

A Heat Shock Transcription Factor with Reduced Activity Suppresses a Yeast HSP70 Mutant

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Strains carrying deletions in both the *SSA1* and *SSA2* HSP70 genes of *Saccharomyces cerevisiae* exhibit pleiotropic phenotypes, including the inability to grow at 37°C or higher, reduced growth rate at permissive temperatures, increased HSP gene expression, and constitutive thermotolerance. A screen for extragenic suppressors of the *ssa1 ssa2* slow-growth phenotype identified a spontaneous dominant suppressor mutation, *EXA3-1* (R. J. Nelson, M. Heschl, and E. A. Craig, *Genetics* 131:277–285, 1992). Here we report that *EXA3-1* is an allele of *HSF1*, which encodes the heat shock transcription factor (HSF). Strains containing the *EXA3-1* allele in a wild-type background exhibit a 10- to 15-fold reduction in HSF activity during steady-state growth conditions as well as a delay in the accumulation of the *SSA4*, *HSP26*, and *HSP104* mRNAs after a heat shock. *EXA3-1*-mediated suppression is the result of a single amino acid substitution of a highly conserved residue in the HSF DNA-binding domain which drastically reduces the ability of HSF to bind to heat shock elements as evaluated by band shift analysis. Together, these results indicate that the poor growth of *ssa1 ssa2* strains is the result, at least in part, of the overproduction of a deleterious heat shock protein(s). This conclusion is supported by the fact that the levels of at least some heat shock proteins are reduced in *ssa1 ssa2* cells containing the *EXA3-1* allele. Surprisingly, strains containing the *EXA3-1* allele in a wild-type HSP70 background grow nearly as well as the wild-type strain over a wide temperature range, displaying only a slight reduction in growth rate at 37°C, indicating that cells contain significantly more HSF activity than is required for growth under steady-state conditions.

The heat shock response, among the most evolutionarily conserved features of living cells, has been found in all organisms in which it has been investigated, including representatives from the eukaryotic, archeal, and eubacterial kingdoms. The cellular response to heat shock and other stresses involves the induction of a small number of proteins collectively referred to as heat shock proteins (hsps). Among these are the Hsp70s, so designated for their approximate molecular mass of 70 kDa. Hsp70s are believed to function in a variety of cellular processes, including protein folding, translocation of proteins across membranes, and regulation of the heat shock response (3, 7, 11).

In the yeast *Saccharomyces cerevisiae*, at least 10 HSP70-like genes have been identified and classified into five families (*SSA* to *SSE*) on the basis of functional criteria and sequence similarity (15, 17, 24, 26). Of special interest to this report is the cytosolically localized *SSA* family. The *SSA* family contains four members designated *SSA1* to *SSA4* (13). Moderate- to high-level synthesis of at least one of the *SSA* gene products is required for cell viability (36). During steady-state growth conditions, the *SSA1* and *SSA2* genes are expressed at moderate levels, while *SSA3* and *SSA4* gene expression is undetectable. Following heat shock, *SSA1* expression increases and *SSA3* and *SSA4* expression is strongly induced (13). Deletion of either *SSA1* or *SSA2* does not result in an obvious phenotype. However, deletion of both genes results in pleiotropic effects. *ssa1 ssa2* double mutants are unable to form colonies at 37°C and exhibit slower growth rates than wild-type strains at all other temperatures tested (8). *ssa1 ssa2* strains also exhibit elevated

levels of hsp synthesis. Indeed, it is high-level expression of *SSA4*, under conditions in which it is not normally expressed, that allows *ssa1 ssa2* strains to survive (36). Interestingly, *ssa1 ssa2* strains exhibit an elevated level of constitutive thermotolerance. *ssa1 ssa2* cells are able to survive exposure to a transient severe heat shock which would kill wild-type cells, presumably as a result of this increased HSP gene expression (36).

HSF1, an essential gene, encodes the heat shock transcription factor (HSF), which is the primary transcriptional activator of the heat shock response in *S. cerevisiae* (32, 37). HSF binds to the promoter regions of heat shock genes at heat shock elements (HSEs) as a homotrimeric complex (21, 30, 38, 39). HSEs are composed of a variable number of 5'-NGAAN-3' repeats arranged in alternating inverted orientation (1, 23). The structure of the DNA-binding domain of the HSF from *Kluyveromyces lactis* has recently been determined and indicates that this region has a helix-turn-helix structure (12). In *S. cerevisiae*, HSF binds DNA constitutively at HSEs, in contrast to higher eukaryotes, in which HSF acquires HSE-binding activity only upon heat shock (14, 28, 39, 41). The cellular response to growth at higher temperature mediated by yeast HSF has two components (19, 29). The first is the classical or transient heat shock response in which HSP gene mRNA synthesis is strongly induced, peaks, and declines. This is followed by a second component, the sustained response, in which a new, higher steady-state level of HSP mRNAs is maintained. However, small temperature changes can result in altered sustained activity without inducing a transient response (29).

The identification and analysis of extragenic suppressors has proven to be a powerful tool in elucidating regulatory mechanisms and pathways. Extragenic suppressors of *ssa1 ssa2* strains have been isolated to facilitate our investigation of the functions of the *SSA* gene products. One of these, *EXA3-1* (extra-

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genic suppressor of *HSP70* subfamily A), was identified as a dominant spontaneous genomic suppressor of the poor-growth phenotype of *ssa1 ssa2* strains at 30°C (18). We show here that *EXA3-1* is an allele of *HSF1*. Strains containing the *EXA3-1* mutation have reduced HSE-driven expression and reduced HSE-binding activity. These results have led us to conclude that the growth of *ssa1 ssa2* strains is impaired, at least in part, as a result of overexpression of some hsp(s) and that the *EXA3-1*-mediated suppression occurs by decreasing HSF activity.

MATERIALS AND METHODS

Yeast strains and transformations. All yeast strains used in this study have the following mutant alleles: *his3-11,15*, *leu2-3,112*, *lys1*, *lys2*, *trp1-Δ1*, and *ura3-52*. JN49 contains the *ssa1-3* and *ssa2-2* alleles (18); JN130 contains the *EXA3-1* allele in addition to *ssa1-3* and *ssa2-2*; MH297 contains the *EXA3-1* allele. After submission of this report, we discovered that the *EXA3-1* strain used for these experiments contained a mutation in the *HSP104* gene as first indicated by a lack of detectable Hsp104. Transformation of an *EXA3-1* strain with a plasmid containing *HSP104* restored synthesis of the protein but as expected had no effect on the expression studies reported here (data not shown and reference 22). *EXA3-1* cells containing the *HSP104* mutation grow slightly faster at 30°C than those having a wild-type *HSP104* gene. Although the reason why an *HSP104* mutation would have any positive effect, even slight, on the growth of an *EXA3-1* strain is not clear, it does explain why this mutation was selected for in the *EXA3-1* cell population. DS13, JN49, JN130, and MH297 are referred to as wild-type, *ssa1 ssa2*, *ssa1 ssa2 EXA3-1*, and *EXA3-1* strains, respectively, throughout this report.

Yeast transformations were performed by electroporation. For platings, direct cell counts were determined with a hemocytometer, and cultures were diluted to equal cell densities. Tenfold serial dilutions of each culture were made in sterile water, and 10 μl of each dilution was spotted onto solid media.

Bacterial strains, library construction, and DNA sequencing. *Escherichia coli* JM109, genotype K-12 F' *traD36 lacI^q Δ(lacZ)M15 proAB/recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ-Δ(lac-proAB)*, was the primary strain used for molecular cloning procedures.

The *HSF1* gene was isolated from wild-type and *EXA3-1* genomic DNA by screening a doubly size-selected library, using an *HSF1* gene fragment as the probe. Libraries were constructed by digesting genomic DNA with *Cla*I and gel purifying DNA fragments of greater than 10 kb. The purified DNA fragments were then digested with *Eco*RI and *Xho*I, and gel DNA fragments in the size range of 3.7 to 4.1 kb were purified from agarose gels and ligated to pUC18 DNA digested with *Eco*RI and *Xho*I. After transformation into *E. coli*, positive clones were identified by colony filter hybridization (2).

The DNA sequence of the wild-type and *EXA3-1 HSF1* clones was determined by the dideoxynucleotide chain termination method, using Sequenase (United States Biochemical). Ten nucleotide polymorphisms were detected in the wild-type sequence compared with the *HSF1* DNA sequence published by Sorger and Pelham (32). For purposes of identification, the polymorphic nucleotides are referenced by the position numbering in the Sorger sequence followed by the change in the nucleotide and amino acid encoded: polymorphism 1, 1217 G to A (Asp to Asn); polymorphism 2, 1403 T to C (Phe to Leu); polymorphism 3, 1413 C to T (Thr to Met); polymorphism 4, 1650 A to G (Asn to Ser); polymorphism 5, 1716 G to T (Ser to Ile); polymorphism 6, 1931 C to A (Gln to Lys); polymorphism 7, 1972 G to A (silent); polymorphism 8, 2691 G to T (Trp to Leu); polymorphism 9, 2697 A to G (Asn to Ser); polymorphism 10, 2787 C to G (Ser to Trp). Polymorphism 3 results in the introduction of a *Bsp*HI site.

Northern (RNA) blotting and quantitation. Northern blotting was performed with total cell RNA isolated from yeast cells grown under appropriate conditions. Heat-shocked cultures were prepared by transferring aliquots of exponentially growing cultures at 23°C to a prewarmed flask in a 39°C bath. Aliquots of 1.5 ml were removed at 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min after the temperature shift and immediately centrifuged for 10 s to pellet the cells. Following centrifugation, the supernatant was removed and the cell pellets were quickly frozen in a dry ice-ethanol bath. RNA was extracted from cell pellets by the heat-freeze method (25). RNA concentrations were determined by measuring the A_{260} and converting to micrograms per milliliter by using the conversion factor 40 μg of RNA per ml per unit of optical density at 260 nm (OD_{260}). Four micrograms of total RNA was electrophoresed per lane in 1% agarose-formaldehyde gels, transferred to GeneScreen membranes (New England Nuclear), and hybridized with random-primer ³²P-labeled restriction fragments, using standard conditions (2). After stringent washing, Northern blots were visualized with a PhosphorImager (Molecular Dynamics) and quantitated by using the ImageQuant software package (Molecular Dynamics). Differences in the amount of RNA present in gels, introduced during loading, were corrected by normalization to the amount of rRNA present in each lane on the membrane. Each blot was stripped, re-probed with a labeled ribosomal DNA fragment, subjected to stringent washing, and then visualized and quantitated as described above. The validity of this method was confirmed in test experiments in which a linear relationship between

the amount of RNA loaded and the signal bound to filters was obtained (2a). We used rRNA as a standard since even mRNAs with long half-lives decay significantly during the heat shock time course, making standardization problematic. For purposes of photographic reproduction, all Northern blots were also subjected to autoradiography.

Extract preparation. Yeast cell extracts were prepared as described previously, using the frozen pellet method with slight modification (14, 20, 40). Briefly, cells grown to an OD_{600} of 1.0 at 23°C were harvested by centrifugation and resuspended in extraction buffer [200 mM Tris-HCl (pH 8.0), 400 mM (NH₄)₂SO₄, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml, 1 μg of pepstatin A per ml, 2 mM 2-mercaptoethanol]. Frozen pellets were prepared by dripping the cell suspension into liquid nitrogen, and the cells were broken by extended processing in a minichopper (Black & Decker) with frequent addition of liquid nitrogen until a fine powder was obtained. After thawing on ice, unbroken cells and particulate matter were removed by centrifugation, and the proteins were precipitated by the addition of (NH₄)₂SO₄ to a final concentration of 60% saturation. The protein pellet was resuspended in protein buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 8.0], 20% glycerol, 0.1 M KCl, 0.2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and dialyzed against protein buffer for 4 h at 4°C with two buffer changes. Protein concentrations were determined by the Bradford method (5) with the Bio-Rad protein assay kit, using bovine serum albumin as a standard.

Gel mobility shift assays. DNA probes *SSA1* HSE2 (5'-TTTTCCAGAACGT TCCATCGGC-3') and *SSA4* HSE (5'-CAATGAAGTACATTCTAGAAAGTTC CTAGAACCCTTATGG AAGCAC-3') were end labeled with Klenow enzyme and [α -³²P]dCTP by standard methods (2). Binding reactions were carried out in 10-μl volumes containing 1 mM MgCl₂, 20 mM HEPES (pH 8.0), 5% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.5 ng of radiolabeled double-stranded DNA, 2 μg poly(dI-dC) · poly(dI-dC), and 0 to 120 μg of protein. Reaction mixes were incubated for 20 min at 23°C and loaded immediately onto 3.75% polyacrylamide gels (acrylamide-bisacrylamide, 40:1) in 10 mM Tris hydrochloride–10 mM H₃BO₃–1 mM EDTA (pH 8.3). Gels were preelectrophoresed for 1 h at 100 V and electrophoresed for 1.5 h at 200 V at room temperature. Gels were dried onto Whatman 3MM paper, visualized with a PhosphorImager, and autoradiographed.

β-Galactosidase assay. β-Galactosidase activity was assayed in yeast cells permeabilized with chloroform and sodium dodecyl sulfate (SDS) as previously described (27). Miller units were calculated as $(OD_{420} \times 1,000)/(OD_{600} \times t \times v)$, where t is incubation time in minutes and v is volume in milliliters (16).

Quantitation of protein by immunodetection. The levels of Hsp70, Hsp104, and HSF proteins were evaluated by immunoblotting techniques. Whole cell extracts were prepared by boiling pelleted yeast cells in 5× SDS-polyacrylamide gel electrophoresis sample buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) for 3 min. Samples were applied to SDS–7.5% polyacrylamide gels and electrophoresed at 200 V for 45 min. The proteins were transferred to nitrocellulose filters, incubated with appropriate polyclonal rabbit antisera, and detected by using a chemiluminescence detection system (Amersham). Relative differences in protein level were determined by scanning densitometric analysis of the exposed films. The validity of this method was confirmed in test experiments in which a linear relationship between the amount of protein loaded and the chemiluminescent signal was obtained through the range of protein concentrations used in these experiments. Equal loading of protein was confirmed by scanning and quantitating Coomassie blue-stained gels loaded in duplicate.

RESULTS

***EXA3-1* is an allele of *HSF1*.** *EXA3-1* was originally identified because of its ability to partially suppress the growth defect of *ssa1 ssa2* strains at 30°C (18). To more carefully evaluate the effect of the *EXA3-1* mutation, the growth characteristics of wild-type, *ssa1 ssa2*, and *ssa1 ssa2 EXA3-1* strains were assessed at 23, 30, 35, and 37°C (Fig. 1). The suppressor (*ssa1 ssa2 EXA3-1*) strain exhibited better growth than the *ssa1 ssa2* double mutant at 23, 30, and 35°C, but the growth rate was not restored to that of the wild-type strain, and the suppressor strain was unable to form colonies at 37°C.

Preliminary genetic analysis indicated linkage of *EXA3-1* and *HSF1*, suggesting *EXA3-1* might be an allele of *HSF1* (18). We took advantage of the fact that *EXA3-1* is dominant to test this possibility. A 2.2-kb *Hind*III *HSF1* gene fragment, completely internal to and comprising 90% of the protein coding region, was isolated from an *EXA3-1* strain and subcloned into a wild-type *HSF1* gene (Fig. 2A). The resulting plasmid was transformed into an *ssa1 ssa2* strain, and the effects of the wild-type and hybrid *HSF1* genes on growth of the *ssa1 ssa2*

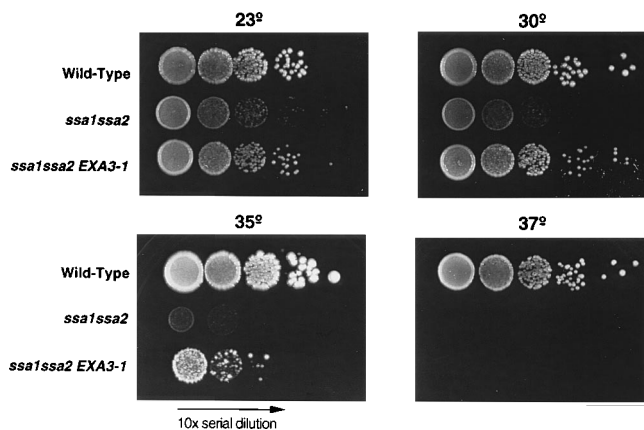


FIG. 1. *EXA3-1*-mediated suppression of the slow-growth phenotype of *ssa1 ssa2* double mutants. Strains of the indicated genotype were grown to log phase in rich liquid media, and dilutions were spotted onto rich solid media. Plates were incubated from 2 to 4 days at the indicated temperatures.

strain at 34°C were compared (Fig. 2B). The *Hind*III fragment from the *EXA3-1* strain suppressed the *ssa1 ssa2* growth defect at 34°C, demonstrating that *EXA3-1* is allelic to *HSF1*.

HSP gene expression is decreased in *EXA3-1* strains. To determine what effect the *EXA3-1* mutation had on the expression of genes under *HSF1* control, we evaluated changes in both the steady-state activity and the heat shock-induced activity of HSF in *EXA3-1* strains by measuring HSE-driven β -galactosidase production in vivo. To do so, we used plasmid constructs pZJ and pZJHSE2-26, in which modified *CYCI* promoters were fused to the *lacZ* gene of *E. coli* (27). pZJ, which contains a *CYCI* promoter in which the normal regulatory element, the upstream activation sequence of *CYCI*, has

been deleted, produces undetectable levels of β -galactosidase under normal growth conditions or after a heat shock (Fig. 3B and reference 27). In plasmid pZJHSE2-26, the *CYCI* upstream activation sequence has been replaced with the HSE2 regulatory element from the *SSA1* promoter, which has previously been shown to drive both basal and temperature-inducible transcription (27). The β -galactosidase activities were determined for the wild-type and *EXA3-1* strains containing plasmid pZJHSE2-26 grown at constant temperatures of 16.5, 23, 30, and 35°C (Fig. 3A). The β -galactosidase activity increased with increasing temperature in both strains. However, at each temperature, the *EXA3-1* strain produced between 10- and 15-fold less β -galactosidase activity than the wild-type strain, indicating that the HSF encoded by *EXA3-1* has substantially reduced sustained activity.

The transient component of the heat shock response was also measured in this assay. β -Galactosidase activities for the wild-type and *EXA3-1* strains containing either pZJ or pZJHSE2-26 were determined at various times after a shift from 23 to 39°C (Fig. 3B). The *EXA3-1* strain exhibited a reduced induction of the heat shock response as measured by this assay.

Since the analysis of HSE-driven reporter constructs suggested that the *EXA3-1* strain accumulates hsp's more slowly than wild-type strains after heat shock, we evaluated the transient component of the heat shock response of three *HSP* genes in the wild-type and *EXA3-1* strains. The responses of the *SSA4*, *HSP26*, and *HSP104* genes, all of which are under HSF control, have low basal levels of expression, and are highly induced after heat shock, were measured by Northern hybridization analysis following a 23 to 39°C heat shock. A temporal delay in the accumulation of each of the *HSP* mRNAs was observed in the *EXA3-1* strain compared with the wild-type strain (Fig. 4A and B). In the wild-type strain, peak mRNA levels occurred 25 to 30 min after the heat shock. In contrast, the accumulation of *HSP* mRNAs in the *EXA3-1* strain was slower, with message levels peaking at 60 to 70 min (Fig. 4B). The *EXA3-1* strain accumulated about 2-fold more *SSA4* mRNA than the wild-type strain, while the magnitudes of the *HSP26* and *HSP104* mRNA responses were about 0.6- to 0.7-fold that of the wild-type strain.

The level of hsp's is decreased in an *ssa1 ssa2 EXA3-1* strain. The observation that *HSP26* and *HSP104* mRNA levels are reduced after heat shock in the *EXA3-1* strain suggested that the *EXA3-1*-mediated suppression of *ssa1 ssa2* is the result of reduced HSF activity. To directly assess the levels of hsp's in *ssa1 ssa2* and *ssa1 ssa2 EXA3-1* strains, antibodies specific for Hsp70 and Hsp104 proteins were used. *ssa1 ssa2* and *ssa1 ssa2 EXA3-1* cells were grown at a constant temperature of either 23 or 34°C, and the levels of *HSP70* and *HSP104* gene products were determined by immunoblot analysis of whole cell extracts. Hsp104 levels were not significantly affected by the *EXA3-1* allele at 23°C (Fig. 5; compare lanes 5 and 6). However, at 34°C, Hsp104 levels in the *ssa1 ssa2 EXA3-1* strain were approximately 55% of levels in the *ssa1 ssa2* strain (Fig. 5; compare lanes 7 and 8).

The level of Ssa3/4 was approximately 40% lower in *ssa1 ssa2 EXA3-1* cells than in *ssa1 ssa2* strains (Fig. 5; compare lanes 1 and 2 and lanes 3 and 4) in cells grown at either 23 or 34°C. The antibody used in this experiment reacts with other Hsp70s. The Ssb1/2 band is most obvious since these proteins are similar in abundance to the *SSA* proteins. In agreement with previously published experiments, we found that the *EXA3-1* allele resulted in increased accumulation of Ssb1/2 (Fig. 5, lanes 1 to 4, and reference 18). The mechanism behind this altered expression of Ssb proteins is under investigation.

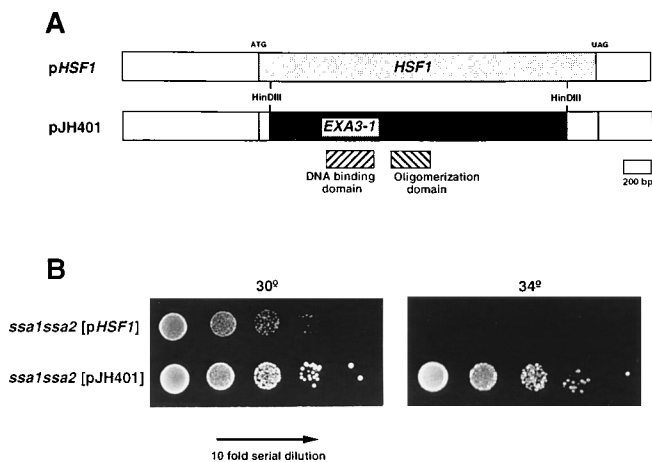


FIG. 2. Suppression of the *ssa1 ssa2* growth defect by an *EXA3-1 HSF1* gene fragment. (A) Schematic representation of plasmids used to demonstrate that *EXA3-1* is an allele of *HSF1*. pHSF1 contains a 3.9-kb *EcoRI-XhoI* restriction fragment containing a wild-type *HSF1* gene on a centromeric plasmid vector. The *HSF1* coding region is indicated by a shaded box, and noncoding regions are indicated by open boxes. In plasmid pJH401, a 2.2-kb *Hind*III fragment from the *HSF1* gene of *EXA3-1* has been substituted for the wild-type *Hind*III fragment. Shown below the plasmid constructs are the locations of the DNA-binding and oligomerization domains (30, 37). (B) Growth of *ssa1 ssa2* strains transformed with either pHSF1 or pJH401. Transformants were grown to log phase in synthetic liquid media to ensure maintenance of plasmids. Direct cell counts and dilutions were performed as described in Materials and Methods. Dilutions were spotted onto appropriate synthetic media, and the plates were incubated at the indicated temperatures for 3 to 5 days.

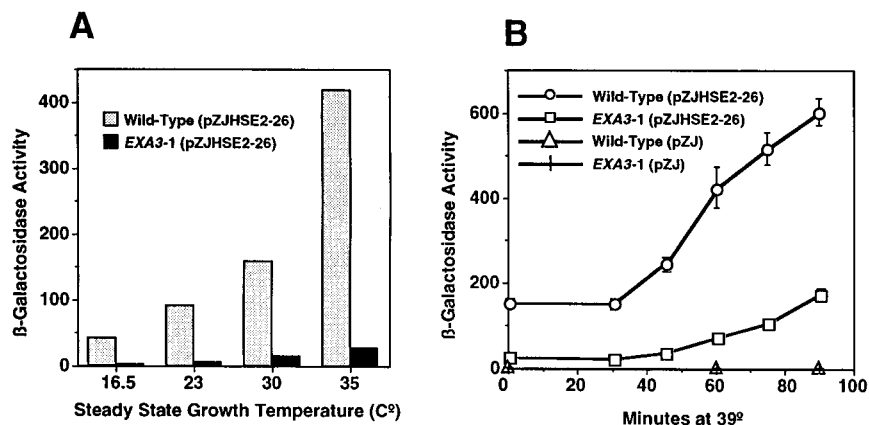


FIG. 3. Steady-state and heat shock-induced *SSA1* HSE2-driven β -galactosidase activity in wild-type and *EXA3-1* cells. (A) The β -galactosidase activity in wild-type and *EXA3-1* cells transformed with pZJHSE2-26 was determined from cultures grown at constant temperature. Cells were grown at the indicated temperatures in synthetic liquid media to mid-log phase. (B) The induction of β -galactosidase activity in wild-type and *EXA3-1* strains transformed with either pZJHSE2-26 or pZJ was determined immediately prior to a 23 to 39°C heat shock and at 30, 45, 60, 75, and 90 min after transfer to 39°C. Note that the levels of β -galactosidase activity produced in the pZJ controls are extremely low and when plotted result in lines which are coincident with the x axis. Activities, presented in Miller units, are the averages of samples processed in triplicate.

EXA3-1 is a point mutation in the DNA-binding domain of HSF. To further localize the *EXA3-1* mutation within the *HSF1* gene, we constructed additional *HSF1* hybrid genes. We found that a 0.95-kb *Bam*HI-*Msc*I fragment encoding amino

acids 66 to 382 from a genomic *EXA3-1* *HSF1* clone was capable of suppressing the *ssa1 ssa2* growth defects (Fig. 6A and B). This region of the *HSF1* gene encodes the DNA-binding domain and part of the oligomerization domain of the HSF

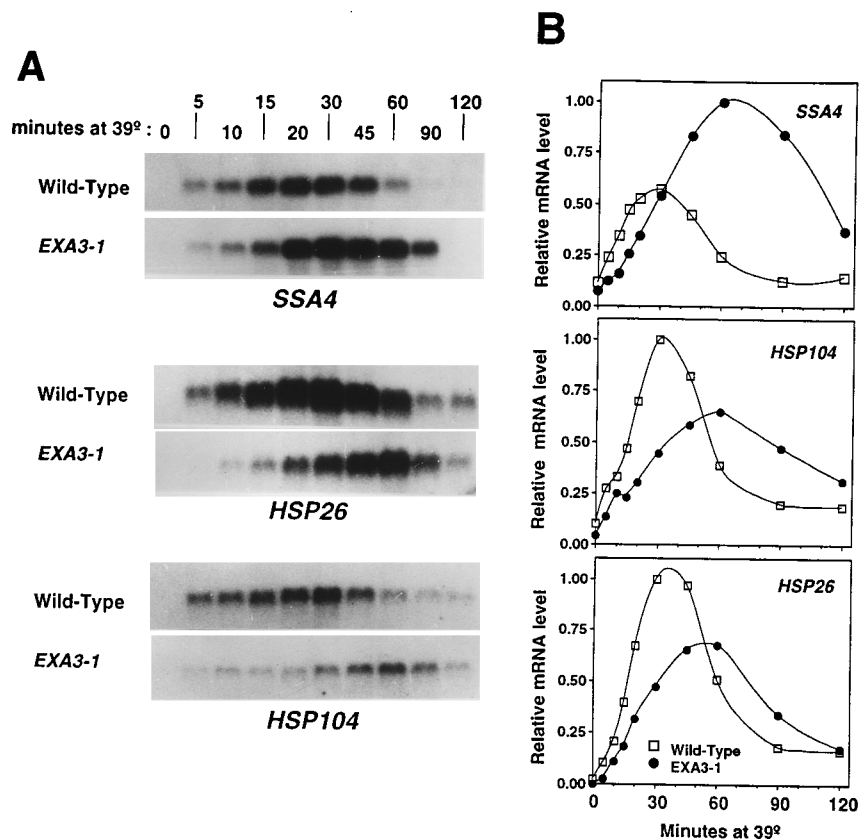


FIG. 4. Induction of *SSA4*, *HSP26*, and *HSP104* mRNAs in wild-type and *EXA3-1* strains after heat shock. (A) Northern hybridization analysis of *HSP* mRNA induction following heat shock. Total RNA was isolated from wild-type and *EXA3-1* cells immediately prior to a 23 to 39°C heat shock and at the times indicated. Four micrograms of yeast RNA was loaded in each lane onto 1% agarose-formaldehyde gels. Following electrophoresis, gels were transferred to GeneScreen hybridization membrane, and the *SSA4*, *HSP26*, and *HSP104* mRNAs were detected by using ³²P-labeled gene fragments as probes. After stringent washing, the membranes were visualized with a PhosphorImager and autoradiographed. (B) Graphical representation of Northern hybridization data after quantitation and normalization to rRNA levels (see Materials and Methods).

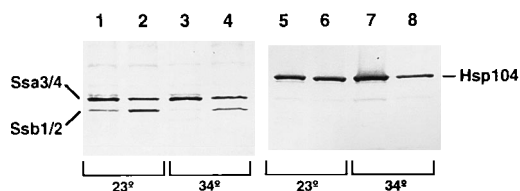


FIG. 5. Hsp70 and Hsp104 protein levels are decreased in *ssa1 ssa2 EXA3-1* cells. *ssa1 ssa2* (lanes 1, 3, 5, and 7) and *ssa1 ssa2 EXA3-1* (lanes 2, 4, 6, and 8) cells were grown at 23 or 34°C; equal amounts of whole cell extracts were separated on SDS-polyacrylamide gels and subjected to immunoblot analysis using antisera specific to Hsp70 (lanes 1 to 4) and Hsp104 (lanes 5 to 8) as described in Materials and Methods. The positions of the Hsp70s (Ssa3/4 and Ssb1/2) and Hsp104 are indicated.

protein, both of which are required for normal activity *in vivo*. We determined the DNA sequence of the *Bam*HI-*Msc*I fragment from the wild-type progenitor and *EXA3-1* mutant strains. As described in Materials and Methods, multiple polymorphisms from the published *S. cerevisiae HSF1* sequence were detected. However, comparison of the DNA sequence from the *EXA3-1* strain with the *HSF1* sequence from the

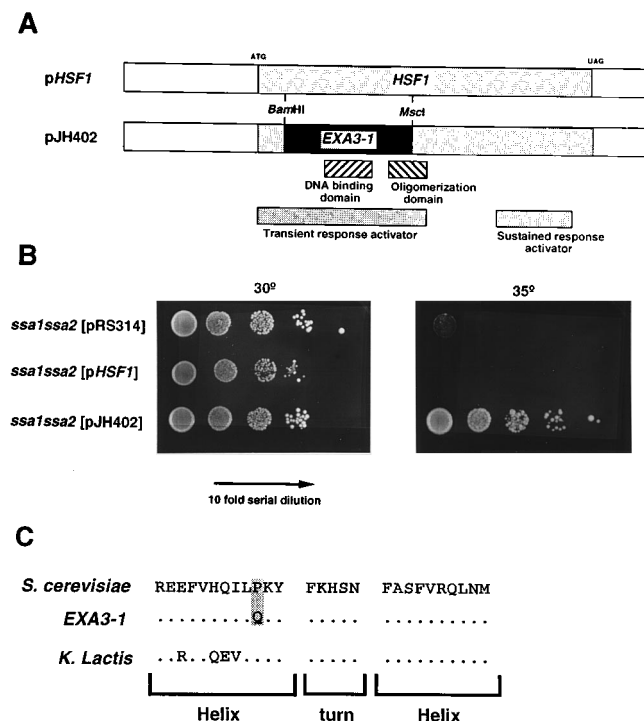


FIG. 6. *EXA3-1* contains a point mutation in the HSF DNA-binding domain. (A) Schematic representation of plasmids used to localize the *EXA3-1* mutation. In plasmid pJH402, a 0.95-kb *Bam*HI-*Msc*I fragment from the *HSF1* gene of *EXA3-1* has been substituted for the corresponding wild-type fragment. Shown below the plasmid constructs are the locations of the DNA-binding, oligomerization, and transient and sustained activation domains (19, 30, 37). (B) Growth of *ssa1 ssa2* strains transformed with either pHSF1, pJH402, or pRS314 (the parent vector of pHSF1 and pJH402). Transformants were grown to log phase in synthetic liquid media to ensure maintenance of plasmids. Direct cell counts and dilutions were performed as described above, and dilutions were spotted onto solid synthetic media. Plates were incubated at the indicated temperatures for 3 to 5 days. (C) Protein sequences of HSF DNA-binding domains. Protein sequences, indicated by the single-letter code, of the *S. cerevisiae* wild-type and *EXA3-1* HSFs are shown. The position of the *EXA3-1* mutation is indicated by a shaded box. The *K. lactis* sequence and the position of the helix-turn-helix motif determined from the crystal structure of the *K. lactis* DNA-binding domain are indicated (12).

progenitor strain revealed a single mutation in the DNA sequence from the *EXA3-1* strain, which results in a proline-to-glutamine substitution at amino acid position 214. Comparison of the sequence of this region with the crystal structure of the *K. lactis* HSF DNA-binding domain indicates that Pro-214 is near the end of the first helix in the helix-turn-helix motif (Fig. 6C).

The *EXA3-1* mutant exhibits a severe defect in DNA binding. The determination that the mutation in *EXA3-1* is within the DNA-binding domain of HSF suggested that DNA binding would be affected in the *EXA3-1* mutant. We evaluated by band shift analysis the HSF DNA-binding activity in protein extracts from the wild-type and *EXA3-1* strains. Specific HSF-HSE DNA binding was detected by the ability of HSF to bind to and retard the migration of 32 P-labeled oligonucleotides containing HSEs. We used HSE2 from the *SSA1* promoter and the *SSA4* HSE as probes for HSF binding. The HSE2 oligonucleotide, used as the upstream activation sequence in plasmid pZJHSE2-26 described above, contains the HSE2 element from the *SSA1* promoter. The *SSA4* HSE oligonucleotide contains the complete HSE from the *SSA4* promoter. This HSE has been shown to be both necessary and sufficient to drive high-level transcription after heat shock (4).

A band shift was detected for the *SSA4* HSE oligonucleotide when it was incubated with either wild-type or *EXA3-1* protein extract (Fig. 7A; compare lanes 3 and 7 with lanes 1 and 5, respectively). However, HSE-binding activity is significantly reduced in the *EXA3-1* extract. Quantitation of a phosphor image of the gel indicates a ninefold reduction in HSF-HSE complexes in the *EXA3-1* extract. The specificity of the HSF-HSE interaction is demonstrated by the observation that the addition of a 300-fold excess unlabeled *SSA4* HSE oligonucleotide essentially eliminated complex formation (Fig. 7A; compare lanes 3 and 4 and lanes 7 and 8). The formation of complexes between HSF and the *SSA1* HSE2 oligonucleotide with the *EXA3-1* extract was affected more severely than was found with the *SSA4* HSE oligonucleotide (Fig. 7B). While not visible on the autoradiograph, complexes were detectable on phosphor images. Quantitation of the wild-type and *EXA3-1* complexes revealed over a 50-fold reduction in complex formation in the *EXA3-1* extract compared with the wild-type extract.

The difference in complex formation between the wild-type and *EXA3-1* extracts was not due to differences in the amount of HSF present in the extracts. Western blotting (immunoblotting) analysis revealed the wild-type and *EXA3-1* extracts contain nearly identical amounts of HSF cross-reacting protein (Fig. 7C). Several bands were detected. The largest is consistent with the size of full-length HSF. The smallest, migrating at 75 kDa, is the size of a frequently observed proteolytic fragment of HSF which retains DNA-binding activity (31, 38). Since the patterns of bands in the two extracts are nearly identical, partial proteolysis cannot explain the large differences observed in HSE-binding activity between the extracts.

An additional copy of *HSF1* is detrimental to *ssa1 ssa2* cells. The determination that the *EXA3-1* mutation causes a decrease in DNA binding, and as a result a decrease in expression of heat shock genes, suggests that overexpression of hsp is in part responsible for the growth defect of *ssa1 ssa2* strains. Therefore, we tested whether additional HSF activity would be detrimental to an *ssa1 ssa2* strain by evaluating the effect of one to two additional copies of the *HSF1* gene on growth. *ssa1 ssa2* strains carrying a wild-type *HSF1* gene on a centromeric plasmid were unable to form colonies at 34°C (Fig. 8A), in contrast to the same strain transformed with the vector alone.

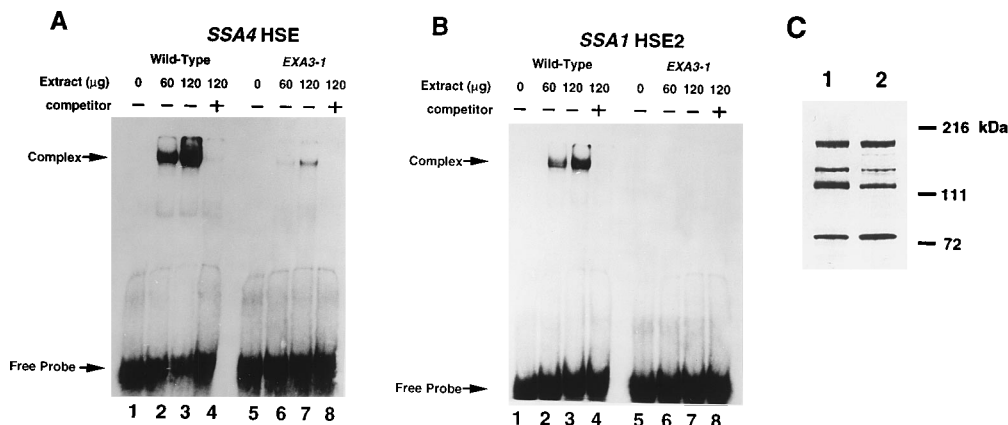


FIG. 7. Detection of HSE-binding activity in wild-type and *EXA3-1* protein extracts. 32 P-labeled oligonucleotide duplex DNAs were incubated with yeast cell extracts and 2 μg of poly(dI-dC) at 23°C to allow complex formation. Complexes were resolved from free probe by native gel electrophoresis. (A) Detection of *SSA4* HSE-binding activity. (B) Detection of *SSA1* HSE2-binding activity. Lanes: 1 to 4, wild type; 5 to 8, *EXA3-1*; 1 and 5, no extract; 2 and 6, 60 μg of extract; 3 and 7, 120 μg of extract; 4 and 8, 120 μg of extract with 300-fold molar excess unlabeled oligonucleotide. The positions of complexed and free probe are indicated. (C) Detection of HSF in wild-type and *EXA3-1* extracts. Equal amounts of protein from the wild-type (lane 1) and *EXA3-1* (lane 2) extracts used in panels A and B were separated on SDS-polyacrylamide gels, and HSF protein was detected by immunoblotting as described in Materials and Methods.

Growth of *EXA3-1* strains. The observation that *EXA3-1* strains exhibit a 10- to 15-fold decrease in HSE-driven transcription under steady-state growth conditions suggested that the growth of these strains might be compromised. Accordingly, we evaluated the growth of an *EXA3-1* strain at 16.5 (data not shown), 23, 30, 35, and 37°C. Surprisingly, the *EXA3-1* strain grew as well as wild-type over a wide temperature range and exhibited only a minor reduction in growth rate at 37°C (Fig. 8B).

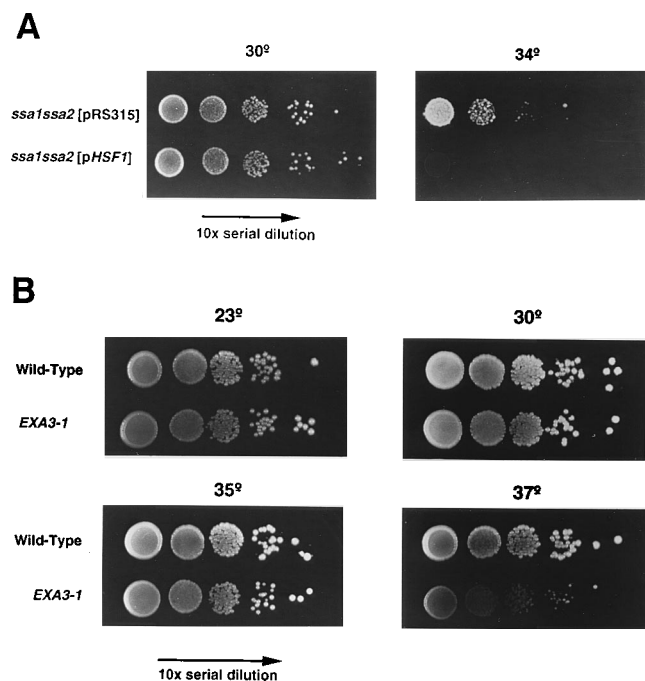


FIG. 8. (A) Growth of *ssa1 ssa2* strains containing one to two extra copies of *HSF1*. *ssa1 ssa2* strains were transformed with either pHSF1 or pRS315 (vector alone). The growth of the transformants at 30 and 34°C on selective media is shown. (B) Growth of an *EXA3-1* strain evaluated at 23, 30, 35, and 37°C on rich media.

DISCUSSION

Cells containing disruptions of both *SSA1* and *SSA2* are unable to form colonies at 37°C and grow more slowly than wild-type cells at lower temperatures. Analysis of *EXA3-1*, an extragenic suppressor of the slow-growth phenotype of *ssa1 ssa2* strains, revealed that it is an allele of *HSF1*, which encodes HSF. Our initial expectation was that the altered HSF would have a higher activity than wild-type HSF, since it was previously demonstrated that overexpression of the heat-inducible *SSA3* and *SSA4* genes can partially suppress the temperature sensitivity of *ssa1 ssa2* strains. However, several lines of evidence presented here indicate that the activity of HSF encoded by *EXA3-1* is less than that of wild-type HSF. First, the sustained level of expression from an HSE-driven promoter was 10- to 15-fold less in an *EXA3-1* strain than in a wild-type strain over a wide spectrum of temperatures. Second, the induction of mRNAs encoded by three different *HSP* genes having very low-level basal expression, *SSA4*, *HSP26*, and *HSP104*, was slower in the *EXA3-1* strain than in the wild-type strain. Third, the levels of Ssa3/4 and Hsp104 were lower in an *ssa1 ssa2 EXA3-1* mutant than in an *ssa1 ssa2* strain. In addition, a deletion mutant of HSF, 40Δ147-583, in which the transient and sustained transcriptional activation domains have been removed or disrupted (29), resulting in an HSF with significantly reduced activity, partially suppressed the growth defect of the *ssa1 ssa2* strain (2a).

It should be noted that preliminary analysis of the *ssa1 ssa2 EXA3-1* strain suggested that the *EXA3-1* mutation caused an increase in Ssa4 expression (18), which was interpreted to mean that *EXA3-1* resulted in a more active form of HSF. These experiments showed that Ssa4 accumulated to higher levels in an *ssa1 ssa2 EXA3-1* strain than in an *ssa1 ssa2* strain 6 h after a shift from 23 to 37°C. This preliminary result is actually in agreement with those reported here. Although steady-state expression levels of Ssa4 are lower in an *ssa1 ssa2 EXA3-1* strain, the heat shock-induced expression of *SSA4* mRNA, while slower, peaks at a higher level, leading to a higher level of Ssa4 upon heat shock (Fig. 4). Interestingly, the level of expression of *HSP26* and *HSP104* mRNAs is decreased in the *EXA3-1* strain. We do not understand this difference in expression among hsp's but suggest that it may be related to the

fact that Ssa proteins are involved in regulation of the expression of heat-inducible genes upon heat stress (33).

Analysis of the DNA sequence of the region responsible for the suppression by *EXA3-1* identified a single mutation within the DNA-binding domain of HSF at amino acid position 214, resulting in a proline-to-glutamine substitution. Pro-214 is absolutely conserved in the HSFs from many organisms, suggesting an important role for the proline residue in the structure of the DNA-binding domain. The recent determination of the structure of this region from the related organism *K. lactis* indicates that Pro-214 introduces a prominent kink near the end of the first helix in the helix-turn-helix motif (12). The glutamine substitution at this position most likely alters the three-dimensional geometry of HSF in this region, thereby disrupting normal protein-DNA interactions. Indeed, band shift analysis indicated a significant decrease of HSE-binding activity in *EXA3-1* protein extracts compared with wild-type extracts. The dominant nature of the *EXA3-1* allele suggests an ability of the mutant protein to form heteromultimeric complexes with the wild-type protein, leading to trimers of HSF with decreased DNA-binding affinity.

Together, the results of these in vivo and in vitro experiments suggest that *EXA3-1* suppresses the poor growth of *ssa1 ssa2* cells by reducing expression of heat shock genes. A corollary of such an argument is that increased expression of hsps would be detrimental to *ssa1 ssa2* cells. Consistent with these ideas, an additional copy of *HSF1* further reduces the growth rate of *ssa1 ssa2* cells. HSF is more active in an *ssa1 ssa2* strain than in a wild-type strain, as illustrated by the unusually high expression of *SSA4* (9). The HSF-binding site of *SSA4*, its HSE, is sufficient to drive high levels of expression from a heterologous promoter in *ssa1 ssa2* but not wild-type strains at 23°C (4). This hyperactivity of HSF is likely a direct result of the lack of the *SSA1* and *SSA2* gene products, as they have been implicated in autoregulation of the heat shock response. Overexpression of *SSA1* causes a reduction in heat-inducible expression from both the *SSA1* and *SSA4* promoters (33). Therefore, we propose that the absence of negative feedback from Ssa1/2 leads to a constitutively high rate of heat shock gene transcription. This response presumably allows *ssa1 ssa2* cells to survive by elevating expression of *SSA3* and *SSA4*, which can substitute for Ssa1/2 (36).

However, the global overexpression of other heat shock genes appears to be detrimental under normal growth conditions. Our data suggest that the *EXA3-1* mutation suppresses the growth defect of *ssa1 ssa2* strains by decreasing the activity of HSF and thereby reducing the overexpression of heat shock genes in this background. Clearly, this suppression indicates that a delicate balance must be struck in these cells to allow sufficient Hsp70 production without detrimental overexpression of other hsps.

Hsp70 has been implicated in the regulation of the heat shock response in prokaryotes as well; evidence exists for the direct interaction of the *E. coli* Hsp70, DnaK, with the heat shock transcriptional regulator σ^{32} (10, 34, 35). Bukau and Walker (6) identified suppressors of the slow-growth phenotype of a *dnaK* deletion mutant at 30°C, a permissive temperature for this slowly growing temperature-sensitive strain. One complementation group contained mutations in the gene encoding σ^{32} , which is responsible for directing RNA polymerase to heat shock promoters after a temperature upshift. These σ^{32} mutations caused a decrease in either activity or stability of the protein, resulting in lower expression levels of hsps. The similarity of these results for *E. coli* to those described here for a eukaryote is striking, pointing to a universality in the regula-

tion of the heat shock response and the importance of the role of Hsp70s as regulators of the response.

Interestingly, the *EXA3-1* strain grew as well as the wild-type strain at most temperatures, showing a growth defect only at 37°C, despite a 10- to 15-fold decrease in HSE-driven expression during steady-state growth conditions. This robust growth is surprising since HSF is required for cell viability, and it suggests that wild-type cells contain significantly more HSF activity than is required for normal growth at moderate temperatures. Presumably, more hsps are required for efficient growth at 37°C and the reduced hsp level in *EXA3-1* strains is responsible for the growth defect at this temperature.

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REFERENCES

- Amin, J., J. Ananthan, and R. Voellmy. 1988. Key features of heat shock regulatory elements. *Mol. Cell. Biol.* **8**:3761-3769.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1989. Current protocols in molecular biology. John Wiley & Sons, New York.
- Baxter, B., and E. A. Craig. Unpublished data.
- Becker, J., and E. A. Craig. 1994. Heat-shock proteins as molecular chaperones. *Eur. J. Biochem.* **219**:11-23.
- Boorstein, W., and E. A. Craig. 1990. Structure and regulation of the *SSA4* gene of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**:18912-18921.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
- Bukau, B., and G. Walker. 1990. Mutations altering heat shock specific subunit of RNA polymerase suppress major cellular defects of *E. coli* mutants lacking the DnaK chaperone. *EMBO J.* **9**:4027-4036.
- Craig, E. A., B. D. Gambill, and R. J. Nelson. 1993. Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol. Rev.* **57**:402-414.
- Craig, E. A., and K. Jacobsen. 1984. Mutations of the heat-inducible 70 kilodalton genes of yeast confer temperature-sensitive growth. *Cell* **38**:841-849.
- Craig, E. A., M. Slater, W. R. Boorstein, and K. Palter. 1985. Expression of the *S. cerevisiae* Hsp70 multigene family, p. 659-668. In R. Calendar and L. Gold (ed.), Sequence specificity in transcription and translation. Alan R. Liss, Inc., New York.
- Gamer, J., H. Bujard, and B. Bukau. 1992. Physical interaction between heat shock proteins DnaK, DnaJ and GrpE and the bacterial heat shock transcription factor sigma32. *Cell* **69**:833-842.
- Georgopoulos, C., and W. Welch. 1993. Roles of the major heat shock proteins as molecular chaperones. *Annu. Rev. Cell. Biol.* **9**:601-634.
- Harrison, C., A. A. Bohm, and H. C. M. Nelson. 1994. Crystal structure of the DNA binding domain of the heat shock transcription factor. *Science* **263**:224-227.
- Ingolia, T. D., M. R. Slater, and E. A. Craig. 1982. *Saccharomyces cerevisiae* contains a complex multigene family related to the major heat shock-inducible gene of *Drosophila*. *Mol. Cell. Biol.* **2**:1388-1398.
- Kingston, R., T. Schuetz, and Z. Larin. 1987. Heat-inducible human factor that binds to a human hsp70 promoter. *Mol. Cell. Biol.* **7**:1530-1534.
- Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. *Annu. Rev. Genet.* **22**:631-677.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Mukai, H., T. Kuno, H. Tanaka, D. Hirata, T. Miyakawa, and C. Tanaka. 1993. Isolation and characterization of *SSE1* and *SSE2*, new members of the yeast HSP70 multigene family. *Gene* **132**:57-66.
- Nelson, R. J., M. Heschl, and E. A. Craig. 1992. Isolation and characterization of extragenic suppressors of mutations in the *SSA* hsp70 genes of *Saccharomyces cerevisiae*. *Genetics* **131**:277-285.
- Nieto-Sotelo, J., G. Wiederrecht, A. Okuda, and C. S. Parker. 1990. The yeast heat shock transcription factor contains a transcriptional activation domain whose activity is repressed under nonshock conditions. *Cell* **62**:807-817.
- Park, H.-O., and E. A. Craig. 1989. Positive and negative regulation of basal

- expression of a yeast HSP70 gene. *Mol. Cell. Biol.* **9**:2025–2033.
21. **Parker, C. S., and J. Topol.** 1984. A *Drosophila* RNA polymerase II transcription factor binds to the regulatory site of an hsp70 gene. *Cell* **37**:273–283.
 22. **Parsell, D. A., and S. Lindquist.** 1994. Heat shock proteins and stress tolerance, p. 457–494. *In* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (ed.), *The biology of heat shock proteins and molecular chaperones*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 23. **Perisic, O., H. Xiao, and J. T. Lis.** 1989. Stable binding of *Drosophila* heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. *Cell* **59**:797–806.
 24. **Rose, M. D., L. M. Misra, and J. P. Vogel.** 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell* **57**:1211–1221.
 25. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **18**:3091–3092.
 26. **Shirayama, M., K. Kawakami, Y. Matsui, K. Tanaka, and A. Toh-e.** 1993. *MSI3*, a multicopy suppressor of mutants hyperactivated in the *RAS*-cAMP pathway, encodes a novel HSP70 protein of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **240**:323–332.
 27. **Slater, M., and E. Craig.** 1987. Transcriptional regulation of an hsp70 heat shock gene in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**:1906–1916.
 28. **Sorger, P., M. Lewis, and H. Pelham.** 1987. Heat shock factor is regulated differently in yeast and HeLa cells. *Nature (London)* **329**:81–84.
 29. **Sorger, P. K.** 1990. Yeast heat shock factor contains separable transient and sustained response transcriptional activators. *Cell* **62**:793–805.
 30. **Sorger, P. K., and H. C. M. Nelson.** 1989. Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell* **59**:807–813.
 31. **Sorger, P. K., and H. R. B. Pelham.** 1987. Purification and characterization of a heat-shock element binding protein from yeast. *EMBO J.* **6**:3035–3041.
 32. **Sorger, P. K., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* **54**:855–864.
 33. **Stone, D. E., and E. A. Craig.** 1990. Self-regulation of 70-kilodalton heat shock proteins in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**:1622–1632.
 34. **Straus, D., W. Walter, and C. Gross.** 1990. DnaK, DnaJ and GrpE negatively regulate heat shock protein gene expression by controlling the synthesis and stability of sigma-32. *Genes Dev.* **4**:2202–2209.
 35. **Tilly, K., N. McKittrick, M. Zyllicz, and C. Georgopoulos.** 1983. The DnaK protein modulates the heat shock response of *Escherichia coli*. *Cell* **34**:641–646.
 36. **Werner-Washburne, M., D. E. Stone, and E. A. Craig.** 1987. Complex interactions among members of an essential subfamily of *hsp70* genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**:2568–2577.
 37. **Wiederrecht, G., D. Seto, and C. Parker.** 1988. Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. *Cell* **54**:841–853.
 38. **Wiederrecht, G., D. Shuey, W. Kibbe, and C. Parker.** 1987. The *Saccharomyces* and *Drosophila* heat shock transcription factors are identical in size and DNA binding properties. *Cell* **48**:507–515.
 39. **Wu, C.** 1984. Two protein-binding sites in chromatin implicated in the activation of heat-shock genes. *Nature (London)* **309**:229–234.
 40. **Young, M. R., and E. A. Craig.** 1993. *Saccharomyces cerevisiae* HSP70 heat shock elements are functionally distinct. *Mol. Cell. Biol.* **13**:5637–5646.
 41. **Zimarino, V., and C. Wu.** 1987. Induction of sequence-specific binding of *Drosophila* heat shock activator protein without protein synthesis. *Nature (London)* **327**:727–730.