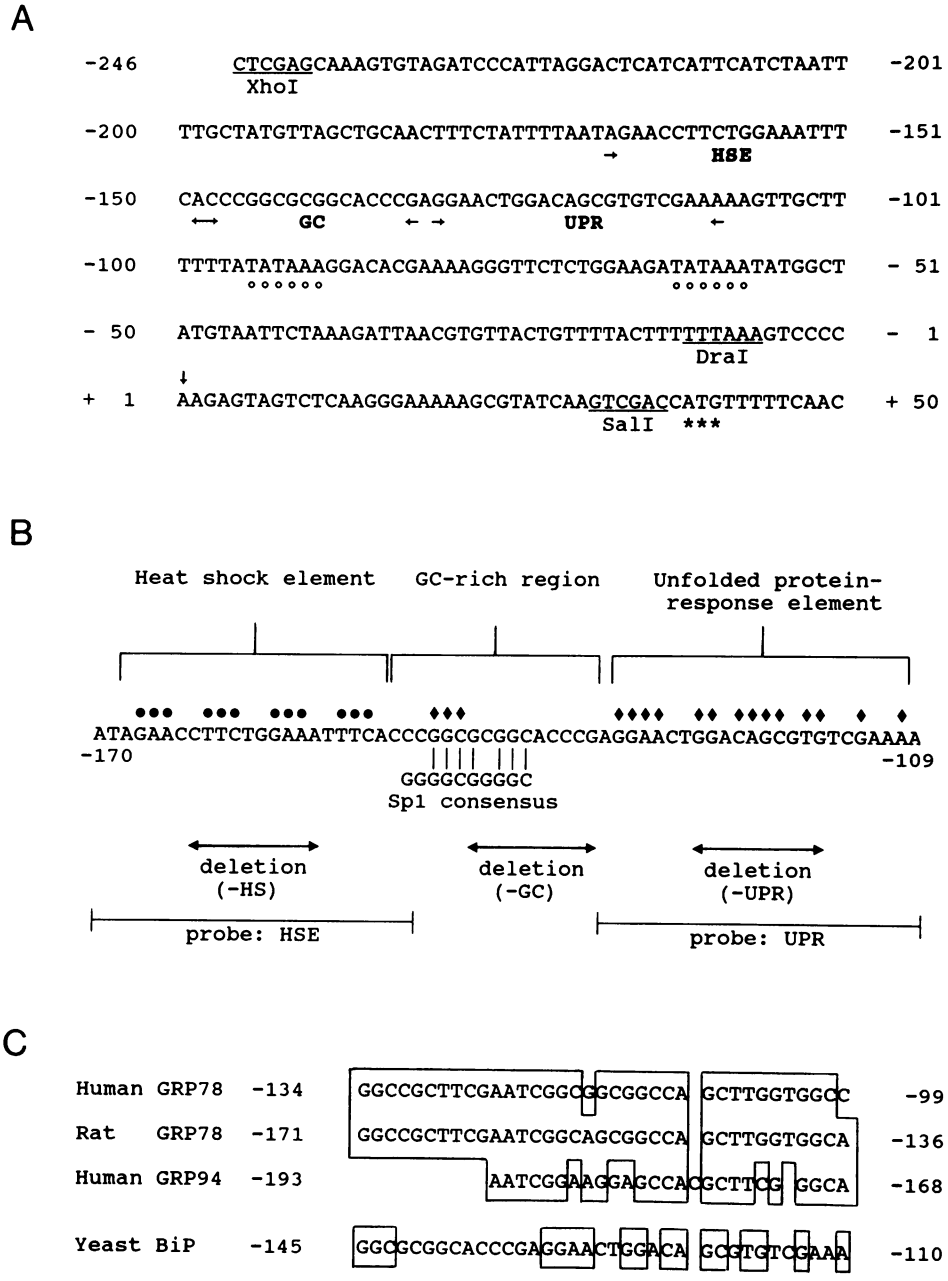

Fig. 1. (**A**) The sequence of the *KAR2* promoter. The *Xho*I−*Sal*I fragment contains all the information required for accurate transcription of the yeast *KAR2* gene, i.e. two TATA boxes (open circles), a heat shock element (HSE), a GC-rich region (GC) and an unfolded protein-response element (UPR). The arrow and asterisks indicate the start sites for transcription and translation, respectively. (**B**) Design of mutant promoters. Closed circles in the heat shock element mark the position of the 20 bp consensus sequence composed of (-GAA- or -TTC-) defined by Lis *et al.* (1990). The consensus sequence for binding of the transcription factor Sp1 (Kadonaga *et al.*, 1986) is aligned with the sequence of the GC-rich region. Closed diamonds in the unfolded protein-response element show the conserved nucleotides in the promoters of yeast BiP and mammalian BiP (GRP78) and GRP94. The locations of the deletions that were used to generate mutant promoters and the sequences of synthetic oligonucleotide probes used for the DNA mobility shift assays (Figures 6 and 7) are indicated by lines. (**C**) Comparison of UPRs. The boxed region displays sequences shared between the yeast *KAR2* promoter and the promoters of the mammalian genes encoding GRP78 and GRP94 (Chang *et al.*, 1989). Each sequence is numbered relative to its own start site for transcription.

tion on *KAR2* that follows exposure of yeast cells to elevated temperatures. A 22 bp, *cis*-acting, regulatory element located between −131 and −110 is involved in the sustained response of *KAR2* to the presence of unfolded proteins in the ER. The element (named UPR) is homologous to the highly conserved sequences present in the promoters of the mammalian BiP and GRP94 genes. Between these two elements lies a GC-rich region (−148 to −133), which appears to contribute the high level of constitutive expression of *KAR2* (K.Kohno, K.Normington, J.F.Sambrook, M.J.Gething and K.Mori, submitted).

In this paper we have used mutants that lack 10 nucleotide segments internal to each regulatory element to show that these *cis*-acting sequences work independently of one another. Furthermore, we show that (i) the 22 bp UPR element is sufficient to confer responsiveness to unfolded proteins on a heterologous promoter, (ii) the HSE and UPR elements each specifically bind distinct sets of proteins, which are present in extracts of both unstressed and stressed yeast cells and (iii) mutant UPR elements that are unable to respond to unfolded proteins *in vivo* bind proteins inefficiently *in vitro*. These findings indicate that a critical step in the induction of BiP mRNA by different forms of stress is selective activation of the appropriate transcription factor.

Results

Comparison of promoter elements

Figure 1A shows the sequence of the promoter region containing three separate elements that control transcription of the yeast *KAR2* gene (K.Kohno, K.Normington, J.F.Sambrook, M.J.Gething and K.Mori, submitted). Figure 1B shows the location of (i) a functional 20 bp heat shock element (HSE, nucleotides −168 to −149) consisting of an array of four 5 bp modular units, each of which conforms to the consensus sequences [-GAA- or -TTC-] defined by Lis *et al.* (1990), (ii) a tract of 16 nucleotides (−148 to −133), 15 of which are G or C residues, that contributes strongly to the high level of constitutive expression of *KAR2* [seven of ten contiguous nucleotides in this region are identical to the consensus sequence for binding of the transcription factor Sp1 (Kadonaga *et al.*, 1986] and (iii) a 22 bp sequence (nucleotides −131 to −110) that is homologous to sequences found in the promoters of the mammalian glucose-regulated genes, GRP78 (BiP) and GRP94 (Figure 1C). These conserved mammalian sequences are important for the induction of BiP and GRP94 that occurs when cells are exposed to calcium ionophores—a treatment that has been postulated to trigger the accumulation of unfolded proteins in the ER (Resendez *et al.*, 1988; Chang *et al.*, 1989). Nucleotides −131 to −110 of the yeast *KAR2* promoter, which are located in the region previously shown to respond to an accumulation of unfolded proteins in the yeast ER (K.Kohno, K.Normington, J.F.Sambrook, M.J.Gething and K.Mori, submitted), show 60% homology to these mammalian conserved sequences. For the remainder of this paper, we refer to this element of the yeast *KAR2* promoter as the UPR (Unfolded Protein-Response element).

Design of promoter mutants

To evaluate the contributions of the three conserved elements to basal and stress-induced expression of the yeast *KAR2*

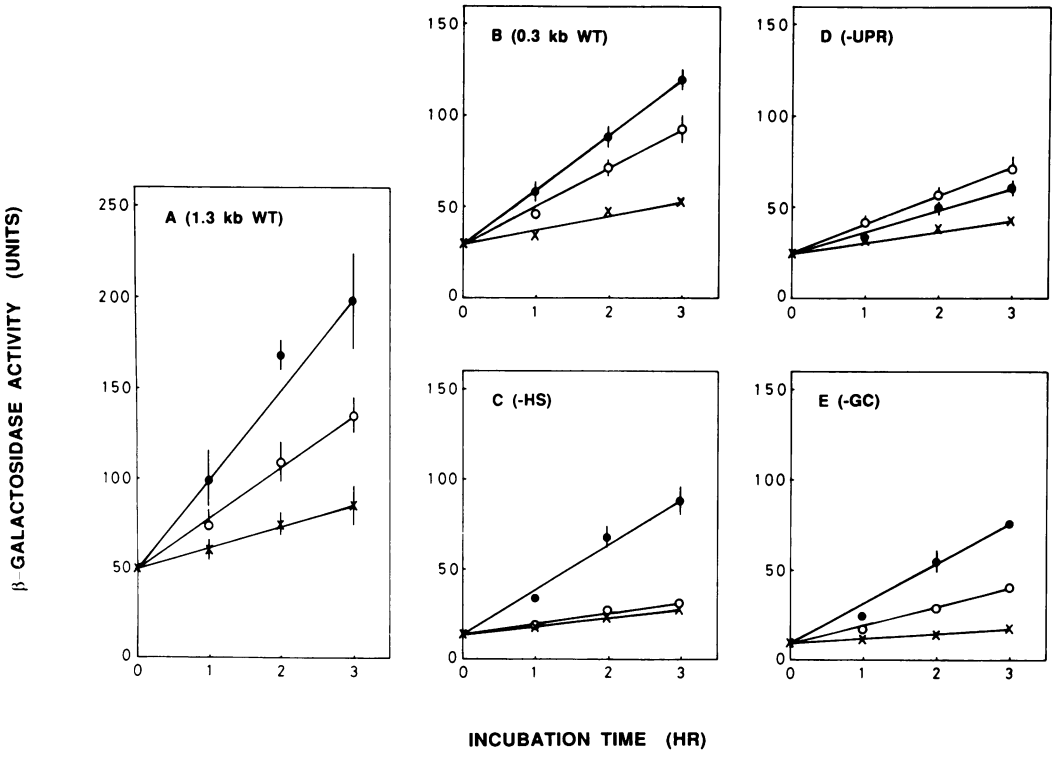

Fig. 2. Expression of β-galactosidase activity from wild-type and mutant *KAR2* promoters in SEC$^+$ cells. SEC$^+$ haploid yeast cells (strain SEY6210) were transformed with plasmids containing fusion genes in which wild-type or mutant versions of the yeast *KAR2* promoter were fused to the coding sequence of the *E.coli lacZ* gene. Transformants were cultured in YNBD-Ura broth at 23°C. An aliquot of the mid-log phase cultures of each transformant was treated with 1 μg/ml tunicamycin at 23°C or was incubated at 37°C for the indicated time. β-galactosidase activities in untreated control cells cultured at 23°C (×), tunicamycin-treated cells (●) or heat-shocked cells (○) were measured as described in Materials and methods. Values are expressed as the means +/− SE (bars) of duplicate determinations of three independent transformants.

Table I. Basal and induced activities of wild-type and mutant *KAR2* promoters in yeast *sec* mutants

Yeast strain	Promoter				
	1.3 kb WT	0.3 kb WT	-HS	-GC	-UPR
Basal activity[a]					
SEC$^+$	161	100	49	34	82
sec53	145	100	51	26	78
sec62	143	100	46	27	79
Induction by stress[b]					
SEC$^+$ TM	2.3	2.3	3.1	4.2	1.4
HS	1.6	1.8	1.1	2.3	1.6
sec53 30°C	3.3	3.0	4.4	6.1	1.5
sec62 30°C	1.4	1.4	1.0	1.7	1.6

[a]The basal activities of wild-type and deleted versions of the *KAR2* promoter were determined by measuring β-galactosidase activity in extracts of cells grown at 23°C. The figures in the table show the amount of β-galactosidase activity expressed by various forms of the *KAR2* promoter relative to the 0.3 kb version of the wild-type promoter.
[b]The figures shown are the ratio of β-galactosidase activity in cells cultured in the presence or absence of stress for 3 h.

gene and to determine whether these elements work independently of one another, we constructed a series of deletion mutants, each of which lacks a segment of 10 nucleotides in one or other of the conserved elements. Because there are ~10 bp per helical turn of double-stranded DNA, these deletions should not cause rotational displacement of the upstream and downstream sequences relative to one another and therefore should minimize disruption of protein binding sites that lie along one face of the helix. The exact sites of deletion within each of the three conserved elements are shown in Figure 1B.

Activities of the mutant promoters

To measure the activities of the mutant *KAR2* promoters, we constructed a series of fusion genes in which the *E.coli* sequences coding for β-galactosidase were placed immediately downstream of wild-type or deleted versions of the *KAR2* promoter in a centrometric single-copy yeast vector (see Figure 9A and Materials and methods). The resulting constructs were used to transform SEC$^+$ ('wild-type') haploid yeast cells (strain SEY6210) and the abilities of the mutant and wild-type promoters to drive expression of *lacZ* were then assayed by measuring β-galactosidase activity in transformants cultured in the presence or absence of stress. Transformants grown at 23°C were either exposed to heat (37°C for up to 3 h) or cultured for up to 3 h at 23°C in the presence of tunicamycin, which inhibits *N*-glycosylation of proteins in the ER by blocking formation of dolichol−PP−GlcNAc (Elbein, 1987). We have previously shown that expression of the yeast *KAR2* gene (i) increases transiently after heat shock and (ii) is elevated in a sustained and dramatic fashion when yeast cells are incubated in the presence of tunicamycin (Normington *et al.*, 1989; Rose *et al.*, 1989). In eukaryotic cells, the failure to glycosylate properly leads to the accumulation of unfolded proteins in the ER (Gething *et al.*, 1986).

As shown in Figure 2A, unstressed yeast cells carrying *lacZ* linked to the 1.3 kb wild-type yeast *KAR2* promoter express significant amounts of β-galactosidase activity. The levels of enzyme activity increase markedly in response to heat shock and to treatment with tunicamycin. Similar results were obtained when the 0.3 kb version of the wild-type promoter was used to drive expression of β-galactosidase (Figure 2B). Table I shows (i) the basal activities of promoters relative to the 0.3 kb wild-type promoter at 23°C and (ii) the extent of induction after treatment of cells with tunicamycin or heat. Although the truncated (0.3 kb) promoter displays a lower level of basal expression in unstressed cells than that of the 1.3 kb wild-type promoter, it retains its ability to respond to stress. These results confirm that all the information required for transcriptional response of *KAR2* to two different forms of stress resides in the 0.3 kb segment of DNA that lies immediately proximal to the start site of transcription.

The -HS promoter, which lacks 10 centrally-located nucleotides from the HSE (Figure 2C), can no longer respond to heat shock but retains its ability to respond to treatment with tunicamycin. The activity of this mutated promoter in unstressed cells is ~50% of that observed with the 0.3 kb wild-type promoter (Table I), indicating that the HSE also contributes to the basal level of expression of the yeast *KAR2* gene. Deletion of 10 nucleotides from the GC-rich region (-GC promoter, Figure 2E) has no effect on the

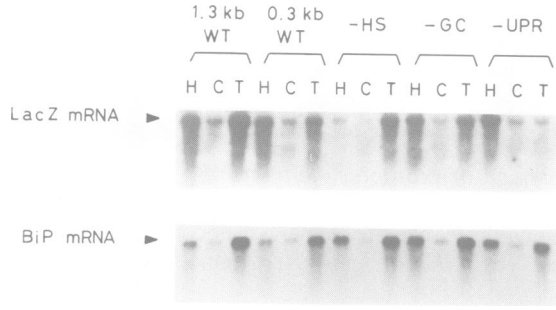

Fig. 3. Expression of *lacZ* mRNA from wild-type and mutant *KAR2* promoters in SEC$^+$ cells. The yeast transformants described in the legend to Figure 2 were cultured in YNBD-Ura broth at 23°C. An aliquot of the mid-log phase cultures of each transformant was treated with 1 μg/ml tunicamycin for 1 h at 23°C or was incubated at 37°C for 10 min. Total RNAs isolated from control cells (C), tunicamycin-treated cells (T) and heat-shocked cells (H) were hybridized with a radiolabeled probe specific for BiP mRNA or *lacZ* mRNA as described in Materials and methods.

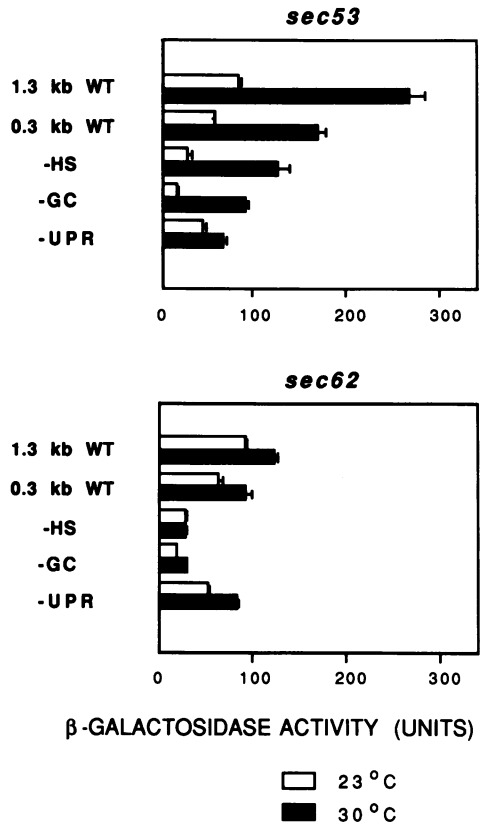

Fig. 4. Expression of β-galactosidase activity from wild-type and mutant *KAR2* promoters in *sec* mutants. Yeast temperature-sensitive secretory mutants (*sec53* and *sec62*) were transformed with plasmids described in the legend to Figure 2. Transformants were cultured in YNBD-Ura broth at permissive temperature (23°). An aliquot of the mid-log phase culture of each transformant was transferred to semi-permissive temperature (30°C) and cultured for 3 h. β-galactosidase activities in control cells cultured at 23°C (open boxes) and cells cultured at 30°C (closed boxes) were measured as described in Materials and methods. Values are expressed as the means +/− SE (*bars*) of duplicate determinations of three independent transformants.

induction of β-galactosidase activity by either heat shock or tunicamycin treatment. However, the level of expression in unstressed cells is reduced to only ~30% of that of the 0.3 kb wild-type promoter, confirming that the GC-rich region contributes to the high level of constitutive expression of *KAR2* (K.Kohno, K.Normington, J.F.Sambrook, M.J.Gething and K.Mori, submitted). The -UPR promoter (Figure 2D), which lacks 10 nucleotides from the UPR element, displays slightly lower activity than the 0.3 kb wild-type promoter in unstressed cells and does not differ significantly in heat inducibility from either the 0.3 kb or the 1.3 kb versions of the wild-type promoter. However, the response of the -UPR promoter to treatment with tunicamycin is reduced to only a few percent of that displayed by the two wild-type promoters and the -HS and -GC mutant promoters.

The ability of the mutant promoters to modulate the levels of mRNA in response to stress was assayed by Northern hybridization. Total RNAs from transformants cultured in the presence or absence of stress were hybridized with a radiolabeled DNA probe specific for *lacZ* sequences (Figure 3). Transformants bearing the 1.3 kb form of the wild-type promoter expressed basal amounts of *lacZ* mRNA in the absence of heat shock or treatment with tunicamycin.

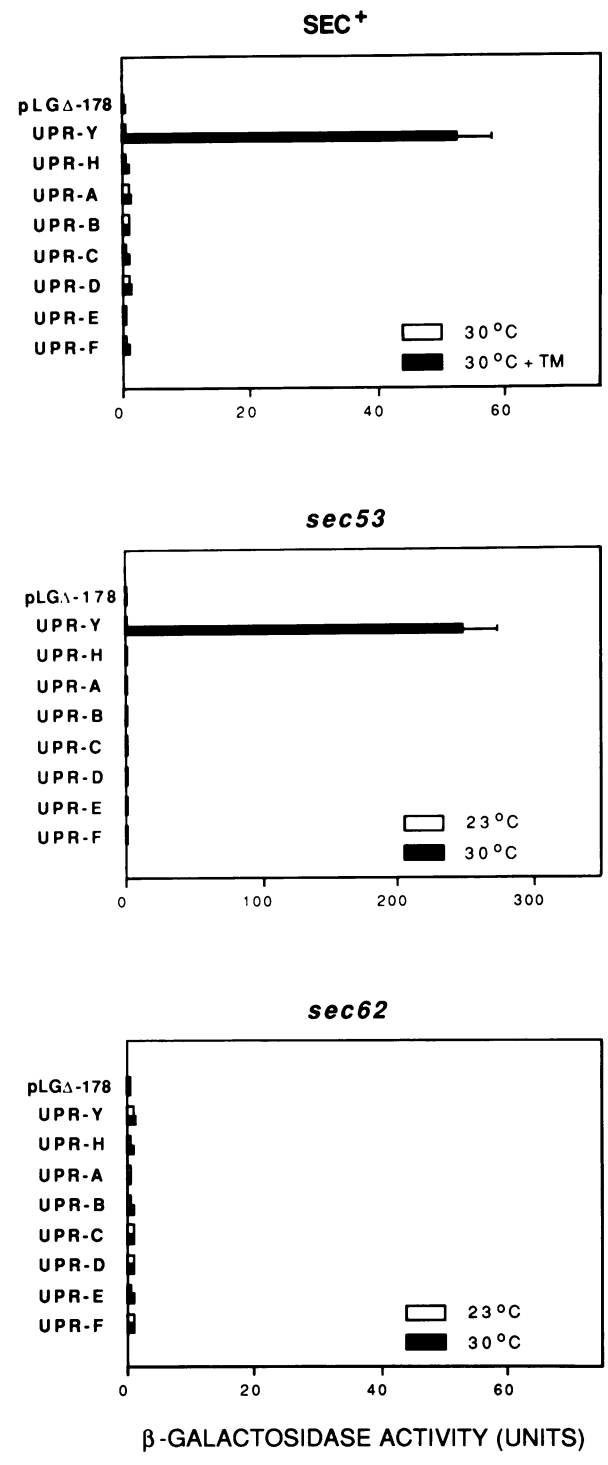

Fig. 5. The 22 bp yeast UPR but not mutant UPR sequence confers stress inducibility to the *CYC1*−*lacZ* fusion gene. Yeast SEC$^+$ (SEY6210), *sec53* and *sec62* cells were transformed with pLGΔ-178 alone and the plasmid containing wild-type or mutant UPR sequence at the *Xho*I site upstream of *CYC1*. Transformants were cultured in YNBD-Ura broth in the absence of stress (30°C for SEC$^+$ and 23°C for *sec53* and *sec62*). An aliquot of the mid-log phase cultures of each transformant was then incubated in the presence of stress for 3 h (addition of 5 μg/ml tunicamycin for SEC$^+$ and a temperature shift-up to 30°C for *sec53* and *sec62*). β-galactosidase activities in cells in the presence (dotted boxes for SEC$^+$ and closed boxes for *sec53* and *sec62*) or absence (open boxes) of stress were determined as described in Materials and methods. Values are expressed as the means +/− SE (*bars*) of duplicate determinations of four independent transformants.

Reduced but detectable amounts of *lacZ* mRNA were also present in unstressed transformants carrying the 0.3 kb wild-type *KAR2* promoter or any of three mutant forms of the promoter. The amount of *lacZ* mRNA increased dramatically in transformants bearing the wild-type promoter or the -GC promoter that had been exposed to heat or treated with tunicamycin. The -HS promoter retained its ability to respond to treatment with tunicamycin but could no longer respond to heat. Conversely, the -UPR promoter lost its ability to respond to treatment with tunicamycin but fully retained its ability to respond to heat. As a control, we used a probe specific for BiP coding sequences. The only source of BiP mRNA in the transformants is the endogenous, chromosomal *KAR2* gene, which has a wild-type promoter. In every case, BiP mRNA was synthesized at a basal rate in unstressed transformants and at an elevated rate in cells exposed to heat shock or treated with tunicamycin. These results are in agreement with those reported previously (Normington *et al.*, 1989; Rose *et al.*, 1989).

The results of Northern analysis of *lacZ* mRNA (Figure 3) are consistent with those obtained from assays of β-galactosidase activity (see Figure 2). They show that the regulatory region of the yeast *KAR2* gene contains at least two independently-acting, stress-responsive elements. The HSE element is essential for the induction of transcription by heat shock but not by tunicamycin; the UPR element responds to treatment with tunicamycin but not to heat shock. The GC-rich element is not required for response to either form of stress. However, it may contribute to the high level of constitutive activity of the *KAR2* promoter, since deletion of the element causes a reproducible reduction in the expression of β-galactosidase (Figure 2 and Table I) and β-galactosidase mRNA (Figure 3). However, even in the absence of the GC-element, basal transcription of *KAR2* occurs at a high level (Figure 3).

Activities of the KAR2 promoters in sec mutants

Transcription of *KAR2* is induced when certain types of temperature-sensitive secretory mutants are incubated at semi- or non-permissive temperature. Enhanced expression is seen in *sec* mutants that accumulate immature precursors of secretory proteins in the ER but is not seen in *sec* mutants that accumulate precursors in the cytosol (Normington *et al.*, 1989; Rose *et al.*, 1989). To measure the contribution of the three *cis*-acting elements in the *KAR2* promoter to induced expression, two strains of yeast carrying different *sec* mutations (*sec53* and *sec62*) were transformed with plasmids carrying wild-type or mutant versions of the *KAR2* promoter upstream of the *lacZ* gene. At the non-permissive temperature *sec53* cells are defective in phosphomannomutase activity (Kepes and Schekman, 1988). This results in an accumulation of incompletely glycosylated and folded precursors of secretory glycoproteins in the ER (Ferro-Novick *et al.*, 1984). By contrast, *sec62* cells are defective in translocation of polypeptides into the ER and thus accumulate unglycosylated precursors on the cytosolic face of the ER membrane (Rothblatt *et al.*, 1989). *SEC62* encodes a transmembrane protein that is a component of the translocation machinery (Deshaies and Schekman, 1990).

The activities of the wild-type and mutant promoters were measured in the two *sec* mutants after incubation at the permissive temperature (23°C) and after shifting to the semi-permissive temperature (30°C). β-galactosidase activity in the transformants of both *sec53* and *sec62* increased for at least 3 h during incubation of cells at 30°C. Whereas incubation at the higher temperature was lethal to *sec53* cells, *sec62* cells displayed the same profiles of promoter activity at both 30°C and 37°C.

As shown in Figure 4 and summarized in Table I, the basal activities of five types of promoters are almost identical in SEC^+ cells and *sec* mutants incubated at 23°C. However, after a temperature shift to 30°C, β-galactosidase activity is markedly induced in *sec53* cells carrying promoters that contain the UPR element. This induction is abolished by deletion of 10 nucleotides from the UPR element (-UPR promoter). The profile of induction in *sec53* cells is almost identical to that in SEC^+ cells treated with tunicamycin (Table I), indicating that the UPR element is involved in both types of induction. By contrast, β-galactosidase activity is only slightly induced in *sec62* cells incubated at 30°C. This increase of activity is most probably a response to an increase in temperature since no induction is observed in cells carrying the -HS promoter and there is no difference between *sec53* and *sec62* cells in the extent of induction mediated by the -UPR promoter. The magnitude of the induction is the same in *sec62* cells as in SEC^+ cells exposed to heat shock (Table I). These results provide strong evidence that the presence of unfolded proteins in the ER is the proximal signal for the induction of the yeast *KAR2* gene.

The UPR element of the yeast KAR2 gene confers stess inducibility to a heterologous promoter

Heat shock elements confer heat inducibility upon heterologous promoters in both mammalian and yeast cells (Pelham and Bienz, 1982; Bienz and Pelham, 1986; Sorger and Pelham, 1987). To test whether the UPR sequence also serves as an upstream activator sequence, we inserted a synthetic, double-stranded oligonucleotide encoding the 22 bp yeast UPR sequence (UPR-Y) into a 2μ-based, multicopy yeast vector containing the *CYC1−lacZ* fusion gene (pLGΔ-178). SEC^+ (SEY6210), *sec53* and *sec62* cells were transformed with the resulting plasmid and β-galactosidase activity was measured in transformants cultured in the presence or absence of stress. The results are shown in Figure 5 and summarized in Table II. Because expression of *lacZ* is under the control of the promoter region of *CYC1*, which lacks all upstream activator sequences (Guarente and Mason, 1983), the plasmid pLGΔ-178 displays no stress inducibility in any type of cell. Insertion of the UPR-Y sequence into the *CYC1* promoter had no significant effect on the basal expression of *lacZ*. However, β-galactosidase activity increased 75-fold when SEC^+ cells were treated with tunicamycin and 207-fold when *sec53* cells were incubated at 30°C. This dramatic difference in inducibility results from the lower level of basal expression of the *CYC1* promoter—compare the high level of β-galactosidase activity obtained with the *KAR2* promoter carried on a single-copy vector (Figures 2 and 4) with the low level obtained from the *CYC1* promoter carried on a multicopy vector (Figure 5). These results demonstrate that the UPR sequence is sufficient to confer stress-induced stimulation of transcription on a foreign promoter. Because β-galactosidase activity is not induced when *sec62* cells are incubated at 30°C, we conclude that the 22 bp UPR element is able to discriminate between the presence of unfolded proteins in the ER and the cytosolic compartment.

Table II. Stess-inducible activities *in vivo* of wild-type and mutant UPR sequences

UPR sequence[a]		Induction by stress (-fold)[b]		
		SEC$^+$	*sec53*	*sec62*
UPR-Y	TCGAGGAACTGGACAGCGTGTCGAAA	75	207	1.2
UPR-H	TCGAGGCGGCGGCCAGCTTGGTGGCC	1.5	1.3	1.4
UPR-A	TCGAGGAACTGGACATCGTGTCGAAA	1.4	1.8	1.0
UPR-B	TCGAGGAACTGGACCTCGTGTCGAAA	1.1	1.4	1.3
UPR-C	TCGAGGAACTGGAACTAGTGTCGAAA	1.1	1.5	1.0
UPR-D	TCGAGGAACTTTACAGCGGTTCGAAA	1.3	1.3	1.0
UPR-E	TCGAGGCGGCGGACAGCGTGGTGGCC	1.3	1.4	1.4
UPR-F	TCGAGGAACTGGCCAGCTTGTCGAAA	1.6	1.7	1.2

[a]Closed circles show nucleotides identical to the sequence of UPR-Y (wild-type yeast UPR). The 10 bp 'core element' in wild-type yeast UPR is underlined.
[b]The figures shown are the ratio of β-galactosidase activity in cells cultured in the presence or absence of stress for 3 h.

To dissect the UPR element, we measured the ability of mutant forms of the UPR element to drive stress-induced expression of the *lac*Z gene in pLGΔ-178 (see Table II). UPR-A, UPR-B, UPR-C and UPR-D carry single or multiple transversions in the 10 bp core region of the yeast UPR sequence. These mutant UPRs did not confer stress-inducibility to the *CYC1*−*lac*Z gene, confirming that the core 10 bp is essential for the UPR function. The promoter of the human BiP gene contains a 22 bp segment (UPR-H, see also Figure 1C) that displays 50% identity with the yeast UPR. Eight of the 10 nucleotides in its core region are identical to the corresponding yeast sequence. However, despite these similarities, UPR-H does not function as an upstream activator sequence in yeast (Table II). To test whether this inability of UPR-H is due to sequence differences in the core region or the flanking region, we assayed the activities of two chimeric UPRs. UPR-E consists of yeast sequences in the 10 bp core region and human sequences in the flanking region while UPR-F consists of human sequences in the core region and yeast sequences in the flanking region. Neither of the two chimeric UPRs conferred stress inducibility to the *CYC1*−*lac*Z fusion gene, indicating that the sequences not only of the core but also of the flanking region are critical to form a UPR that can function as an upstream activator sequence *in vivo*.

Yeast cells contain distinct HSE- and UPR-specific DNA binding proteins

To search for *trans*-acting factors that bind specifically to the stress-responsive elements of *KAR2*, we compared the electrophoretic mobilities of double-stranded DNAs corresponding with the HSE and UPR elements before and after incubation with extracts of yeast cells. The results of these DNA mobility shift assays are shown in Figure 6. In initial experiments, proteins extracted from cells of an unstressed protease-deficient strain of yeast (BJ926) were precipitated with 10−70% saturated $(NH_4)_2SO_4$, dialyzed and incubated with ^{32}P-labeled, 24 bp, synthetic, double-stranded DNAs corresponding to the HSE or UPR elements (shown in Figure 1B). As reported previously by Sorger and Pelham (1987), HSE−protein complexes were detected as three closely spaced bands (lane 2). By contrast, UPR−protein complexes (lane 17) migrated as three widely spaced bands. No complexes could be detected in the absence of added cell extracts (lanes 1 and 16). These results show that unstressed yeast cells contain proteins capable of binding to both HSE and UPR elements.

To confirm the specificity of binding, competition experiments were performed in which different quantities of unlabeled DNA were included in the reaction mixtures. The amount of competition observed was in all cases a function of the amount of unlabeled, homologous competing DNA present in the binding reaction. The results obtained when the competing DNAs were present at 100-molar excess are shown in Figure 6. The ability of ^{32}P-HSE to form complexes (lane 2) was abolished by an excess of unlabeled HSE (lane 3), but was unaffected by TUP (lane 4) or UPR (lane 5). TUP is a multiple transversion of UPR in which (i) all A and G residues have been replaced with C and T and (ii) all C and T residues have been replaced with A and G, respectively. By contrast, the ability of ^{32}P-UPR to form complexes (lane 17) was inhibited by unlabeled UPR (lane 20) but not by HSE (lane 18) or TUP (lane 19).

To analyze further the properties of the proteins that bound specifically to the HSE and UPR elements, cell extracts were fractionated by differential precipitation with $(NH_4)_2SO_4$. Proteins that precipitated between 40 and 50% saturation, or between 50 and 60% saturation with $(NH_4)_2SO_4$ were prepared separately (designated 40−50% and 50−60% in Figure 6). The protein giving rise to one of the three HSE-specific bands (HSF-1) was detectable only in the 50−60% fraction (lane 12), while the protein giving rise to HSF-2 was present in both fractions (lanes 7 and 12). The protein giving rise to HSF-3 was detected only in the 40−50% fraction (lane 7). In the case of binding to UPR, proteins giving rise to two of the bands (UPRF-2 and UPRF-3) were present predominantly in the 40−50% fraction (lane 22) while the protein giving rise to the third band (UPRF-1) was present

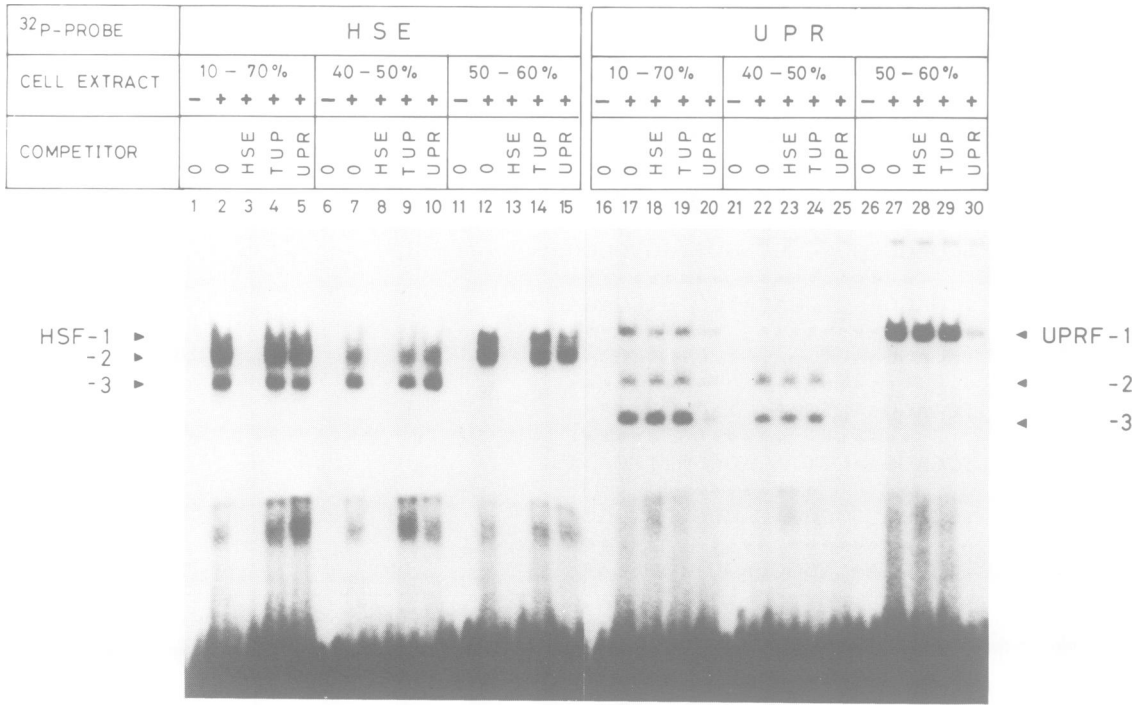

Fig. 6. Proteins that bind specifically to the HSE and UPR elements of the *KAR2* promoter can be detected by DNA mobility shift assays in extracts of unstressed yeast cells. Extracts were prepared from control (unstressed) cultures of the protease-deficient diploid yeast cells (BJ926) and soluble proteins were fractionated by differential precipitation with $(NH_4)_2SO_4$. ^{32}P-labeled, synthetic, double-stranded oligonucleotides corresponding to the HSE or the UPR (see Figure 1B) were mixed with aliquots of the $(NH_4)_2SO_4$ fractions containing either 40 μg of protein (10–70% fraction) or 20 μg of protein (40–50% fraction and 50–60% fraction) in the presence or absence of 100-fold molar excess of unlabeled competitor DNA. Samples were separated by electrophoresis through a 5% polyacrylamide gel as described in Materials and methods. The locations of HSE–protein complexes (HSF-1,2,3) and UPR–protein complexes (UPRF-1,2,3) are indicated.

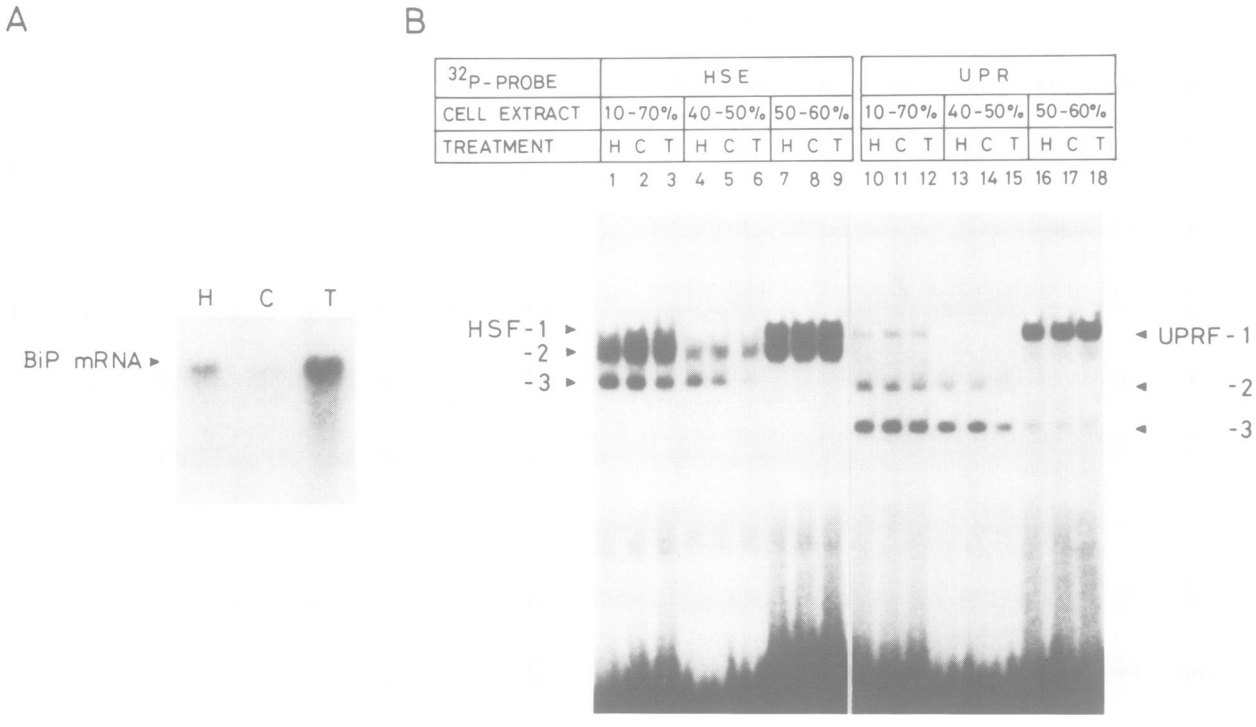

Fig. 7. Detection of HSE- and UPR-binding proteins in stressed yeast cells. Aliquots of a mid-log phase culture of BJ926 cells were treated with 1 μg/ml tunicamycin at 30°C for 1 h or incubated at 37°C for 20 min. **A.** Total RNAs isolated from control cells (C), tunicamycin-treated cells (T), or heat-shocked cells (H) were separated by agarose gel electrophoresis and hybridized with a radiolabeled probe specific for BiP mRNA as described in Materials and methods. **B.** ^{32}P-labeled, synthetic, double-stranded oligonucleotides corresponding to the HSE or the UPR were mixed with aliquots of the $(NH_4)_2SO_4$ fractions containing either 40 μg of protein (10–70% fractoin) or 20 μg of protein (40–50% fraction and 50–60% fraction), each of which had been prepared from control cells (C), tunicamycin-treated cells (T) and heat-shocked cells (H). Samples were separated by electrophoresis through a 5% polyacrylamide gel as described in Materials and methods. The locations of HSE–protein complexes (HSF-1, 2, 3) and UPR–protein complexes (UPRF-1, 2,3) are indicated.

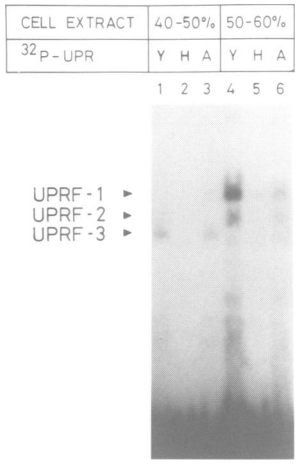

Fig. 8. Correlation of *in vivo* inducible activities of wild-type and mutant UPRs to their binding activities to protein factors in cell extracts. ^{32}P-labeled, synthetic, double-stranded oligonucleotides corresponding to the UPR-Y, UPR-H and UPR-A (for their sequences see Table II) were mixed with aliquots of the $(NH_4)_2SO_4$ fractions containing 20 μg of protein (40−50% fraction and 50−60% fraction). Samples were separated by electrophoresis through a 5% polyacrylamide gel as described in Materials and methods. The locations of UPR−protein complexes (UPRF-1,2,3) are indicated.

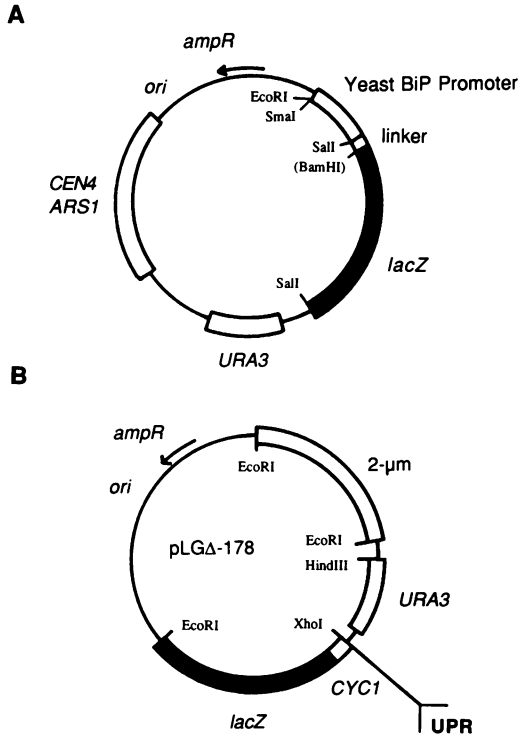

Fig. 9. Structures of plasmids. **A**. Wild-type and mutant *KAR2* promoters were inserted into pSEYc102, a centromere-based, single-copy yeast vector as described in Materials and methods. The *Bam*HI site in parentheses was abolished by insertion of a synthetic linker.

The following series of plasmids contains wild-type or mutant versions of the *KAR2* promoter fused to the *E.coli lacZ* gene: plasmid pSZLWT contains the 1.3 kb wild-type *KAR2* promoter; pSZWT contains the 0.3 kb wild-type *KAR2* promoter. Plasmids pSZ-HS, pSZ-GC and pSZ-UPR contain mutant versions of the *KAR2* promoter that lack 10 bp from the HSE, GC-rich and UPR elements, respectively. For the exact locations of these deletions, see Figure 1B. **B**. Synthetic, double-stranded 26 bp oligonucleotides corresponding to wild-type and mutant UPRs (listed in Table II) were inserted into the *Xho*I site of pLGΔ-178, a 2μ-based, multicopy yeast vector containing the *CYC1−lacZ* fusion gene.

exclusively in the 50−60% fraction (lane 27). The 50−60% fraction apparently contains more UPRF-1 than the 10−70% fraction (compare lane 17 and 27). However, mixing experiments showed that the 10−70% and 40−50% fractions both contain material(s) that interfere(s) with the binding of UPRF-1 to the ^{32}P-labeled probe. $(NH_4)_2SO_4$ fractionation is therefore a critical step in obtaining preparations that show strong and reproducible binding of UPRF-1 to the UPR. The behavior of UPRF-2 varied slightly from preparation to preparation. In most cases, UPRF-2 was recovered in the 40−50% fraction. In some cases, however, the binding activity was recovered chiefly in the 50−60% fraction (data not shown). All binding by the fractionated proteins to the radiolabeled UPR was specific, as judged by competition experiments.

We next examined the effect of exposing cells to stress on the HSE- and UPR-specific binding activities. Extracts were prepared from control, heat shocked and tunicamycin-treated cells and proteins were fractionated by differential precipitation with $(NH_4)_2SO_4$. As shown in Figure 7, BiP mRNA was induced by both heat shock and treatment with tunicamycin. However, the profile of the HSE-specific DNA binding activity was not detectably altered by exposure to heat shock. This result, which suggests that induction of transcription by heat does not result simply from a change in the concentration of HSE-binding protein, is in agreement with that previously reported by Sorger *et al.* (1987). Similarly, exposure to stress does not affect either the mobility of the UPR−protein bands or the amount of UPR-specific binding activity detected in any of the fractions obtained by differential precipitation with $(NH_4)_2SO_4$.

To test whether there is a correlation between the binding of putative *trans*-acting factors to the UPR *in vitro* and stress-induced transcription *in vivo*, we tested the ability of the previously described UPR-like sequences to bind proteins (see Figure 5 and Table II). Biologically active UPR-Y, inactive UPR-H and UPR-A (an inactive point mutant of UPR-Y) were labeled with ^{32}P and incubated with proteins in the 40−50% and 50−60% $(NH_4)_2SO_4$ fractions. As shown in Figure 8, UPRF-1 exhibited little binding to UPR-A (lane 6) and did not bind detectably to UPR-H (lane 5). Thus the ability of UPR-like sequences to bind UPRF-1 *in vitro* correlates well with their ability to mediate induction *in vivo*. The only binding activity detectable in freshly prepared 50−60% $(NH_4)_2SO_4$ fraction was UPRF-1 (Figures 6 and 7). However, UPRF-2 appeared after the fraction had been stored for extended periods at −70°C (Figure 8, lane 4). UPRF-2 displays binding properties toward UPR-Y, UPR-H and UPR-A that are indistinguishable from UPRF-1. It therefore seems likely that UPRF-2 is a proteolytic fragment of UPRF-1 that retains binding activity. Treatment of cells with tunicamycin prior to preparation of cell extracts did not change the binding profiles or patterns of fractionating of either UPRF-1 or UPRF-2 (data not shown). Finally, UPRF-3 bound with equal efficiency both to UPR-Y, which is biologically active (lane 1) and to UPR-A, which is not (lane 3). These results indicate that the binding of UPRF-3 is unlikely to have biological relevance.

Taken together, these results indicate that UPRF-1 is a transcription factor that binds specifically to the 22 bp segment of the *KAR2* promoter that responds to the presence

of unfolded proteins in the ER. Like its counterpart in the heat shock system, HSF (Sorger and Pelham, 1987), UPRF-1 may activate transcription by virtue of modifications that cannot be detected by shifts in the mobility of specific DNA–protein bands.

Discussion

BiP, like several other proteins of the stress-70 family (Craig, 1990), is synthesized constitutively under conditions of normal cellular growth. In both yeasts and mammalian cells, the expression of BiP increases in response to the presence of unfolded proteins in the ER (Kozutsumi *et al.*, 1988; Normington *et al.*, 1989). In addition, in yeasts (but not in mammalian cells), BiP is transiently expressed at a higher level after a temperature upshift (Normington *et al.*, 1989; Rose *et al.*, 1989). In this paper we have shown that three different *cis*-acting elements in the *KAR2* promoter contribute to this complex pattern of behavior independently of each other. It seems likely that induction of *KAR2* by two different forms of stress is mediated by selective activation of the interaction between the appropriate *trans*-acting factor and the *cis*-element.

The UPR sequence of the yeast *KAR2* promoter is homologous to the highly conserved regulatory domains present in the promoters of the mammalian GRP78 (BiP) and GRP94 genes (Figure 1C). These regulatory domains were originally identified by Lee and co-workers using 5′ deletion analysis and DNA footprinting as sequences required for the induction of the GRP78 and GRP94 genes that occurs when cells are exposed to calcium ionophores (Resendez *et al.*, 1988; Chang *et al.*, 1989). However, more recent studies show that transcriptional regulation of the mammalian BiP gene is complicated (Wooden *et al.*, 1991). No one *cis*-acting element is essential for promoter activity and the responsiveness of the promoter to events in the ER is affected neither by linker-scanning mutations nor by internal deletions in the conserved regulatory domain. While induction is attenuated when a proximal region containing a CCAAT motif is mutated by linker-scanning, the CCAAT motif alone is not sufficient for promoter activity. A possible interpretation of these results is that the mammalian BiP promoter contains multiple control elements and the highly conserved domain is functionally redundant (Wooden *et al.*, 1991).

In yeast cells, transcriptional regulation of *KAR2* appears to be simpler. Whereas the heat shock element in the *KAR2* promoter regulates both basal and stress-responsive expression of yeast BiP mRNA, the 22 bp UPR sequence responds chiefly to the accumulation of unfolded proteins in the ER. Deletion of the central 10 bp from the UPR element (i) reduces the level of basal expression only slightly, (ii) completely eliminates the increase in transcription of *KAR2* that occurs when cells are incubated in the presence of tunicamycin (Figure 2 and 3) and (iii) abolishes the sustained response of the *KAR2* promoter that occurs when certain yeast *sec* mutants are incubated at the non-permissive temperature (Figure 4). The *sec53* mutation, which affects processes in the ER is able to induce transcription of *KAR2 via* the UPR element. The *sec62* mutation, which interrupts the secretory pathway at an earlier stage has no effect on expression of *KAR2* (Normington *et al.*, 1989; Rose *et al.*, 1989) but instead induces the synthesis of cytosolic stress proteins (Normington *et al.*, 1989). This result strongly indicates that the ER of yeasts, like that of mammalian cells (Kozutsumi *et al.*, 1988), must be equipped with a signaling system that monitors the state of proteins in the organelle, transmits information across the membrane of the ER and adjusts the level of expression of the BiP gene accordingly. While the details of many steps in this signaling pathway are unknown, it now seems likely that in yeast, modulation of transcription can be achieved solely by the interaction of the *trans*-acting factor with the UPR element of the *KAR2* promoter. The UPR sequence can be transplanted into a heterologous promoter to drive stress-induced stimulation of transcription (Figure 5) and specifically binds to a protein factor(s) present in yeast cell extracts (Figure 6). Analysis of mutant UPR sequences shows that binding to the *trans*-acting factor *in vitro* correlates well with transcriptional activity *in vivo* (Figure 8). As is the case with the heat shock transcription factor HSF, UPRF-1 is present in approximately equal concentrations in both stressed and unstressed cells (Figure 7). Thus, a crucial step in signaling pathway that triggers sustained activation of the yeast *KAR2* gene in response to the accumulation of unfolded proteins in the ER must involve modification of a pre-existing pool of the *trans*-acting factor that binds specifically to the UPR element.

We have taken advantage of the dramatic induction mediated by the UPR element to select yeast mutants that are unable to respond to the presence of malfolded proteins in the ER (K.Mori, M.J.Gething and J.F.Sambrook, unpublished results). We hope that analysis of these mutants will enable us to define the biochemical events in the pathway by which signals are transmitted from one intracellular compartment to another.

Materials and methods

Strains and microbial techniques

S.cerevisiae strain SEY6210 (*MATα ura3*-52 *leu2*-3, 112 *his3*-Δ200 *trp1*-Δ901 *lys2*-801 *suc2*-Δ9) was obtained from S.Emr (California Institute of Technology). Strain BJ926 (*MATa*/α *trp1*/+*his1*/ + *prc1*-126/− *pep4*-3/−*prb1*-1122/− *can1*-11/− *gal2*/−) was provided by B.Fishel (Southwestern Medical Center). Strain RSY12/SEY5536α (*MATα ura3*-52 *leu2*-3,112 *sec53*-6) and RDM50-94C/YFP329 (*MATα ura3*-52 *leu2*-3,112 *his4 sec62*) were kindly provided by R.Schekman (University of California, Berkeley). The compositions of rich broth medium (YPD) and selective medium for transformants (YNBD-Ura) are described elsewhere (Sherman *et al.*, 1986). Transformation of yeast was performed by the lithium acetate method (Ito *et al.*, 1983). Recombinant DNA Techniques were carried out as described in Sambrook *et al.* (1989 and references therein).

Site-directed mutagenesis and construction of plasmids

To manipulate the promoter and coding region of *KAR2* separately, we created a *Sal*I site immediately upstream of the translational start site (see Figure 1A). The 277 bp *Xho*I–*Sal*I fragment containing the promoter was then cloned into M13mp18 and used as a template for oligonucleotide-mediated site-directed mutagenesis as described by Kunkel (1985). After the sequences of the mutants had been confirmed, the wild-type or mutant promoters were recovered from the RFs of recombinant M13 bacteriophages by digestion with *Sma*I and *Sal*I and inserted between the *Sma*I and *Bam*HI sites of pSEYc102, a centromere-based, single-copy yeast vector (the gift of M.Douglas, University of North Carolina). To ensure that the downstream coding sequences of *lac*Z would be in frame and would be expressed under the control of the *KAR2* promoter, we also inserted a 21 bp synthetic oligonucleotide, which encodes the five N-terminal amino acids of yeast BiP and whose 5′- and 3′-termini are complementary to protruding termini generated by *Sal*I and *Bam*HI, respectively:

5′TCGACCATGTTTTTCAACAGA3′
3′ GGTACAAAAAGTTGTCTCTAG5′

The 1.3 kb *Eco*RI–*Sal*I fragment spanning the wild-type *KAR2* promoter was inserted between the *Eco*RI and *Bam*HI sites of pSEYc102 in a similar fashion (see Figure 9A).

Plasmid pLGΔ-178 is a 2μ-based, multicopy yeast vector containing the *CYC1*−*lacZ* fusion gene (see Figure 9B), which was kindly provided by L.Guarente (Massachusetts Institute of Technology). Because all of the upstream activator sequences have been deleted from the promoter region of the *CYC1* (iso-1-cytochrome c) gene, the basal level of expression of *lacZ* is extremely low (Guarente and Mason, 1983). Synthetic, 26 bp, double-stranded oligonucleotides encoding wild-type and mutant UPRs with sequences at both ends complementary to protruding termini generated by *Xho*I were inserted into the *Xho*I site upstream of *CYC1*. Constructs were confirmed by dideoxy sequencing of double-stranded DNA templates (Sambrook *et al.*, 1989).

Enzyme assays

Assays of β-galactosidase activity in extracts of yeast cells were carried out as described (Slater and Craig 1987; Park and Craig 1989). Units are defined as $[OD_{420} \times 10^3]/]OD_{600} \times tv]$, where t = incubation time in minutes and v = volume in milliliters.

Northern hybridization

Total yeast RNA was isolated as described by Lindquist (1981) Electrophoresis of denatured RNA (5 μg) and Northern hybridization in 50% formamide were carried out as described (Sambrook *et al.*, 1989), using radiolabeled probes specific for yeast *KAR2* (the central 1.07 kb *Eco*RI fragment) and *E.coli lacZ* (the central 2.12 kb *Bg*lI fragment).

Yeast cell extracts

These were prepared from the protease-deficient strain BJ926 as described (Sorger and Pelham, 1987; McDaniel *et al.*, 1989) with some modifications. The cells were cultured at 30°C to mid-log phase (OD_{600} ~0.6) and divided into 200 ml aliquots. As appropriate, aliquots were treated with tunicamycin (1 μg/ml) for 1 h or incubated at 37°C for 20 min. The cells were harvested, washed once with breakage buffer (200 mM Tris−Cl pH 8.0, containing 10 mM $MgCl_2$, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 μg/ml leupeptin and 5 μg/ml aprotinin) and suspended in 2 pellet volumes of breakage buffer at 4°C. The cells were disrupted with glass beads (0.5 mm diameter) and extracted at 4°C for 30 min with shaking in the presence of 10% $(NH_4)_2SO_4$. The homogenate was centrifuged at 10 000 *g* for 10 min and the supernatant was obtained by further centrifugation at 100 000 *g* for 1 h. Into an aliquot of the supernatant, saturated $(NH_4)_2SO_4$ was added to a final concentration of 70% and the precipitated proteins were used as whole cell extracts [10−70% $(NH_4)_2SO_4$ fraction]. In some cases, the proteins in this extract were further fractionated by differential addition of saturated $(NH_4)_2SO_4$ to final concentrations of 40%, 50% and 60%. Proteins precipitated between 40% and 50% saturation and between 50% and 60% saturation were collected separately. The precipitates were dissolved in a minimum volume of dialysis buffer (20 mM HEPES pH 7.9 containing 50 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol and 0.5 mM PMSF) and dialyzed extensively at 4°C. The protein concentration in typical extracts ranged between 6 and 12 mg/ml, as determined by the method of Bradford (1976).

DNA mobility shift assay

The 5′-termini of synthetic, 24 bp, double-stranded DNAs (HSE and UPR in Figure 1B) or the 3′-termini of synthetic, 26 bp, double-stranded DNAs (UPR-Y, UPR-H, and UPR-A in Table II) were radiolabeled with polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol) or the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (3000 Ci/mmol) respectively and purified by electrophoresis through polyacrylamide gels. For binding of proteins to the synthetic HSE, the buffer consisted of 20 mM HEPES pH 7.9 containing 60 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol and 2% Ficoll. The same buffer was used for binding of protein to the synthetic UPR element, except that the concentrations of KCl and EDTA were 50 mM and 0.25 mM respectively and Mg^{2+} was omitted. Binding reactions were initiated by addition of cell extracts into binding buffer containing 0.2−0.3 ng of radiolabeled double-stranded oligonucleotide (8−10 000 c.p.m.), 1 μg of poly(dI-dC):poly(dI-dC) (Pharmacia) and 1−2 μg of denatured salmon sperm DNA. Where appropriate, unlabeled competitor DNA was included in the binding buffer before addition of cell extracts. After incubation for 10 min at 4°C in a final volume of 20 μl, samples were immediately loaded onto a non-denaturing 5% polyacrylamide gel (the acrylamide:bisacrylamide ratio was 30:0.8) containing 0.5% Ficoll. Gels were pre-run for 3 h and samples were electrophoresed for 3.5 h at 150 V at 4°C in 0.5 × TBE (Sambrook *et al.*, 1989). The gels were then dried and exposed to X-ray film.

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