

## The *Saccharomyces cerevisiae* HSP12 Gene Is Activated by the High-Osmolarity Glycerol Pathway and Negatively Regulated by Protein Kinase A

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Received 12 July 1995/Returned for modification 9 August 1995/Accepted 25 August 1995

The *HSP12* gene encodes one of the two major small heat shock proteins of *Saccharomyces cerevisiae*. Hsp12 accumulates massively in yeast cells exposed to heat shock, osmotic stress, oxidative stress, and high concentrations of alcohol as well as in early-stationary-phase cells. We have cloned an extended 5'-flanking region of the *HSP12* gene in order to identify *cis*-acting elements involved in regulation of this highly expressed stress gene. A detailed analysis of the *HSP12* promoter region revealed that five repeats of the stress-responsive CCCCT motif (stress-responsive element [STRE]) are essential to confer wild-type induced levels on a reporter gene upon osmotic stress, heat shock, and entry into stationary phase. Disruption of the *HOG1* and *PBS2* genes leads to a dramatic decrease of the *HSP12* inducibility in osmotressed cells, whereas overproduction of Hsp12 produces a fivefold increase in wild-type induced levels upon a shift to a high salt concentration. On the other hand, mutations resulting in high protein kinase A (PKA) activity reduce or abolish the accumulation of the *HSP12* mRNA in stressed cells. Conversely, mutants containing defective PKA catalytic subunits exhibit high basal levels of *HSP12* mRNA. Taken together, these results suggest that *HSP12* is a target of the high-osmolarity glycerol (HOG) response pathway under negative control of the Ras-PKA pathway. Furthermore, they confirm earlier observations that STRE-like sequences are responsive to a broad range of stresses and that the HOG and Ras-PKA pathways have antagonistic effects upon CCCCT-driven transcription.

The response of the yeast *Saccharomyces cerevisiae* to osmotic stress has recently been the focus of attention. It has been found that, in this organism, shifts to higher osmolarities trigger a signal transduction pathway consisting of a cascade of mitogen-activated protein (MAP) kinase homologs (13). This discovery prompted many studies indicating that the same molecular strategy is conserved in other yeasts as well as in mammals (25, 29, 67, 75; for reviews, see references 8 and 31). In addition, evidence suggesting that heat stress activates similar pathways in a number of mammalian cell lines has been presented (22, 59).

In *S. cerevisiae*, two genes, *HOG1* and *PBS2*, coding for components of the osmosensing pathway have been isolated (9, 13). Hsp12 is a member of the MAP kinase family, whereas Pbs2 is a MAP kinase kinase homolog. Upon osmotic stress, Hsp12 is tyrosine phosphorylated in a Pbs2-dependent manner (13). Simultaneously, glycerol accumulates in order to increase the internal osmolarity and counteract cell dehydration (7, 46). *hog1* and *pbs2* mutants show an osmosensitive phenotype, accumulating low amounts of glycerol and failing to grow on high-osmolarity media. The *PBS2-HOG1* cascade was therefore called the high-osmolarity glycerol (HOG) response pathway (13). Other genes encoding products interacting with and presumably controlling this protein kinase cascade have been cloned (44, 51). However, in contrast with *HOG1* and *PBS2*, disruption of the latter genes does not produce an osmosensi-

tive phenotype (44). This result suggests the existence of an alternative pathway operating upstream of Pbs2.

*GPD1*, coding for an isotype of the NAD<sup>+</sup>-dependent glycerol-3-phosphate dehydrogenase, and *CTT1*, encoding the cytosolic catalase T, are among the target genes of the HOG pathway identified so far (1, 42, 65). Both genes are induced upon a shift to high-salt media, and inactivation of either *HOG1* or *PBS2* results in lower amounts of *CTT1* mRNA in osmotressed cells. Other genes induced upon osmotic shock, as identified by Northern (RNA) and two-dimensional protein analyses, are those encoding the heat shock proteins (HSPs) Hsp26 and Hsp12 (80). Although the function of these unrelated yeast small HSPs remains elusive, the massive accumulation of both polypeptides upon heat shock and osmotic stress is intriguing (52, 55, 73, 80). Small HSPs of *Drosophila melanogaster*, mammalian, and tomato cells form large cytoplasmic complexes called heat shock granules (2, 3, 5, 50) and, in general, show limited homology with the eye lens protein  $\alpha$ -B-crystallin (17). Recently, Jakob and coworkers (36) have demonstrated that mammalian small HSPs and  $\alpha$ -B-crystallin molecules exhibit chaperone activity. Although Hsp26 forms aggregates similar to heat shock granules and its intracellular location has been determined (58), a function has yet to be assigned to this protein (62). Unlike Hsp26, Hsp12 does not show homology with  $\alpha$ -B-crystallin (55, 71). Instead, an identity of 47% has been found between the N-terminal regions of Hsp12 and Wh11, a 7.8-kDa polypeptide encoded by a gene differentially expressed in the budding phase and hyphal-forming cells of *Candida albicans* (70).

Apart from heat shock and osmotic stress, Hsp12 is expressed in *S. cerevisiae* cells exposed to ethanol and oxidative stress, in cells undergoing glucose depletion or entering stationary

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TABLE 1. *S. cerevisiae* strains used

Strain	Relevant genotype	Source or reference
BJ 2168	<i>MAT<math>\alpha</math> leu2 ura3-52 trp1 prc1-407 prb1-1122 pep4-3</i>	YGSC <sup>a</sup>
YPH102	<i>MAT<math>\alpha</math> ura3 leu2 his3 ade2 lys2</i>	13
JBY13	<i>MAT<math>\alpha</math> ura3 leu2 his3 ade2 lys2 hog1-<math>\Delta</math>1::TRP1</i>	Derived from YPH102
JBY43	<i>MAT<math>\alpha</math> ura3 leu2 his3 ade2 lys2 pbs2-<math>\Delta</math>1::URA3</i>	Derived from YPH102
MYY290	<i>MAT<math>\alpha</math> leu2 his3 phoC phoE ura3</i>	68
MYY385	<i>MAT<math>\alpha</math> leu2 his3 phoC phoE ura3 hsf1-m3</i>	Congenic to MYY290
SEY6210	<i>MAT<math>\alpha</math> leu2 ura3 lys2 trp1 his3</i>	85
SM10	<i>MAT<math>\alpha</math> leu2 ura3 lys2 trp1 his3 yap1-<math>\Delta</math>1::HIS3</i>	Derived from SEY6210
YAW10	<i>MAT<math>\alpha</math> leu2 ura3 lys2 trp1 his3 yap1-<math>\Delta</math>1::HIS3 cad1-<math>\Delta</math>1::hisG</i>	Derived from SEY6210
SP1	<i>MAT<math>\alpha</math> leu2 his3 trp1 ade8 can1 ura3</i>	14
TK161-R2V	<i>MAT<math>\alpha</math> leu2 his3 trp1 ade8 can1 ura3 RAS2<sup>Val-19</sup></i>	Derived from SP1
S18-D	<i>MAT<math>\alpha</math> leu2 his3 trp1 ade8 ura3 tpk1<sup>w1</sup> tpk2::HIS3 tpk3::TRP1</i>	Derived from SP1
RS13-58A-1	<i>MAT<math>\alpha</math> leu2 his3 trp1 ade8 ura3 tpk<sup>w1</sup> tpk2::HIS3 tpk3::TRP1 bcy1::LEU2</i>	Derived from SP1
S7-7A	<i>MAT<math>\alpha</math> leu2 his3 trp1 ade8 ura3 tpk2::HIS3 tpk3::TRP1</i>	Derived from SP1
S13-3A	<i>MAT<math>\alpha</math> leu2 his3 trp1 ade8 ura3 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2</i>	Derived from SP1

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phase, and in mutants harboring a defective adenylate cyclase (37, 54, 55, 71; this paper). This complex pattern of expression and the unusual strength of the *HSP12* promoter has prompted us to map the regulatory *cis*-acting elements and determine the role of diverse signal transduction pathways in the regulation of this gene. The stress-responsive element (STRE) present in the *CTT1* gene is the only osmo-responsive *cis*-acting sequence characterized as yet (47, 65). This element appears to coincide with the CCCCT element found in the promoter region of *DDR2*, a gene induced by heat shock and DNA damage (39, 40). The heat shock element (HSE)-heat shock transcription factor pair is another *cis-trans* pair able to mediate transcriptional activation in eukaryotic cells upon heat shock as well as other stresses (for a review, see reference 45). Band shift analyses suggest, however, that a *trans*-acting factor distinct from Hsf binds to the CCCCT motif (39, 40).

Here we show that five repeats of the CCCCT motif are necessary to confer wild-type stress inducibility on the *HSP12* gene. Disruption of the second most proximal CCCCT motif (STRE2) causes a dramatic decrease in transcriptional activity in heat-shocked and salt-stressed cells. Likewise, this promoter allele is unable to confer inducibility on a reporter gene during the diauxic shift. We also demonstrate that the *HSP12* gene is modulated by the HOG pathway, and we extend early observations indicating that this gene is down-regulated by the Ras-cyclic AMP-dependent protein kinase (PKA) pathway. A possible interaction between both signal transduction pathways is discussed.

## MATERIALS AND METHODS

**Strains, growth conditions, exposure to stress, and DNA manipulation.** All yeast strains used in this work are listed in Table 1. *S. cerevisiae* BJ2168 was used to integrate and determine the transcriptional activity of the *HSP12* promoter mutant alleles fused to the  $\beta$ -glucuronidase (GUS) reporter gene. Unless stated otherwise, yeast strains were grown aerobically on Sabouraud dextrose (SD) medium (0.67% yeast nitrogen base [Difco], 2.0% glucose) containing the necessary supplements at 30°C. Heat shock was imposed by shifting exponentially growing cells from 23 to 37°C in a waterbath set at 65°C. The thermal shift took less than 15 s as monitored by a thermometer in direct contact with the cell suspension. Exposure to salt stress was carried out as described previously (80). For all DNA manipulations, *Escherichia coli* SURE {*mcra*  $\Delta$ (*mcra*-*hsdRMS*-*mrs*)171 *supE44 thi-1 l-gyrA96 relA1 lac recB recJ sbcC unuC::Tn5 (kan<sup>r</sup>)* *uvrC* [*F'* *proAB lacI<sup>q</sup>ZDM15 Tn10 (Tet<sup>r</sup>)*]; Stratagene) was used. Bacterial transformation and plasmid DNA isolation were performed according to procedures described elsewhere (35, 61).

**Construction of fusion genes and probes and genomic DNA library screening.** Because of the absence of suitable restriction sites to fuse the *HSP12* promoter region to the GUS gene, a *SalI*-*Bam*HI fragment comprising the nucleotides

between -606 and +13 (relative to the translation start site) of the *HSP12* gene was PCR generated. This fragment was fused in frame with the ATG start codon of the GUS reporter gene (64) by digestion and ligation at the *Bam*HI site. Proper transcript termination was achieved by placing an *SstI*-*Bgl*II linker and a 0.4-kb *Bgl*II-*Hind*III fragment containing the *PGK* terminator (32) downstream of the GUS gene. This construct was subsequently subcloned into YIplac211 (27) as a 3.1-kb *SalI*-*Hind*III fragment producing pKV2. Another version of this clone (pKV3) (Fig. 1) was constructed with a YIplac211 vector where the *Bam*HI site of the multicloning site had been previously destroyed by end filling and religation. To generate pKV3-d0 (Fig. 1), the 346-bp *Xba*I fragment containing the nucleotides between -606 and -261 was removed from pKV2 by digestion and religation. For construction of a fusion gene carrying an extended *HSP12* promoter region, a YEp13-based yeast genomic DNA library (49) (a kind gift of W. Mulder and L. Grivell, University of Amsterdam, Amsterdam, The Netherlands) was screened with the 0.6-kb *SalI*-*Bam*HI fragment present in pKV3 (see above) as a probe. Detection of positive clones was performed by *in situ* lysis on filters and subsequent hybridization as described elsewhere (61). A 3.0-kb *Xba*I fragment containing sequences upstream of the -260 position was isolated from a 4.8-kb genomic DNA clone (pBV3) and inserted into the unique *Xba*I site of pKV3-d0. A clone carrying the *Xba*I fragment in the correct orientation was selected by digestion analysis and named pKV1 (Fig. 1). pKVc (Fig. 1) was produced by removal of the remaining 260 nucleotides of the *HSP12* promoter region from pKV3-d0 by *Bam*HI digestion and religation.

**Generation of deletions and site-directed mutagenesis.** Progressive 5' deletions of pKV3 were generated with the Erase-a-Base kit (Promega) starting at the *SalI* site. To prevent exonuclease III action upon upstream vector sequences, pKV3 was predigested with *Kpn*I. Mutant alleles of the *HSP12* promoter harboring internal deletions or mutations were constructed by means of mutagenic primers (Table 2) and PCR amplification (33). The names of the primers are identical to those of the *HSP12* mutant alleles generated. Constructs containing multiple mutations were created by using suitable mutant alleles as DNA templates. Mutagenized PCR products were digested with *SalI* and *Bam*HI, cloned into pKV3, and verified by sequencing with a Sequenase kit (United States Biochemical Corp.).

**Chromosome integration and copy number determination.** All constructs were digested with *Stu*I and integrated at the *URA3* locus. Proper integration and copy number was determined by Southern analysis. Yeast transformation, isolation of yeast genomic DNA, and Southern analysis were carried out by established procedures (38, 61, 72).

***HOG1* and *HSP12* gene overexpression.** The *HOG1* gene (13) was reisolated by a pMA3a-based clone complementing the *hog1* osmosensitive phenotype (pFJ0; a kind gift of P. Bossier and C. Rodrigues-Pousada, Gulbenkian Institute of Science, Oeiras, Portugal). pMA3a is a multicopy plasmid (>50 copies per cell) containing a *LEU2d* selective marker (69). The identity of the pFJ0 clone was confirmed by Southern and restriction analyses (not shown). YPH102 cells (Table 1) were subsequently transformed with pFJ0 or with a pMA3a plasmid bearing a full-length *HSP12* gene (pFJ1), isolated as a 1.9-kb *Bam*HI DNA fragment from pBV3 (Fig. 1). As negative control, an empty pMA3a vector was used.

**Northern analysis and determination of transcriptional activity.** Northern (RNA) analysis was performed as described previously (80), except for the electrophoretic separation of RNA. Formaldehyde-agarose gels were used instead, according to the method of Ausubel and colleagues (4). Signal quantitation was performed by phosphorimaging (PhosphorImager 425; Molecular Dynamics), and, unless stated otherwise, the mean value for at least two independent experiments was determined. GUS mRNA levels were used as a measure of transcriptional activity. For the correction of differences in RNA

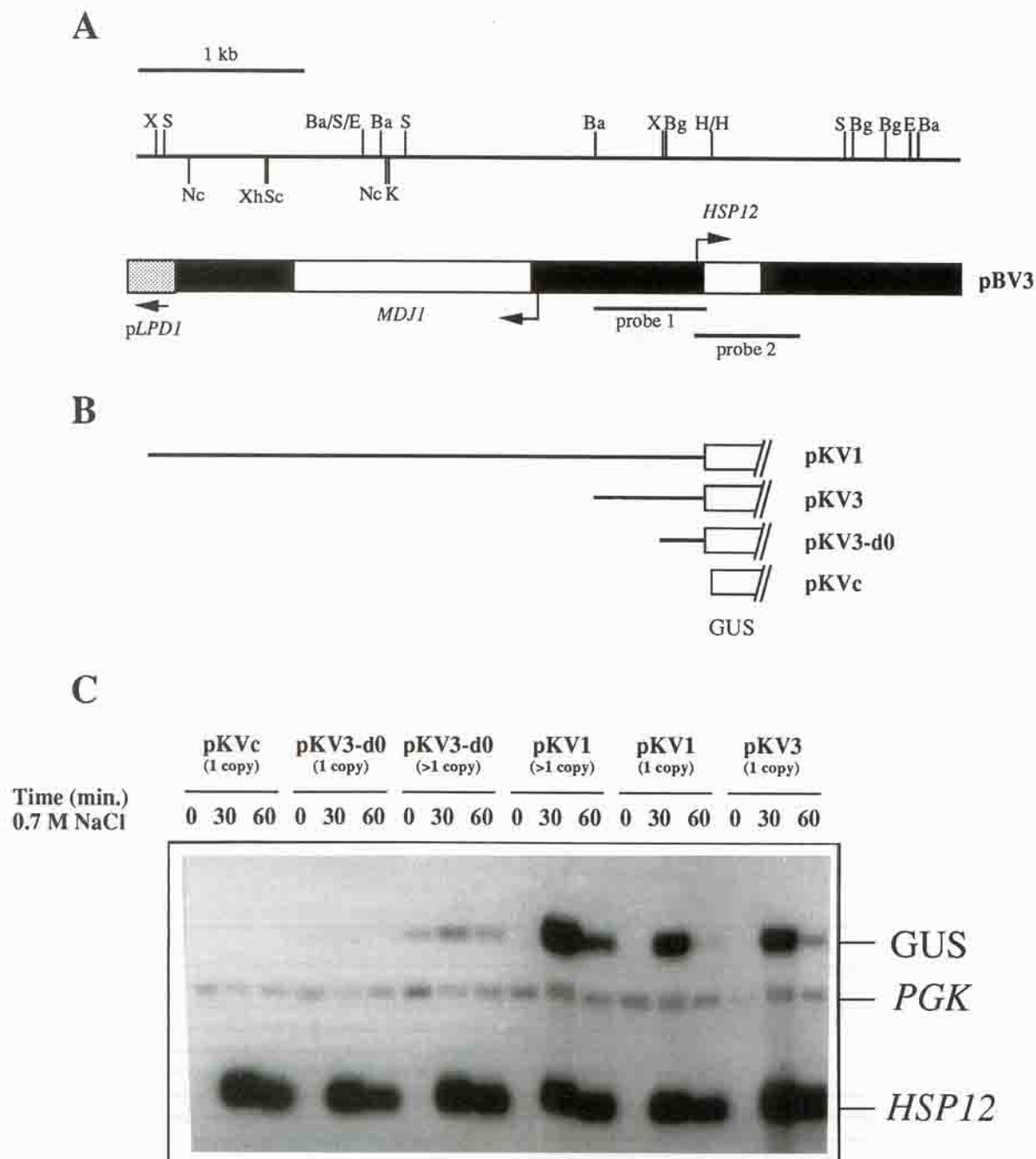


FIG. 1. 5' deletion analysis of an extended *HSP12* 5'-flanking region. (A) Localization of the *HSP12* and *MDJ1* open reading frames (see the text) in the genomic DNA clone pBV3. Black and white bars represent intragenic regions and open reading frames, respectively. The gray bar corresponds to the partial promoter region of the *LPD1* gene present in pBV3. Above pBV3, the restriction map of the clone is given. Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; Nc, *Nco*I; S, *Sal*I; Sc, *Sac*I; X, *Xba*I; Xh, *Xho*I. (B) *HSP12*-GUS fusion genes used to dissect the *HSP12* promoter region. Sequences of the pBV3 clone (see panel A) fused to the GUS reporter gene are depicted as black lines. (C) Northern analysis of cells carrying pKVc, pKV3-d0, pKV1, and pKV3 exposed to a high salt concentration (0.7 M NaCl) for 0, 30, and 60 min. The levels of *PGK* and *HSP12* mRNA (wild-type endogenous copy) were used as internal controls for RNA loading and exposure to stress, respectively.

loading, a 2.5-kb *Hind*III fragment containing the *PGK* open reading frame as gene-specific probe was used (32). The slight inducibility of the *PGK* gene upon 30 min of heat stress (53) was taken into account by setting separate 100% values for stressed and nonstressed cells. Standard deviations ranged from 3 to 12%.

## RESULTS

The *HSP12* 5'-flanking region contains multiple copies of putative STREs interspersed with matches to the Abf1- and

**Rap1-binding sites.** Isolation of a genomic clone containing up to 609 nucleotides of the *HSP12* 5'-flanking region relative to the translation start site has been reported previously (55). To verify whether this DNA fragment was sufficient to confer wild-type regulation to a GUS reporter gene, we screened a YEpl3-based yeast genomic DNA library for clones possessing a longer 5'-flanking region. After two rounds of in situ screening, we isolated two identical clones (pBV2 and pBV3) with a



TABLE 2. Primers used for site-directed mutagenesis

Primer <sup>a</sup>	Sequence <sup>b</sup>	Location <sup>c</sup>
d100	5'-CCCCTCTTGATCTctgcaGTCTTTCTCTAAATC-3'	-190 to -158
d101	5'-AGGTTCCGGTTCGTCaagCTtaTGATCTGAAAGG-3'	-204 to -172
d102	5'-GTAAGTATAGATCTACAaagCTtaGAAAATCGTTTG-3'	-248 to -215
d103	5'-CACAGACCAGAAGCACACAGCCCCCTGAAAATCG-3'	-276 to -261 () -236 to -219
d104	5'-CCAGAAGCACTCTAGtatGAGAGTAACTAGATC-3'	-270 to -238
d105	5'-GCTGGCATCTGTTAAGGGAAAAGGAAAAGGAAAAGG-3'	-449 to -434 () -368 to -349
d106	5'-CAGCTGGCGGTAGCAATGGCTCTTGGGACAAGGAC-3'	-553 to -536 () -513 to -497
d107	5'-GAAGCACTCTAGACGaAttTAAGTATAGATCTACAG-3'	-267 to -233
GUS-inv	5'-GTTTCTACAGGACGTAACATAAGG-3'	
EV2	5'-CGCCAGGGTTTTCCAGTCAGGAC-3'	

<sup>a</sup> To facilitate annealing during the amplification of the final product, oligodeoxynucleotides d100 through d107 were used in conjunction with complementary primers (33). GUS-inv and EV2 are, respectively, primers annealing with the sense strand of the GUS open reading frame and with the vector sequences (antisense strand) upstream of the *HSP12* promoter region on the Y1plac211-based constructs used for amplification in combination with the mutagenic primers.

<sup>b</sup> Nucleotides which differ from the wild-type sequence are given in lowercase.

<sup>c</sup> Location (5'→3') of the sequences in the *HSP12* promoter region annealing to the primer (Fig. 3). (), internal deletion.

4.8-kb insert hybridizing with a promoter region-specific probe (Fig. 1A, probe 1) and a 0.6-kb cDNA fragment containing the *HSP12* open reading frame (Fig. 1A, probe 2). Restriction and sequencing analysis confirmed the identity of the clones. An EMBL/GenBank database search revealed that the extended *HSP12* 5'-flanking region comprises the *MDJ1* gene (60) and, further upstream, part of the *LPD1* promoter region (57). The *HSP12* and *MDJ1* genes have been assigned to chromosome VI and are located between the *SUF9* and *CDC4* genes (60, 71).

The intragenic sequence shared by the *HSP12* and *MDJ1* promoter regions contains seven copies of the CCCCT motif (40) in different orientations (Fig. 2). Five CCCCT repeats as well as two putative heat shock elements are located in the proximal 606 bp of the *HSP12* 5'-flanking region (Fig. 3). In addition, a match to the Abf1-binding site consensus sequence,

similar to the one present in the gene encoding ribosomal protein S33 (21), is centered at position -260 relative to the *HSP12* translation start site. Further upstream, a match to the Rap1 consensus sequence (84) spans the nucleotides between -566 and -554 (Fig. 3 and 4).

The proximal 606 bp of the *HSP12* 5'-flanking region are necessary and sufficient to confer wild-type regulation on an *HSP12*-GUS fusion gene upon osmostress. To map the functional stress-responsive *cis*-acting elements present in the *HSP12* promoter region, different *HSP12*-GUS fusion genes were constructed and integrated at the *URA3* locus (see Materials and Methods). pKV1, a construct bearing approximately 3,300 bp of the *HSP12* 5'-flanking region (Fig. 1B), caused the accumulation of GUS mRNA upon a shift to 0.7 M NaCl, which paralleled the induction of the endogenous *HSP12* gene (Fig. 1C). Similar results were obtained when

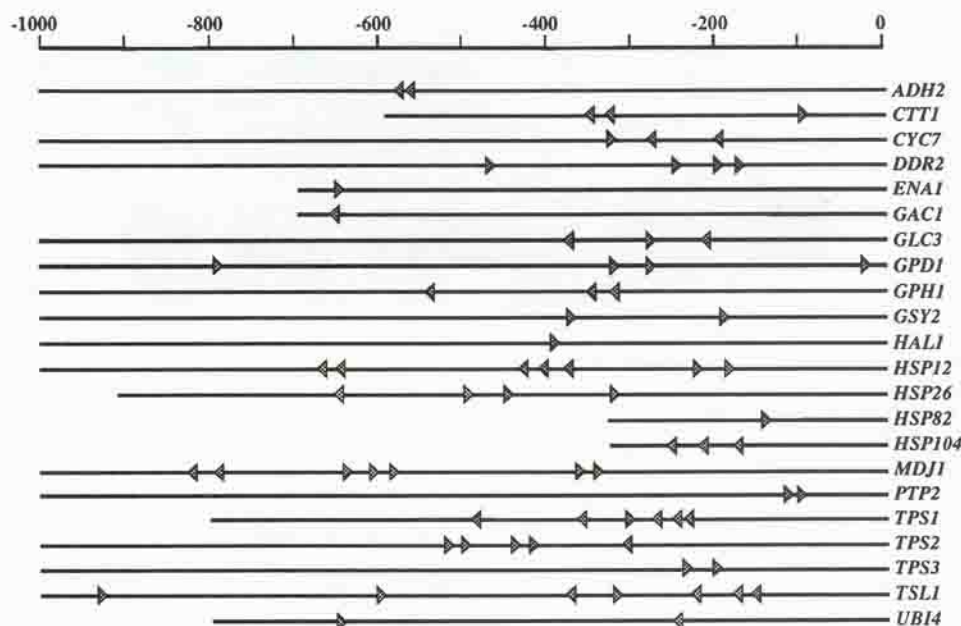


FIG. 2. Schematic representation of stress-inducible promoters containing STRE-like sequences. Promoters of genes known to be induced by heat shock, salt stress, and/or entry into stationary phase were searched for the presence of the CCCCT motif in both strands. Lines represent the length of the 5'-flanking region searched. Arrowheads indicate the location of the motifs. Arrowheads pointing right and left represent CCCCT motifs on the sense and antisense strands, respectively. Sequences are from the GenBank and EMBL databases. For further information, see the text and references 1, 6, 18, 39, 41, 45-47, 52, 55, 60, 62, 76, 80, 82, and 83.





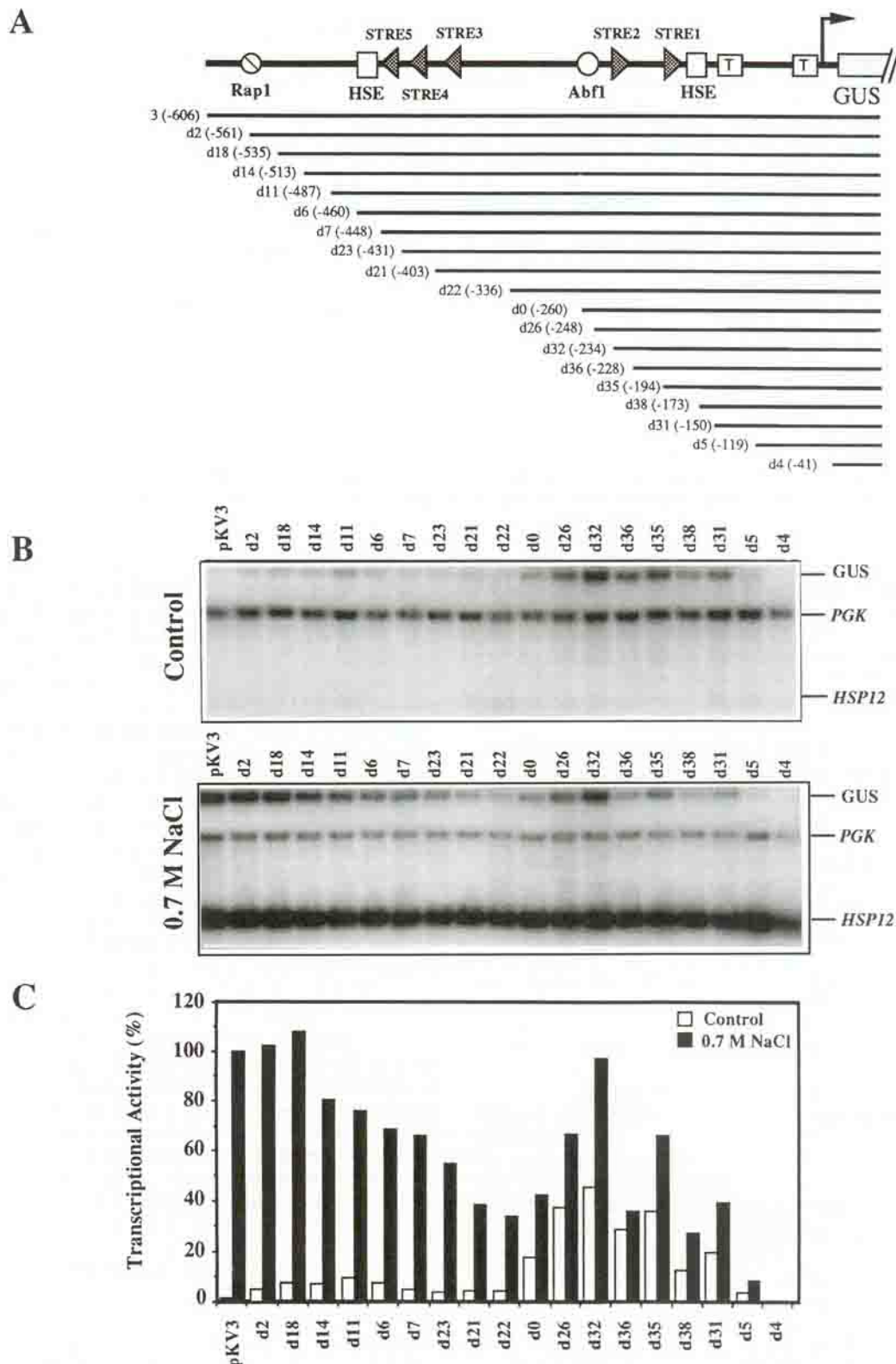


FIG. 4. 5' deletion analysis of the *HSP12* promoter region. (A) Exonuclease III-generated *HSP12*-GUS fusion genes. The 5'-end locations of the different *HSP12* promoter region alleles are indicated on the left of each construct. T, TATA box. Arrowheads pointing right and left represent CCCCT motifs on the sense and antisense strands, respectively. (B) Northern analysis of single-copy integrants growing exponentially in SD minimal medium and exposed to 0.7 M NaCl for 30 min. (C) Quantification of GUS mRNA levels shown in panel B, corrected for RNA loading variations (see Fig. 1 and Materials and Methods). The induced level of pKV3, the wild-type *HSP12*-GUS allele, was set at 100%.

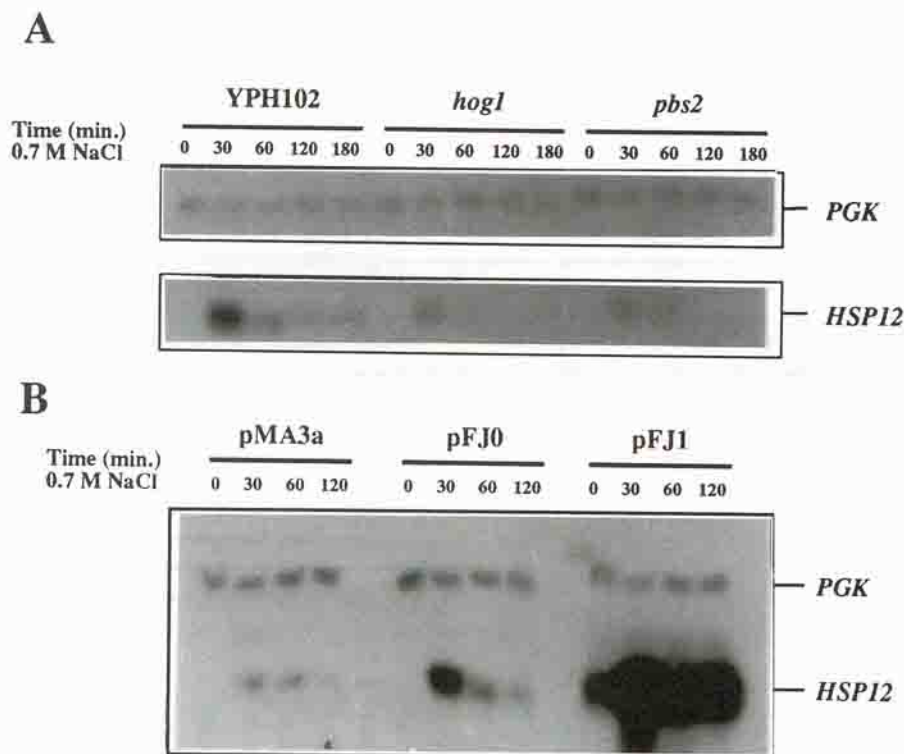


FIG. 5. Modulation of the *HSP12* gene expression by the HOG pathway. (A) Northern analysis of YPH102, JBY13 (*hog1*), and JBY43 (*pbs2*) cells growing exponentially in SD minimal medium and exposed to 0.7 M NaCl for 30, 60, 120, and 180 min. Quantification of the signals obtained (30-min samples) indicated a fourfold reduction of *HSP12* mRNA levels in the mutant strains compared with levels in YPH102 cells. (B) Northern analysis of YPH102 cells transformed with the multicopy plasmid pMA3a as well as plasmids pFJ0 and pFJ1 (pMA3a derivatives carrying the *HOG1* and *HSP12* genes, respectively). These strains were subsequently exposed to 0.7 M NaCl for 0, 30, 60, and 120 min. In this case, approximately 1/10 of the usual specific activity for the *HSP12* probe was used. This explains the weak signal obtained with RNA isolated from wild-type cells.

**The putative Abf1-binding site is not essential for the regulation of basal and salt-induced *HSP12* gene expression.** To determine whether the putative Abf1-binding site plays a role in the regulation of the *HSP12* gene expression, we examined the effect of a mutation in the highly conserved ACG trinucleotide at the 3' end of the Abf1 consensus sequence (Fig. 6B, clone d104). Although Abf1 is unable to bind to this mutant sequence (21), the pattern of gene expression in cells carrying d104 was indistinguishable from that in wild-type transformants (pKV3). Comparable results were obtained by mutating sequences immediately downstream of the Abf1-binding site (Fig. 6B, d107) or deleting 22 nucleotides between positions -261 and -240 (Fig. 6B, d103), which encompass most of the Abf1-binding site and sequences upstream of STRE2.

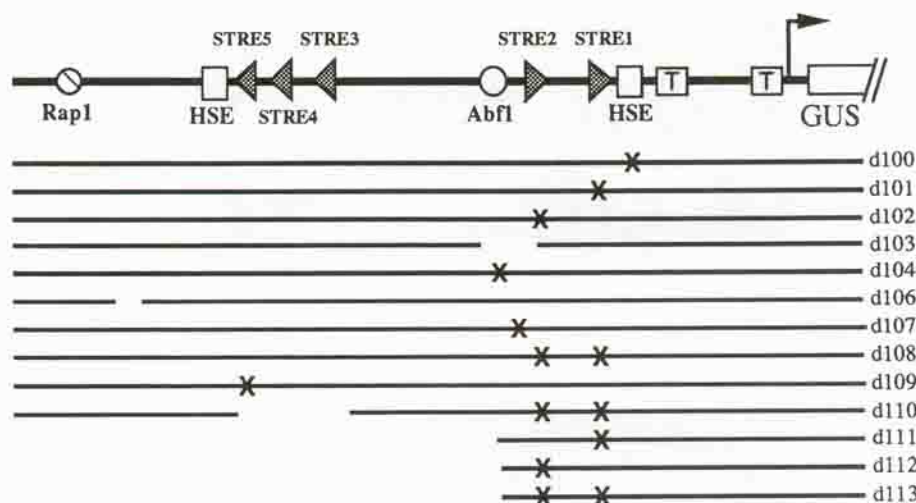
**The CCCCT elements (STREs) are essential for the induction of *HSP12* gene expression upon heat stress and entry into stationary phase.** Both the CCCCT motif and the HSE have been implicated in the activation of heat-induced genes (39, 40, 45, 47). To assess the relative importance of the two putative HSE sequences and the different STRE repeats present in the *HSP12* promoter region, we heat shocked cells carrying d100, d102, d108, and d110 constructs (Fig. 7). Mutation of the proximal HSE (d100) did not affect either basal or induced levels of the GUS mRNA. In contrast, the accumulation of GUS transcript was severely impaired in transformants harboring d102, d108, and d110 clones upon heat shock (Fig. 7), suggesting that the CCCCT elements rather than the HSEs are involved in the activation and derepression of the *HSP12* gene expression in heat-shocked cells. This conclusion seems to be supported by results obtained with a temperature-sensitive al-

lele of the heat shock transcription factor (*hsf1-m3*) (68). Upon a shift to the restrictive temperature, similar levels of the *HSP12* mRNA accumulated in *hsf1-m3* and congenic wild-type cells (data not shown).

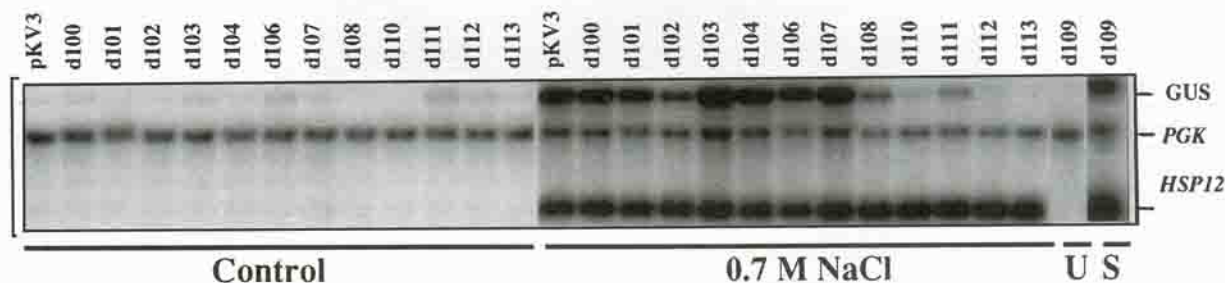
Upon glucose exhaustion and entry into stationary phase, yeast cells adjust their pattern of gene expression, repressing and inducing various genes (for a review, see reference 83). The latter comprise genes encoding several stress proteins, for example, *SSA3* (10), *HSP26* (41), and *HSP12* (55). To verify whether the CCCCT motifs and/or the HSEs were involved in the induction of *HSP12* gene expression during the diauxic shift, the set of strains described above was grown on SD minimal medium until stationary phase (Fig. 8A). As in heat-shocked cells, only mutations in the STREs, particularly STRE2, abolished the *HSP12* inducibility (Fig. 8B). The involvement of CCCCT motifs in gene activation and derepression upon entry into stationary phase may be in agreement with data obtained previously during the dissection of the *SSA3* promoter (see Discussion).

***HSP12* gene expression is modulated by the osmosensing HOG pathway.** It has been shown that a number of salt-induced yeast genes are regulated via the HOG pathway (1, 65), a cascade of MAP kinases showing homology with other yeast and animal protein kinases responding to extracellular signals (13, 31, 76). To examine whether the HOG pathway is involved in signal transduction leading to the activation of *HSP12* gene expression, cells mutated in genes encoding two components (*Hog1* or *Pbs2*) of this pathway were salt shocked and the accumulation of the *HSP12* mRNA was monitored by Northern analysis (Fig. 5A). In wild-type cells the *HSP12* tran-

A



B



C

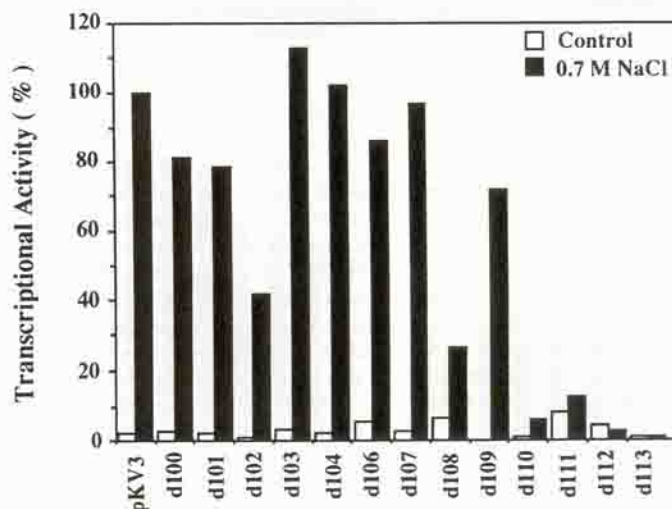


FIG. 6. Dependence of *HSP12* salt inducibility upon the CCCCT elements. (A) Point (X) and internal deletion (gaps) analysis of the *HSP12* promoter region performed by site-directed mutagenesis (see Materials and Methods). The mutagenic primers used are listed in Table 2. T, TATA box. (B) Northern analysis of single-copy integrants growing exponentially in SD minimal medium and exposed to 0.7 M NaCl for 30 min. For clone d109, U and S denote control (unshocked cells) and salt samples, respectively. (C) Quantitation of GUS mRNA levels shown in panel B, corrected for RNA loading variations.



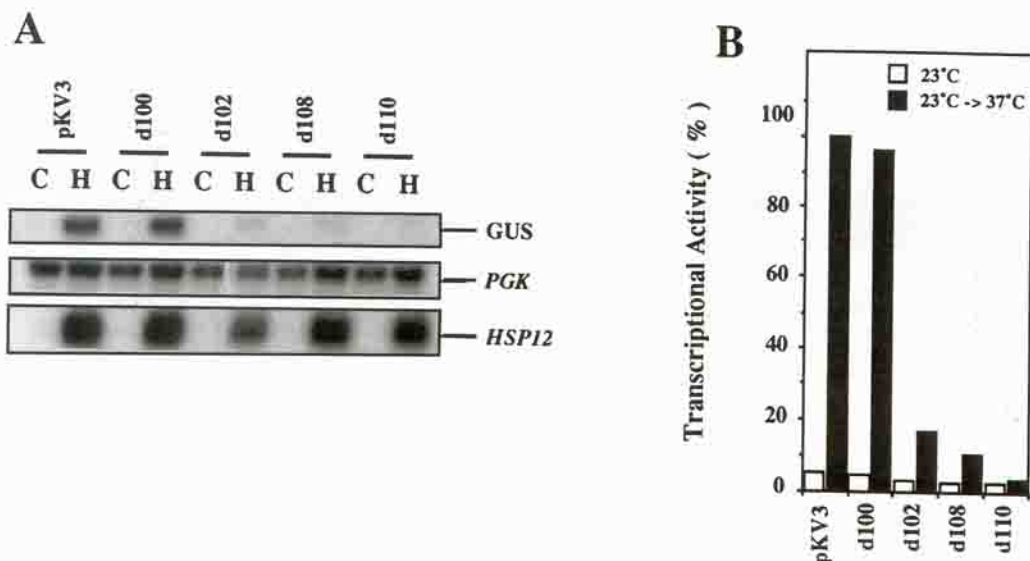


FIG. 7. Heat-induced *HSP12* gene expression. (A) Northern analysis of single-copy integrants growing exponentially in SD minimal medium at 23°C and shifted to 37°C for 30 min. C, control cells; H, heat-shocked cells. (B) Quantitation of GUS mRNA levels shown in panel A, corrected for RNA loading variations.

script levels increased normally 30 min after stress onset. *hog1* and *pbs2* cells, however, exhibited only a slight induction of *HSP12* transcription (Fig. 5A).

Modulation of the *HSP12* gene expression by the HOG pathway upon osmotic stress was confirmed by the observation that induced levels increased approximately fivefold in cells overexpressing *HOG1* compared with levels in cells carrying an empty vector (Fig. 5B). Higher levels were observed in cells harboring pFJ1, a derivative of the multicopy plasmid pMA3a carrying the *HSP12* gene (Fig. 5B). Despite the enormous accumulation of the *HSP12* transcript, these cells did not show improved growth on salt plates (data not shown), in accordance with results obtained earlier with *hsp12* and *hsp12 hsp26* null mutants (80).

***HSP12* gene expression is negatively regulated by the Ras-PKA pathway.** Since *HSP12* basal expression increases upon glucose deprivation (Fig. 8) (71) and with low adenylate cyclase activity (55), we investigated the role of the Ras-PKA pathway in the regulation of this gene upon salt and heat stress. For this purpose, cells carrying mutations in genes coding for the catalytic (*TPK1*, *TPK2*, and *TPK3*) or regulatory (*BCY1*) subunits of PKA or containing a dominant mutation in a stimulator of the adenylate cyclase (*RAS2<sup>Val-19</sup>*) (for a review, see reference 76) were exposed to stress, and gene expression was determined by Northern analysis (Fig. 9A). Cells possessing only one active isotype of the catalytic subunit of PKA (*S7-7A*) exhibited a pattern of *HSP12* expression similar to that in the wild-type strain (Fig. 9B, bars 1 and 5). However, in a congenic strain bearing an additional mutation in the *BCY1* gene (*S13-3A*), resulting in high PKA activity, accumulation of the *HSP12* mRNA was partially or totally abolished upon exposure to salt or heat stress, respectively (Fig. 9B, bars 6). A less pronounced reduction was observed in *RAS2<sup>Val-19</sup>* cells under heat stress (Fig. 9B, bars 2). *tpk1<sup>wt</sup> tpk2 tpk3* strains, either bearing the *bcy1* lesion or not, showed high basal levels, poor inducibility upon heat stress, and a three- to fourfold increase in *HSP12* mRNA levels upon salt stress (Fig. 9B, bars 3 and 4). Taken together, these results suggest that the *HSP12* gene expression is repressed by high PKA activity. On the other hand, an overstimulated adenylate cyclase (*RAS2<sup>Val-19</sup>* cells) seems to produce a less noticeable effect (Fig. 9B, bars 2).

**Yap1 and Yap2/Cad1 are not essential for the induction of *HSP12* gene expression in stressed cells.** A recent report has implicated the Yap1 transcriptional activator in the regulation of stress-induced genes through the CCCCT element (28). In order to test whether *HSP12* gene expression is regulated via Yap1 or its close homolog, Yap2/Cad1 (11, 85), *yap1* and *yap2* disruption mutants were heat and salt shocked and exposed to oxidative stress ( $H_2O_2$ ). Northern analysis showed no significant difference in the pattern of gene expression between a congenic wild-type strain and the mutant strains exposed to salt, heat, or oxidative stress (Fig. 10). Hence, in contrast with transcription activation of the *TPS2* gene (28), STRE-mediated transcription activation of *HSP12* appears to be independent of Yap1 and Yap2.

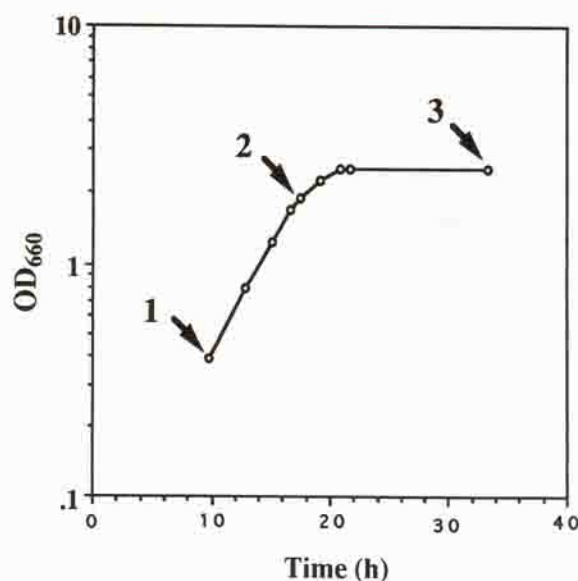
## DISCUSSION

We have cloned an extended 5'-flanking region of the *HSP12* gene and identified the regulatory *cis*-acting elements able to confer stress inducibility on a reporter gene. Furthermore, we have shown that *HSP12* gene expression is modulated by the osmosensing HOG and the Ras-adenylate cyclase pathways.

Two findings provided evidence that the HOG pathway stimulates *HSP12* gene expression in cells under osmotic stress (Fig. 5). First, disruption of the *HOG1* or *PBS2* gene results in a fourfold decrease in the levels of *HSP12* mRNA in salt-stressed cells. On the other hand, cells overproducing *Hog1* show higher induced levels upon a similar shift to high salt concentrations. The activation in osmotressed cells of *HSP12* and genes other than *GPD1* via the HOG pathway indicates that this MAP kinase cascade, apart from being involved in osmoregulation, plays an important role in processes not primarily related to the stimulation of glycerol synthesis (1, 13, 65). Consistent with this, *hog1* and *pbs2* cells exhibit physiological disturbances such as abnormal budding patterns and a defective cytoskeleton repositioning upon recovery from osmotic stress (12).

Although *Hsp12* is massively induced upon osmotic stress as well as other stresses, the function of this protein is still unclear. The apparent wild-type phenotype of *hsp12* and *hsp12*

A



B

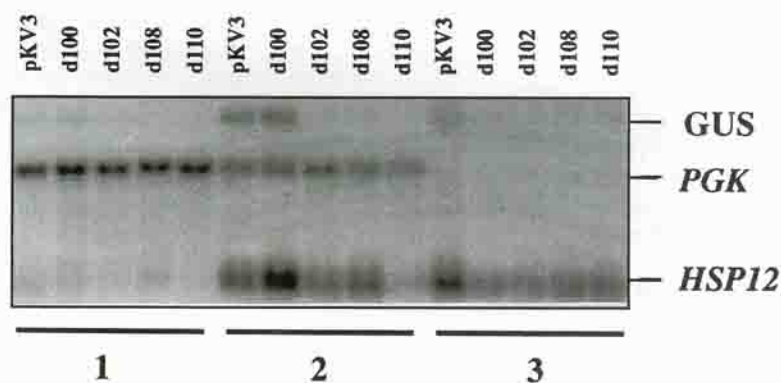


FIG. 8. Post-diauxic shift-induced *HSP12* gene expression. (A) Optical density at 660 nm ( $OD_{660}$ ) of cultures sampled at different times. Arrows correspond to lane numbers in panel B. (B) Northern analysis of batch cultures growing exponentially in SD minimal medium at 30°C. Lanes: 1,  $OD_{660} \approx 0.37$ ; 2,  $OD_{660} \approx 2.0$ ; 3,  $OD_{660} \approx 2.5$ . Low levels of endogenous *HSP12* mRNA in sample d110 are considered an experimental artifact.

*hsp26* cells under heat and osmotic stress (55, 80) is in agreement with the fact that *HSP12* overexpression does not improve growth on high-osmolarity media (Fig. 5 and data not shown). Functional overlap among stress proteins, masking their involvement in stress tolerance acquisition, has been recently uncovered (62). In order to address this possibility, a synthetic lethality screening for mutants requiring the *HSP12* gene on high-osmolarity media is under way.

The high stress inducibility of the *HSP12* gene is likely to be accounted for by the presence of multiple CCCCT elements in the *HSP12-MDJ1* intragenic region. Deletion or introduction of point mutations in any of the five most proximal STREs led to a decrease in transcriptional activity in stressed cells (Fig. 1, 3, 4, and 6 through 8). A more drastic drop in the overall stress inducibility of this promoter was observed when the CCCCT repeat positioned at -232 (STRE2) is mutated. However, when STRE2 was the only intact CCCCT motif (Fig. 6C, clone d111), induction of transcription by osmotic stress was virtually

abolished. Thus, although all CCCCT motifs downstream of position -606 appear to contribute to the overall stress inducibility of *HSP12*, STRE2 seems to be the most important element for attainment of high induced levels. On the other hand, these results indicate that STRE2 is functional only in combination with the three distal STREs (STRE3 to STRE5) and the proximal STRE, STRE1. This suggests that a high degree of synergism among different repeats is needed for efficient transcriptional activation. This conclusion is supported by previous results obtained with artificial promoters bearing one or more CCCCT repeats in both orientations (40, 47). Promoters containing one CCCCT motif were induced two- to eightfold upon stress imposition; however, the introduction of two or more STREs generated 10- to 32-fold induction ratios.

The essential role played by the *HSP12* CCCCT elements in transcription activation upon exposure to a broad range of stresses contrasts with the seeming dispensability of the putative HSEs (Fig. 4 and 6 through 8). HSE sequences have been



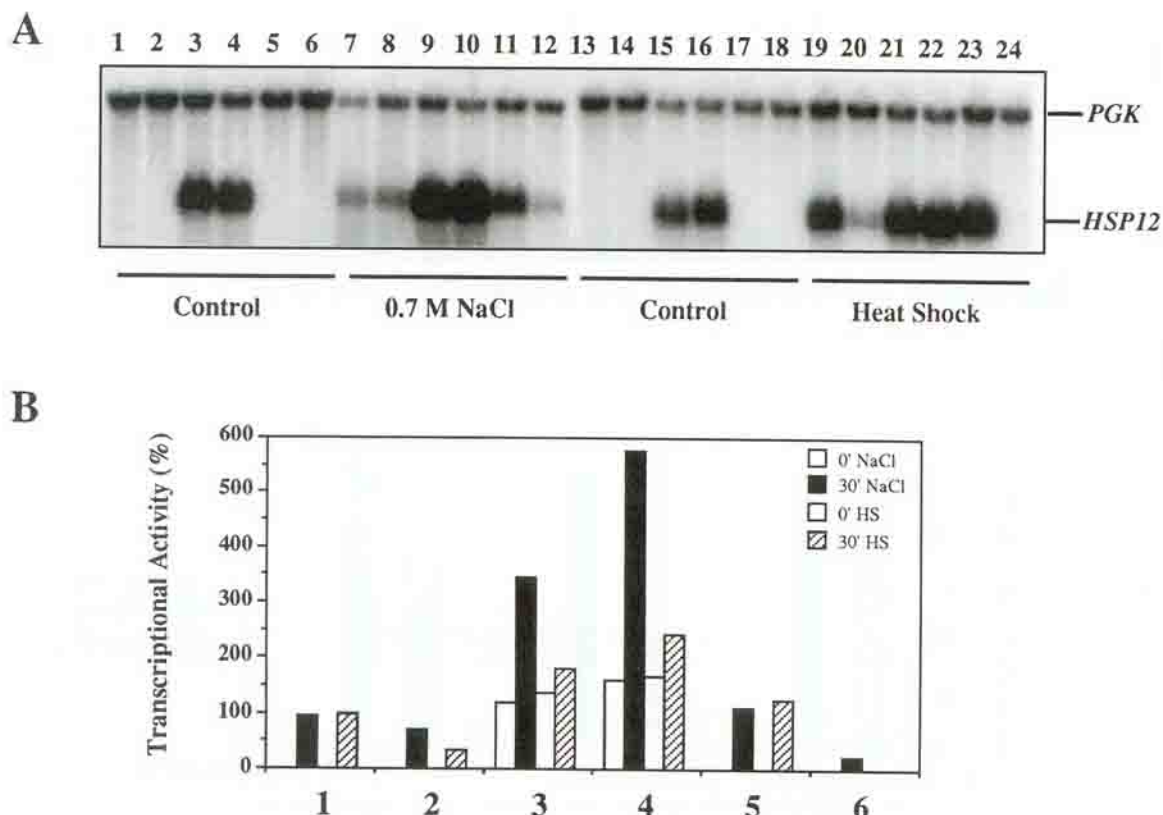


FIG. 9. Effect of the Ras-PKA pathway on *HSP12* gene expression. (A) Northern analysis of SP1 (lanes 1, 7, 13, and 19), TK161-R2V (*RAS2<sup>Y40-19</sup>*) (lanes 2, 8, 14, and 20), S18-D (*tpk1<sup>ts</sup> tpk2 tpk3*) (lanes 3, 9, 15, and 21), RS13-58A-1 (*tpk1<sup>ts</sup> tpk2 tpk3 bcy1*) (lanes 4, 10, 16, and 22), S7-7A (*tpk2 tpk3*) (lanes 5, 11, 17, and 23), and S13-3A (*tpk2 tpk3 bcy1*) (lanes 6, 12, 18, and 24) cells exposed to 0.7 M NaCl for 0 min (lanes 1 through 6) and 30 min (lanes 7 through 12). Concomitantly, the same set of strains was shifted from 23 to 37°C for 0 min (lanes 13 through 18) and 30 min (lanes 19 through 24). (B) Quantification of *HSP12* mRNA levels shown in panel A, corrected for RNA loading variations. Bars 1, SP1; bars 2, TK161-R2V; bars 3, S18-D; bars 4, RS13-58A-1; bars 5, S7-7A; and bars 6, S13-3A.

implicated in the regulation of several heat-induced genes (45). On the other hand, HSEs appear to be unresponsive or extremely dependent on the promoter context for an efficient response to stress signals such as glucose starvation or nutrient depletion (10, 74). This raises the question of whether the broad stress responsiveness of STRE-like sequences may explain cross-protection and the partial overlaps observed among stress responses. For example, it is known that a yeast exposed to heat shock, osmotic stress, and nutrient depletion accumulates trehalose and a variable set of HSPs as well as other proteins, depending upon the kind of stress imposed (34, 45, 46, 83). Furthermore, all these stresses have been shown to enhance thermotolerance (43, 63, 78, 80). Although mechanisms leading to acquisition of stress tolerance are complex and may vary according to the physiological state of the cell, the accumulation of stress proteins and/or trehalose has been proposed to contribute to enhanced survival under stressful conditions (19, 43, 62, 79). When several 5'-flanking regions of genes induced by heat shock, nutrient starvation, and/or osmotic stress were searched for sequences matching the CCCCT motif, we observed that most of them contain multiple STRE-like sequences (Fig. 2). As in the *HSP12-MDJI* intragenic region, *TPS1*, *TPS2*, and *TSL1* promoters contain five or more CCCCT repeats. Interestingly, these genes code for different subunits of the trehalose synthase-phosphatase complex (6, 18, 81), which are induced by heat shock (*TPS1* and *TPS2*) as well as osmotic stress (*TPS2*) (6, 18, 28). Likewise, all subunits accumulate in a coordinate fashion in stationary-phase cells (81). Although promoter studies of these genes are still lacking, the

observed broad stress responsiveness suggests that STRE-like sequences may play an important role in the coordinate expression of genes apparently involved in stress tolerance acquisition.

The presence of STREs in stress-inducible promoters does not preclude the involvement of other sequences. In some cases the role of an (defective?) STRE-like sequence may depend on the combinatorial action with another *cis*-acting element. An example of this may be the *SSA3* promoter, in which a CCCCT-like element (post-diauxic shift element) has been found to be dependent on a downstream HSE for efficient transcriptional activation upon nutrient limitation (10). On the other hand, heat-inducible promoters failing to respond to stationary-phase signals, such as *SSA1* or *SSC1* (82), may be regulated via HSEs rather than STRE-like sequences.

Despite our detailed analysis of the *HSP12* promoter, the data obtained so far did not disclose the precise location of negative regulatory elements. Regions overlapping or flanking the putative Abf1- and Rap1-binding sites appear to confer some degree of repression (Fig. 4). However, site-directed mutagenesis of the Abf1-binding site and flanking sequences did not produce any change in basal levels (Fig. 6). This indicates the existence of redundant mechanisms for negative regulation of *HSP12* transcription. Inactivation of the factor(s) binding to the STRE by posttranslational modification via PKA is a possibility. Our results (Fig. 9) as well as studies with artificial promoters containing STRE sequences (47) strongly suggest that the CCCCT motif is under negative control of PKA. Alternatively, negative regulation of a factor (Yap1)

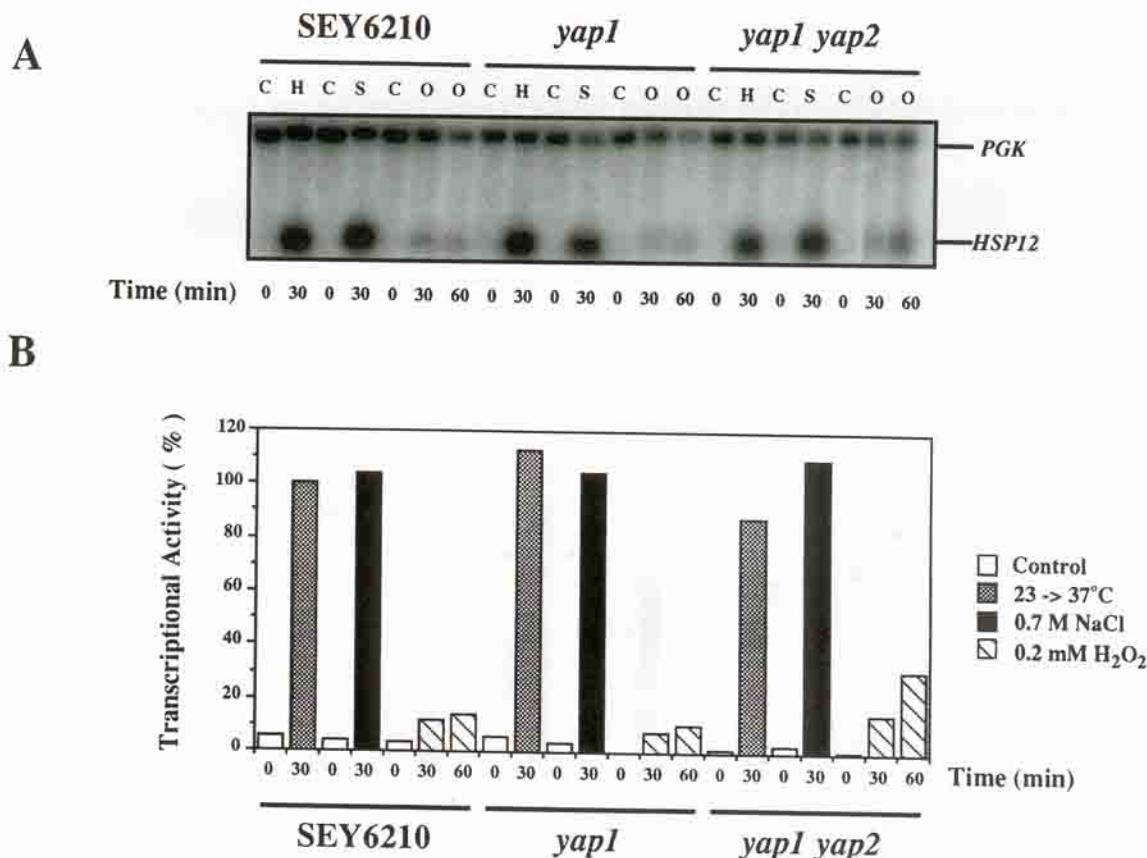


FIG. 10. Effect of the Yap1 and Yap2/Cad1 transcriptional activators on *HSP12* gene expression. (A) Northern analysis of SEY6210, SM10 (*yap1*), and YAW10 (*yap1 yap2*) cells shifted from 23 to 37°C for 0 and 30 min. In addition the same set of strains was exposed to 0.7 M NaCl for 0 and 30 min as well as to 0.2 mM H<sub>2</sub>O<sub>2</sub> for 0, 30, and 60 min. C, control; H, heat shock; S, 0.7 M NaCl; O, 0.2 mM H<sub>2</sub>O<sub>2</sub>. (B) Quantification of *HSP12* mRNA levels shown in panel A, corrected for RNA loading variations. The bars in this panel correspond to the lanes in panel A.

binding to or operating upstream of the STRE-binding factor(s) by PKA has been postulated (28). *HSP12* gene expression appears, however, to be largely Yap1 and Yap2 independent (Fig. 10). The observed difference may be due to the presence or absence of ancillary sequences modulating STRE-driven transcription in the *HSP12* and *TPS2* promoters. The context specificity of such an interaction is consistent with differences found between a STRE-*lacZ* reporter construct and the *CTT1* gene (65a). Although the artificial promoter showed a severe dependence upon the *YAP1* gene, similar levels of *CTT1* mRNA were observed in wild-type and *yap1* cells. A third possibility of negative regulation of the *HSP12* gene is via Mig1-like sequences. Close inspection of STRE2 and flanking sequences (tttccAGGGG; lower- and uppercase letters represent nucleotides flanking and encompassing STRE2, respectively) revealed a striking resemblance with the Mig1 consensus sequence (WWWWSYGGGG [20]; W = A or T, S = C or G, Y = C or T). Mig1 has been implicated in the down-regulation of genes under glucose repression (56). It remains to be investigated whether Mig1 or related *trans*-acting factors may contribute to the maintenance of low levels of *HSP12* mRNA in glucose-containing media (55, 71).

Despite the seeming redundancy of repressing mechanisms, the down-regulation of stress genes exerted by PKA may explain in part the *HSP12* inducibility upon nutrient limitation. Unlike exponentially growing yeast cells, post-diauxic shift and stationary-phase cells contain low levels of cyclic AMP (24). This drop in cyclic AMP concentration may be the trigger for

activation of transcription or derepression. However, growing evidence suggests the involvement of cyclic AMP-independent pathways in nutrient sensing as well as thermotolerance acquisition (14–16, 76, 77). This does not exclude the involvement of PKA from modulating the different stress responses. Indeed, the involvement of a free catalytic subunit(s) of PKA in the induction of thermotolerance and repression of stress genes during refeeding of nutrient-starved cells has been proposed (15, 76). The notion that free PKA catalytic subunits are responsive to signals other than variations of intracellular levels of cyclic AMP predicts a new role for this second messenger. Cyclic AMP may solely be a measure for growth potential, varying the pool of Bcy1-free PKA catalytic subunits according to the physiological state of the cell. This free pool may subsequently be activated by alternative (cyclic AMP-independent) pathways (76). The mechanism proposed would provide a way to integrate signals triggered by diverse extracellular events, such as stress, with the need of the cell to determine whether the conditions are suitable for growth and mitosis (see below).

Cross-talk of the Ras-PKA pathway with other signal transduction pathways may be illustrated by the opposite effects of PKA and the HOG pathway upon *HSP12* gene expression (Fig. 5 and 9). It has been proposed that the HOG pathway may interact with a target downstream of Ras1 and Ras2 (9). Recent genetic evidence suggests, however, that both pathways operate in parallel rather than in a colinear fashion (65). Nevertheless, the observed antagonism may explain differences in



the effect of mutations in the Ras-PKA pathway on *HSP12* inducibility upon osmotic stress and heat shock (Fig. 9). Wild-type cells or cells containing one functional *TPK* gene show similar induced levels upon both stresses. However, in mutant cells containing high or low PKA activity, levels induced upon osmotic stress are generally higher than levels induced upon heat shock. Since the HOG pathway appears to be specifically activated by osmotic stress (65), this suggests that the Pbs2-Hog1 cascade is able to partially override the negative effect of high PKA activity in cells shifted to high-osmolarity media. On the other hand, a defective PKA may be unable to counteract the effect of the HOG pathway, resulting in abnormally high levels of *HSP12* mRNA upon osmotic stress.

The involvement of the Ras-PKA and other pathways previously implicated in growth control or nutrient signalling in the regulation of stress genes as well as stress tolerance acquisition may indicate that responses to stress are intimately coupled to processes controlling cell growth and mitosis (23, 26, 30, 66, 76, 77) (see above). The finding that the stress-responsive CCCCT motif is under the regulation of cyclic AMP and a MAP kinase cascade may provide the molecular basis for such a connection. Because of the apparent conservation of molecular mechanisms for signalling stress among eukaryotes (see the introduction and reference 48), it is tempting to suggest that similar mechanisms of transcriptional regulation may occur in metazoans, including mammals.

#### ACKNOWLEDGMENTS

We are indebted to P. Bossier and C. Rodrigues-Pousada (Lisbon, Portugal), M. Gustin (Houston, Tex.), H. Ruis (Vienna, Austria), W. S. Moye-Rowley (Iowa City, Iowa), J. Thevelein (Leuven-Heverlee, Belgium), and M. Yaffe (La Jolla, Calif.) for providing strains and sharing unpublished data. We thank W. Mulder and L. Grivell for the YEp13-based yeast genomic DNA library. Undergraduate students C. van Beekvelt, P. Boon, F. Kessler, and H. de Kruijff and graduate students A. Goverde and J. Schotting made important contributions to this work.

This work was supported in part by the Junta Nacional de Investigação Científica e Tecnológica (Portugal) through a grant to J.C.S.V.

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