The EMBO Journal vol.11 no.6 pp.2357 - 2364, 1992

Hsp104 is required for tolerance to many forms of stress

Yolanda Sanchez[1], John Taulien, Katherine A.Borkovich[2] and Susan Lindquist

The Howard Hughes Medical Institute and The Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637, USA

[1]Present address: Instituto de Microbiologia Bioquimica, Universidad de Salamanca, Salamanca, Spain
[2]Present address: Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston, TX 77225, USA

Communicated by M.Ashburner

Heat-shock proteins (hsps) are induced by many types of stress. In *Saccharomyces cerevisiae*, a mutation in the *HSP104* gene, a member of the highly conserved *hsp100* gene family, reduces the ability of log-phase fermenting cells to withstand high temperatures after mild, conditioning pretreatments. Here, we examine the expression of hsp104 and its importance for survival under many different conditions. Hsp104 is expressed at a higher level in respiring cells than in fermenting cells and is required for the unusually high basal thermotolerance of respiring cells. Its expression in stationary phase cells and spores is crucial for the naturally high thermotolerance of these cell types and for their long-term viability at low temperatures. The protein is of critical importance in tolerance to ethanol and of moderate importance in tolerance to sodium arsenite. Thus, the *hsp104* mutation establishes the validity of a long-standing hypothesis in the heat-shock field, namely, that hsps have broadly protective functions. Further, that a single protein is responsible for tolerance to heat, ethanol, arsenite and long-term storage in the cold indicates that the underlying causes of lethality are similar in an extraordinary variety of circumstances. Finally, the protein is of little or no importance in tolerance to copper and cadmium, suggesting that the lethal lesions produced by these agents are fundamentally different from those produced by heat.
Key words: ethanol/heat-shock proteins/heavy metals/respiration/thermotolerance

Introduction

Exposure of cells and organisms to elevated temperatures triggers the synthesis of a small group of proteins called the heat-shock proteins (hsps) (Lindquist and Craig, 1988; Morimoto *et al.*, 1990; Nover, 1991). Certain of these proteins and their close relatives, play essential roles in growth and metabolism at normal temperatures. Their increased expression at moderately high temperatures allows growth and metabolism to continue at the upper end of the cells' normal temperature range. In most cases increased expression of hsps is also accompanied by increased tolerance to more extreme temperatures. For example, when log-phase *Saccharomyces cerevisiae* cells are shifted directly from 25 to 50°C, <1% of the cells survive after 5 min. When a matched culture is pre-incubated at 37°C for 30 min, >95% of the cells survive the 50°C treatment. This phenomenon is called 'induced thermotolerance'. It has been observed in a wide variety of plants, animals, fungi and bacteria (Lindquist and Craig, 1988; Morimoto *et al.*, 1990; Nover, 1991).

Hsps are also induced by an extraordinary variety of stress agents; ethanol, heavy metals and transition metals being among the most common (Lindquist and Craig, 1988; Nover, 1991). The proteins' induction is often associated with increased tolerance, both to the immediate inducing agent and to other types of stress. For example, pretreatments with moderate concentrations of ethanol induce tolerance to yet higher concentrations of ethanol and, at the same time, tolerance to high temperatures. Similarly, mild heat treatments induce tolerance to both higher temperatures and to high concentrations of ethanol (Plesset *et al.*, 1982; Li and Laszlo, 1985; Hahn and Li, 1982, 1990; Watson and Cavicchioli, 1983). This phenomenon is called 'cross tolerance'. It has long been postulated that the hsps are responsible for it.

Hsp inducers are bewildering in their variety, but many have in common the capacity to produce protein damage (Hightower, 1980; Pelham, 1986). The biochemical analysis of hsp function has revealed that certain hsps participate in protein assembly and folding pathways (Pelham, 1986; Rothman, 1989). It has been proposed, therefore, that the common signal for hsp induction is protein denaturation and that one function of the induced proteins is to prevent or repair denaturation damage (Pelham, 1986; Gross and Craig, 1991). Pleasing as this hypothesis may be, many investigators have questioned whether hsps play any role at all in protecting cells from many types of stress, heat among them (Hall, 1983; Watson *et al.*, 1984; Hallberg *et al.*, 1985; Widelitz *et al.*, 1986; Carper *et al.*, 1987; Barnes *et al.*, 1990; Watson, 1990). Some suggest that the role of hsps is restricted to certain physiological states (Guy *et al.*, 1986; Aujame and Firko, 1988). Others have postulated two states of tolerance, one induced by moderate stress and not requiring hsps, another induced by more severe stress and requiring them (van Wijk and Boon-Niermeijer, 1986; Laszlo, 1988). Still others propose that hsps play a role in protecting cells from heat and ethanol but not from arsenite and cadmium (Hahn and Li, 1982; Li and Laszlo, 1985).

We have recently reported that the *HSP104* gene (a member of the highly conserved *hsp100* gene family) is required for induced tolerance to heat in log-phase fermenting cells of *S.cerevisiae* (Sanchez and Lindquist, 1990). Cells carrying an *HSP104* deletion mutation grow at the same rate as wild-type cells in glucose at both 25 and 37°C. They also die at the same rapid rate when shifted directly to 50°C. However, when pretreated at 37°C, a temperature which induces the expression of hsp104 and other hsps, mutant and wild-type cells behave very differently. Both acquire

© Oxford University Press

tolerance, but this tolerance is extremely transient in the mutant. Within 10 min of a shift to 50°C, a 1000-fold difference in the viability of mutant and wild-type cells is apparent. Thus, this yeast member of the hsp100 protein family plays a very important role in induced thermotolerance, but other factors can provide at least transient thermotolerance in its absence. We have now used this deletion mutation to assess the relative importance of hsp104 and other factors in protecting yeast cells from many types of stress under a variety of physiological conditions.

The role of hsp104 in respiring cells

We previously investigated the role of hsp104 in cells growing on glucose. With this carbon source, respiratory metabolism is largely repressed and cells grow primarily by fermentation (Kappeli, 1986; Lagunas, 1986). To determine if the protein plays an equally important role in respiring cells, late stationary phase glucose cultures were diluted into acetate medium. To our surprise, mutant cells grew to higher stationary phase plateau densities (Table I). This phenomenon was observed in three different strain backgrounds (W303, DS10 and DBY747) and also in both rich and minimal acetate media.

This result prompted us to examine the expression of hsp104 in acetate media. As may be seen in Figure 1A, the protein is constitutively expressed at a much higher level in acetate than in glucose. In glucose, mutant and wild-type cells grow at the same rate and have similar, low levels of basal thermotolerance (Sanchez and Lindquist, 1990). To determine if the higher constitutive levels of hsp104 expression in acetate media were accompanied by higher constitutive levels of thermotolerance, log phase cells growing at 25°C were exposed directly to 50°C. As previously reported, basal tolerance in glucose was the same for mutant cells and wild-type cells (Figure 2). In contrast, basal tolerance in acetate was much higher for wild-type *HSP104* cells than for isogenic mutant cells. The difference in viability was typically 50- to 100-fold.

Similar experiments were performed with galactose, a fermentable sugar which supports a higher rate of respiratory metabolism than does glucose (Lagunas, 1986). In this medium, hsp104 expression was intermediate between that of cells grown in glucose and cells grown in acetate (Figure 1A). The growth rates of mutant cells and wild-type cells were indistinguishable in galactose. Basal thermotolerance, however, was higher in the wild-type strain than in the mutant (Figure 2). Thus, differences in basal thermotolerance correlate with differences in hsp104 expression rather than with differences in the growth rates of mutant and wild-type cells. We conclude that the much higher levels of basal thermotolerance observed in cells growing in acetate or galactose depend upon the constitutive expression of hsp104.

To examine induced thermotolerance, log phase cells growing on acetate, glucose and galactose were given a mild inducing treatment (37°C for 30 min) before being shifted to 50°C. A dramatic increase in tolerance was observed in both mutant and wild-type cells in all media (Figure 2), but in each case the tolerance of mutant cells was very transient. Within 30 min, a 100- to 1000-fold difference in survival was apparent. Thus, for induced thermotolerance, hsp104 plays a crucial role in both fermenting cells and respiring cells.

Table I. Stationary phase densities (cells/ml) in YPAC

Strain	HSP104	Δhsp104
W303	1.4×10^8	1.9×10^8
DS10	6.0×10^7	1.0×10^8
DBY747	6.2×10^7	1.5×10^8

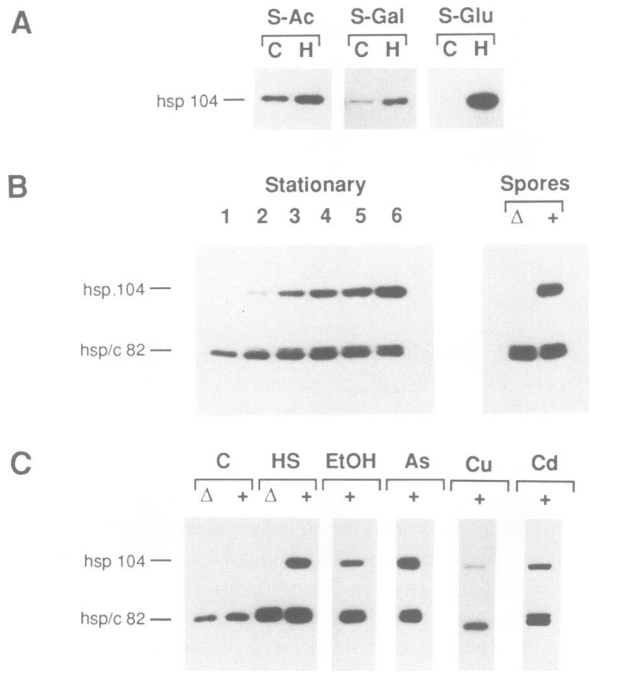

Fig. 1. Expression of hsp104 under different growth and stress conditions. Total cellular proteins were prepared by glass bead lysis in ethanol. After electrophoretic separation on 7.5% SDS–polyacrylamide gels, proteins were transferred to Immobilon membranes. A, B and C were reacted with an hsp104 antiserum, B and C were also reacted with an hsp82 antiserum. Immune complexes were visualized by reaction with ^{125}I-labelled protein A. (**A**) Wild-type cells (+) of strain W303 were grown at 25°C to mid-log phase in liquid medium: S-Glu, S-Gal, S-Ac. The cultures were divided and maintained at 25°C or heat-shocked at 39°C for 30 min. (**B**) Left, wild-type cells were grown from log phase to late stationary phase in rich glucose media (YPDA) (Petko and Lindquist, 1986). Proteins were harvested at densities of: 2×10^7 cells/ml (1), 7.3×10^7 cells/ml (2), 1×10^8 cells/ml (3), 1.5×10^8 cells/ml (4), 1.8×10^8 cells/ml (5) and 2.5×10^8 cells/ml (6). Proteins from 1.5×10^6 cells were loaded in each lane. Right, mutant (Δ) and wild-type cells (+) were sporulated in liquid culture (1% potassium acetate) (Petko and Lindquist, 1986). After 4 days asci were digested with 0.6 mg/ml of zymolyase 20T and mature spores were collected in a Percoll gradient as described (Esposito *et al.*, 1991). Proteins from 2×10^6 spores were loaded in each lane. (**C**) Mutant (Δ) and wild-type cells (+) were grown to mid-log phase in YPDA and maintained at 25°C (C) or exposed to 37°C (HS) for 30 min, 6% ethanol for 1 h (EtOH), 0.75 mM sodium arsenite for 1 h (As), 11 mM cupric sulfate for 30 min (Cu), or 1.5 mM cadmium sulfate for 30 min (Cd).

Hsp104 in stationary phase cells and spores

Next, we examined the role of hsp104 in two cell types that are naturally thermotolerant, stationary phase cells and spores (Schenberg-Frascino and Moustacchi, 1972; Plesset *et al.*, 1987). Some hsps—hsp26 (Kurtz *et al.*, 1986; Petko and Lindquist, 1986), hsp82 (Borkovich *et al.*, 1989) and certain members of the hsp70 family (Kurtz *et al.*, 1986; Werner-Washburne *et al.*, 1989)—are induced in yeast cells as they enter stationary phase or begin to sporulate. Increased thermotolerance may be related to this induction, but the

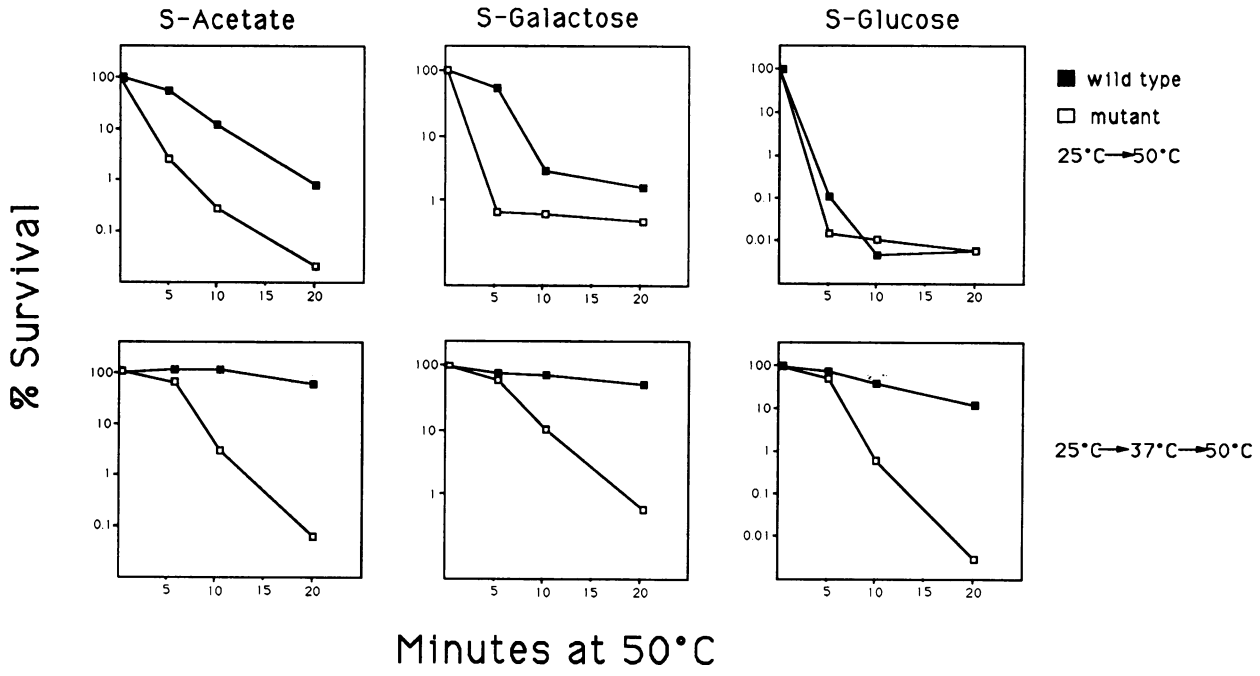

Fig. 2. Hsp104 is required for both basal and induced thermotolerance in respiring cells. Cells were grown at 25°C to mid-log phase ($1-3 \times 10^6$ cells/ml) in S-Ac, S-Gal or S-Glu. Prior to exposure to 50°C, matched cultures were either maintained at 25°C (top panels) or pre-incubated at 37°C for 30 min (bottom panels). Following heating at 50°C, cells were transferred to ice, diluted in ice-cold YPDA, and immediately plated on YPDA.

mutations tested to date (*hsp26* and *hsp82*) have no effect on the thermotolerance of either stationary phase cells or spores (Petko and Lindquist, 1986; Borkovich *et al.*, 1989).

A strong induction of hsp104 was observed in stationary phase cells right after the diauxic shift, when glucose levels decline (Figure 1B, lane 2). The induction paralleled that of hsp82, which migrates just above hsc82. As expected, stationary phase cells were far more thermotolerant than log phase cells (compare Figures 2 and 3). Thermotolerance in the *hsp104* mutant was much lower than in the wild-type. After 60 min at 50°C, a 100-fold difference in survival was observed (Figure 3A). As previously reported for log phase cells (Sanchez and Lindquist, 1990), a plasmid carrying the *HSP104* gene rescues the thermotolerance defect, confirming that the difference in tolerance is due to the absence of hsp104.

In sporulating cells, hsp104 was strongly induced very early (within 4 h of the shift to nitrogen-free medium; data not shown) and remained at a high level in mature spores (Figure 1B). We first asked whether the *hsp104* mutation had any effect on the sporulation process itself. Homozygous mutant diploids sporulated at nearly the same rate as wild-type diploids and to the same extent. Levels of sporulation varied between experiments (60–75%) but, within any given experiment, mutant cells and wild-type cells behaved similarly. Further, wild-type spores and mutant spores germinated at similar rates and displayed equally high viability when dissected at 25°C.

In thermotolerance tests, wild-type spores showed very high levels of thermotolerance (Figure 3B). Mutant spores were much more sensitive to heat. Within 90 min at 51°C, the viability of mutant spores was 100-fold less than that of wild-type spores. Thus, although spores are generally thought to have many mechanisms for protection against heat, the activity of hsp104 is crucial.

Another difference between mutant cells and wild-type cells became apparent during long-term maintenance of the cultures. For stationary phase cells, viability was maintained at 25°C for several days with no apparent difference between the mutant and the wild-type. However, after ~10 days, when cells began to die, mutant cells died 10–15 times more rapidly than wild-type cells (Table II). Even in spores stored at 4°C, a difference in viability was detected. After 6 months, no loss of viability was observed in wild-type spores. In the same period, 50–75% of mutant spores died (Table II).

The role of hsp104 at different temperatures

Our previous experiments examined tolerance at 50°C, which kills cells in minutes. At lower temperatures, cells survive for many hours. At higher temperatures, they die in seconds. Whether the lethal lesions produced by very slow killing are related to those produced by very rapid killing is not known. Mutant and wild-type cells were grown in glucose to log phase, maintained at 25°C or pre-treated at 37°C, and then shifted to either 44°C or 58°C. At both temperatures, the 37°C pretreatment increased survival. However, while 1.5% of wild-type pretreated cells remained viable after 1 min at 58°C, <0.01% of mutant cells remained so. At 44°C, the survival of wild-type pre-treated cells declined slowly, from 71% after 3 h to 1% after 24 h. At nearly every time point examined, the viability of wild-type cells was 10-fold greater than that of mutant cells (data not shown).

The role of hsp104 in other forms of stress

Ethanol induces hsps in yeast, as well as in most other organisms. Under our culture conditions, the strongest hsp inductions were observed after 1 h in 6% ethanol (Figure 1C). As shown in Figure 4A, the hsp104 protein

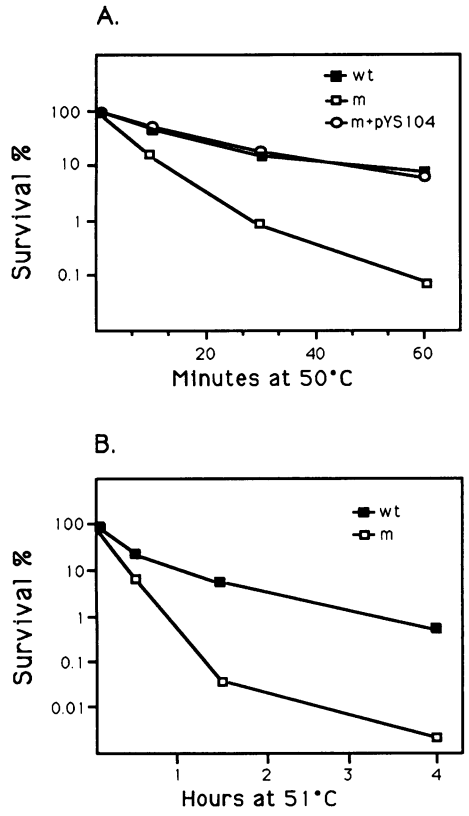

Fig. 3. Mutation of *HSP104* greatly reduced the naturally high thermotolerance of stationary phase cells and spores. (**A**) Cultures from the *hsp104* mutant (m), the wild-type (wt) and a mutant carrying an episomal copy of the *HSP104* gene (m+pYS104) were inoculated at 2×10^4 cells/ml in S-Glu media and cells were grown at 25°C for 72 h. To measure thermotolerance, cells were shifted directly to 50°C. (**B**) Mutant (m) and wild-type (wt) spores prepared as described in the legend of Figure 1B were resuspended in 0.1% Triton X-100 to prevent clumping during plating. Thermotolerance was tested by heating the spores at 51°C.

induced by ethanol was functional in tolerance to heat. Pretreatments with ethanol induced thermotolerance in both mutant and wild-type cells, but the thermotolerance of mutant cells was 100-fold less than that of wild-type cells. To examine the function of hsp104 in tolerance to ethanol, log-phase cells were exposed to 20% ethanol (Figure 4B). Without pretreatment at 37°C, both mutant and wild-type cells were killed extremely rapidly. With pretreatment, wild-type cells showed high levels of tolerance. The *hsp104* mutant showed none. At lower concentrations of ethanol (17–19%), pretreatments induced some tolerance, but mutant cells still died at 100 times the rate of wild-type cells (data not shown).

Another common inducer of the heat-shock response is sodium arsenite. Although a previous report indicated that arsenite does not induce most hsps in *S.cerevisiae* (Chang *et al.*, 1989), it did so reliably in our experiments. After 60 min of incubation in 0.75 mM arsenite, hsp104 was induced to a level comparable with its level of induction by heat (Figure 1C). In fact, all of the hsps were induced by this treatment, as determined by the incorporation of radiolabeled amino acids (data not shown). The arsenite pretreatments greatly increased thermotolerance in both mutant and wild-type cells (Figure 5A), but tolerance in the wild-type was 100-fold greater than in the mutant. Thus,

Table II. Long-term viability

	HSP104	Δhsp104
Stationary phase viability at 25°C		
3 days	5.9×10^7	5.9×10^7
20 days	4.8×10^7	3.6×10^7
55 days	1.8×10^4	1.5×10^3
66 days	1.6×10^3	1.0×10^2
Spore viability at 4°C		
1 day	1.2×10^8	1.3×10^8
6 months	1.2×10^8	0.3×10^8

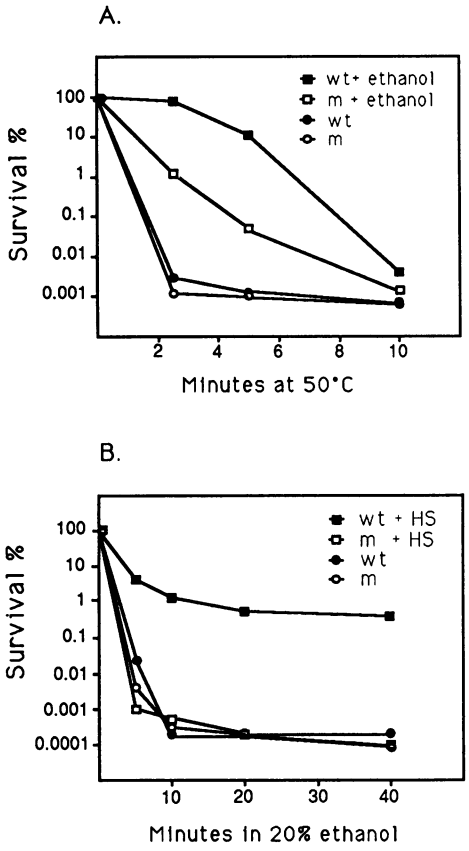

Fig. 4. Mutation of *HSP104* greatly reduced both ethanol-induced tolerance to heat and heat-induced tolerance to ethanol. Mutant and wild-type cells were grown to mid-log phase in YPDA. (**A**) Cells were incubated with or without 6% ethanol at 25°C for 1 h and equal portions of the culture were exposed to 50°C for various lengths of time. (**B**) Cells were maintained at 25°C or incubated at 37°C for 30 min (HS) before being exposed to 20% ethanol.

arsenite-induced hsp104 is fully capable of functioning in thermotolerance.

To examine the role of hsp104 in protection against arsenite, we first determined the concentration of arsenite required to kill 99.9% of the cells in <3 h. Cells were then exposed to this concentration of arsenite (100 mM) with or without a 37°C pretreatment. A 5-fold difference in the survival of mutant cells and wild-type cells was seen after arsenite stress, even without a pretreatment (Figure 5B). Presumably, hsp104 induced during the course of the incubation provided partial tolerance as the incubation continued. Pretreatments at 37°C induced high levels of

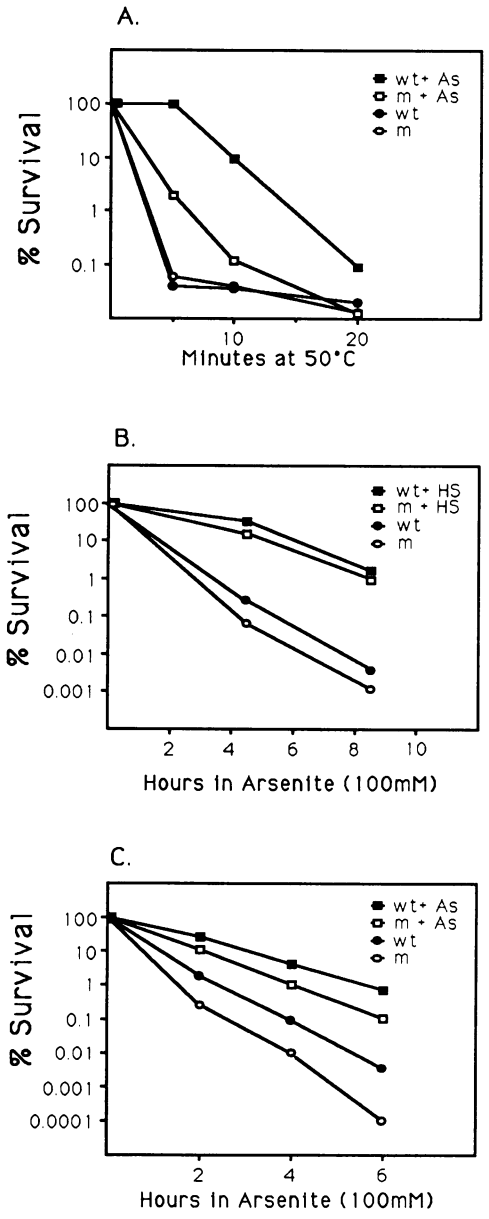

Fig. 5. Mutation of *HSP104* strongly affects arsenite-induced tolerance to heat but has a more modest effect on tolerance to arsenite. Cells were grown to mid-log phase in YPDA. (**A**) Cells were maintained at 25°C in the presence or absence of 0.75 mM arsenite for 30 min, washed, resuspended in fresh media and exposed to 50°C. (**B**) Cells were maintained at 25°C or incubated at 37°C for 30 min. Arsenite was added to 100 mM and cultures were maintained with vigorous aeration. (**C**) Cells were preincubated with or without 0.75 mM arsenite for 30 min at 25°C and were then exposed to 100 mM arsenite as in (B).

tolerance to arsenite, but here the difference between mutant cells and wild-type cells was only 2- to 3-fold.

We then examined the effects of arsenite pretreatments on arsenite tolerance (Figure 5C). With and without the pretreatment, the mutant died faster than the wild-type. In this case, differences were of the order of 10-fold. Thus, although the protective effects of hsp104 are not nearly as dramatic for arsenite as for heat and ethanol, they are substantial.

Finally, we examined tolerance to copper and cadmium. These transition metals induce hsps in many organisms (Nover, 1991). The induction of hsp104 was weaker with copper than with the other inducers tested here (Figure 1C). Induction was maximal at 5–11 mM; higher concentrations (15–20 mM) did not improve induction and killed a substantial fraction of the cells. Cadmium was a much better inducer; at 0.5–1.5 mM, levels of induction were similar to those obtained with heat or ethanol. (These concentrations are well beyond the range where metallothionein provides protection.) When wild-type cells were treated with inducing concentrations of copper or cadmium, their ability to tolerate heat increased 100- to 1000-fold (Figure 6A and D). Little or no thermotolerance was observed in the mutant. Thus, as was the case for the other stresses tested here, the hsp104 protein induced by transition metals is fully functional in thermotolerance.

In contrast to the other inducers, however, heat and metal pretreatments were only slightly beneficial in improving tolerance to copper, and were actually detrimental for tolerance to cadmium (Figures 6B, C, E and F). Furthermore, the *hsp104* mutation did not reduce survival during exposure to copper or cadmium. In fact, with copper, the mutation actually improved survival. Very similar results were obtained in each of several independent experiments.

Discussion

Our results define the importance of hsp104 in the normal growth and development of *S.cerevisiae* as well as during exposure to stress. In many organisms hsp expression varies in development and in the course of normal physiological fluctuations. The biological significance of these changes in expression has been unclear (Petko and Lindquist, 1986; Borkovich *et al.*, 1989; Werner-Washburne *et al.*, 1989). We find that hsp104 is induced as cells enter stationary phase or begin to sporulate and that this induction is vital in both cell types. Mutant cells are not only severely compromised in their ability to survive high temperatures, they are also unable to maintain viability over the long term at low temperatures.

We also find that hsp104 is constitutively expressed in cells growing on two media that support high rates of respiration, acetate and galactose. Cells growing on these media also exhibit much higher levels of basal thermotolerance and this basal thermotolerance requires hsp104. The constitutive expression of this protein, therefore, provides a selective advantage. However, at least in acetate media, higher basal levels of hsp104 expression also confer a disadvantage, reducing plateau phase densities. This apparent trade-off might be weighted in favor of hsp104 expression for a simple reason. The preferred carbon source of yeast cells is glucose and the primary product of its fermentation is ethanol. Yeast cells normally switch to respiratory metabolism when glucose is exhausted and ethanol levels are high. Ethanol, however, is toxic, and hsp104 mitigates this toxicity. Thus, hsp104 is commonly needed for protection when cells are growing by respiratory metabolism. Perhaps the increased protection is worth a slight reduction in growth. In examining a number of strains isolated from the wild, and from breweries and wineries, we find that hsp104 is always expressed at a much higher level in respiring cells than in fermenting cells, but the absolute level of respiratory expression varies over a broad range (F.Rosenberg and S.Lindquist, manuscript in preparation). We suggest these variations in hsp104 expres-

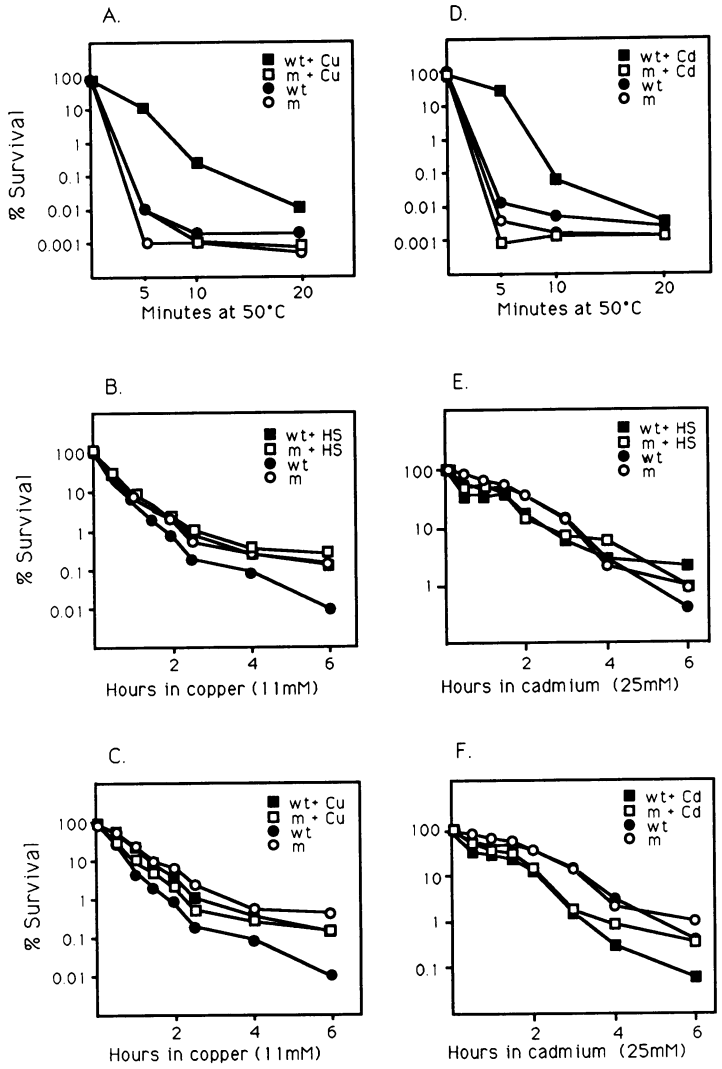

Fig. 6. Mutation of *HSP104* affects copper- and cadmium-induced tolerance to heat but does not reduce tolerance to copper or cadmium. Cells were grown to mid-log phase in YPDA. (**A** and **D**) Cells were maintained at 25°C in the presence or absence of 5 mM cupric sulfate (A) or 1.5 mM cadmium chloride (D) for 30 min, washed, resuspended in fresh media and exposed to 50°C. (**B** and **E**) Cells were maintained at 25°C or incubated at 37°C for 30 min. Cupric sulfate was added to 11 mM (B) or cadmium chloride to 15 mM (E). (**C** and **F**) Cells were preincubated with or without 5 mM cupric sulfate (C) or 1.5 mM cadmium chloride (F) for 30 min at 25°C and were then exposed to 11 mM cupric sulfate (C) or 25 mM cadmium chloride (F). Experiments B and C were performed at the same time, as were experiments E and F. Graphs for mutant and wild-type cells without a pre-treatment are repeated for internal reference.

sion contribute to the broad variations in basal thermotolerance and growth rates that typify these strains.

Hsp104 is not beneficial for growth under any conditions we have examined (including high temperatures) and, as discussed above, may even be slightly detrimental under some conditions. This makes the variety of stressful circumstances in which hsp104 is critical for survival all the more remarkable. The protein protects cells from long exposures at temperatures just beyond their normal growth range and from short exposures at very extreme temperatures. Furthermore, it protects cells against ethanol, sodium arsenite and, most unexpectedly, from long-term storage at low temperatures. Since the *hsp104* mutation has no detectable effect on the pattern of normal cellular protein synthesis or on the induction of other hsps (Sanchez and Lindquist, 1990; Y.Sanchez, E.A.Craig, D.A.Parsell, J.Taulien and S.Lindquist, in preparation), differences in tolerance between mutant cells and wild-type cells are most likely a direct consequence of hsp104 activity. The lethal lesions induced by these different stresses, then, must be similar.

Mutating the *HSP104* gene uncovers the action of other tolerance factors, revealing their relative importance under different circumstances. During exposure to 20% ethanol, 1000- to 10 000-fold differences in survival are seen between mutant cells and wild-type cells. Under such conditions, pretreated mutant cells display no residual tolerance. At temperatures of 50–58°C or at ethanol concentrations of 17–19%, 100-fold differences in survival are observed. In these cases, pretreated mutant cells show at least a transient ability to resist killing. At lower temperatures (44°C) and at lower concentrations of ethanol (15%), the differences in tolerance between the mutant and wild-type are most modest, 5- to 10-fold. Here mutant cells that have been given tolerance-inducing pretreatments show substantial and sustained tolerance compared with untreated cells. Thus, as the degree of stress decreases, the importance of hsp104 relative to other inducible tolerance factors also decreases.

One of these other tolerance factors must be hsp70. Experiments in many different organisms support a role for this protein in thermotolerance (Hahn and Li, 1982; Li and Laszlo, 1985; Lindquist and Craig, 1988; Riabowol *et al.*, 1988; Li *et al.*, 1991; Nover, 1991; Solomon *et al.*, 1991). Deletion of the heat-inducible forms of hsp70 does not compromise thermotolerance in yeast cells carrying a wild-type *HSP104* gene (Craig and Jacobsen, 1984). However, it greatly reduces the residual thermotolerance of cells carrying the *hsp104* mutation (Y.Sanchez, E.A.Craig, D.A.Parsell, J.Taulien and S.Lindquist, in preparation). Furthermore, overexpression of hsp70 (*SSA1*) in large part compensates for the deletion of *HSP104* in thermotolerance (Y.Sanchez, E.A.Craig, D.A.Parsell, J.Taulien and S.Lindquist, in preparation).

These results indicate a close relationship between the functions of hsp70 and those of hsp104. Hsp70 proteins use the energy of ATP to promote the proper unfolding, folding, assembly and disassembly of other proteins (Pelham, 1986; Rothman, 1989; Skowyra *et al.*, 1990). Thus, if the signal for hsp induction is protein denaturation, protecting proteins against denaturation and aggregation provides a logical extension of known hsp70 functions (Pelham, 1986).

The biochemical functions of hsp104 are just beginning to be unravelled. Like hsp70, it is an ATPase (D.A.Parsell and S.Lindquist, unpublished observations). It contains two nucleotide binding sites and both are required for thermotolerance (Parsell *et al.*, 1991). Hsp104 is distributed throughout the cell, with more intense concentration in the nucleus (D.A.Parsell and S.Lindquist, unpublished observations), and presumably affects a large number of biochemical processes. To date, the only process examined in detail is RNA splicing. Splicing is blocked in most organisms at high temperatures but is protected by mild heat pretreatments. It is protected by pretreatments to nearly the same extent in mutant *hsp104* and wild-type cells. However, once splicing is disrupted (as by a sudden severe heat shock), it recovers much more rapidly in the wild-type than in the mutant (Yost and Lindquist, 1991). It may be that the major role of other heat-inducible tolerance factors is to protect vital processes from disruption by heat (Hall, 1983; Pelham, 1986; Lindquist and Craig, 1988; Rothman, 1989), while hsp104 is more closely specialized to restore those processes once they have been disrupted. If so, it would explain why hsp104 is most crucial under the most extreme circumstances.

The hsp104 mutation provides a genetic tool for dissecting the underlying causes of lethality with different types of stress. Here we have confirmed a previous suggestion that the damage caused by copper and cadmium is, in some fundamental way, different from the damage caused by heat and ethanol (Hahn and Liu, 1982; Li and Laszlo, 1985). Hsp104 is of no value in protecting cells against cadmium and is actually slightly deleterious during exposure to copper. Little is known about the critical lethal lesions produced by copper and cadmium, but they are postulated to damage proteins oxidatively (Aust *et al.*, 1985; Goldstein and Czapski, 1986; Stadtman, 1990). The particular targets they damage may be different from those damaged by heat. Alternatively, the structural perturbations they produce may be sufficiently different from those caused by heat and ethanol that they are not repaired by the same system. The hypothesis that all hsp inducers function through the common pathway of protein denaturation is not contradicted, but can be refined. If both transition metals and heat induce in this way, then different types of protein damage must be able to create the signal even though they require different processes for protection or repair.

Previous hypotheses concerning hsp functions must be modified in the light of these studies. The notion that hsps do not function in thermotolerance, or function only in certain special circumstances, is no longer tenable. The hypothesis that hsps are required for heat and ethanol protection, but not for arsenite and cadmium protection, must be redrawn. Full protection against arsenite requires hsp104. These points illustrate the advantages of genetic analysis. Hsp104 provides a 10-fold difference in survival during slow killing at moderately warm temperatures and only a 3- to 10-fold difference in survival during exposure to arsenite. Although its role in survival under these circumstances is substantial, it would be difficult to establish by previous correlative methods.

We have recently reported that hsp104 belongs to a highly conserved family of proteins which we suggested should be designated the hsp100 family (Parsell *et al.*, 1991). Closely related, heat-inducible genes and proteins have been detected in mammals, *Drosophila*, trypanosomes, plants and bacteria (Gottesman *et al.*, 1990; Parsell *et al.*, 1991). *Escherichia coli* produces two proteins in the hsp100 class and mutations in one of these impairs the ability of the cells to tolerate high temperatures (Squires *et al.*, 1991). Given the conservation of function observed with other hsps, we expect that the ability of most organisms to tolerate a wide variety of stresses will depend in large part upon hsp100 proteins. Since hsp104 plays an important role in establishing basal levels of thermotolerance, manipulating the expression of this protein offers a prospect for manipulating the stress tolerance of many species.

Materials and methods

Yeast strains and media

The yeast strains used in this study, the wild-type (W303a) and the *hsp104* mutant (W303aΔhsp104) were described previously (Sanchez and Lindquist, 1990). Cells were grown at 25°C in rich medium (1% yeast extract, 2% bactopeptone, 2% glucose and 40 mg/l of adenine sulfate) or in minimal media: S-Glu (synthetic glucose; 0.7% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% glucose plus essential amino acids and nucleotides), S-Gal (same as S-Glu except that glucose was replaced by 2% galactose) and S-Ac (glucose replaced by 1% potassium acetate).

Protein extractions and immunological analysis

Cells were grown in YPDA or the indicated minimal media at 25°C to a density of 5×10^6 per ml. For stress treatments a portion of the culture was maintained at 25°C with vigorous aeration and the remainder was exposed to stress, also with vigorous aeration, for times indicated in the figure legend. Total cellular proteins from equal numbers of cells were prepared by glass bead lysis in ethanol. After electrophoretic separation on 7.5% SDS–polyacrylamide gels, proteins were transferred to Immobilon (Amersham International) membranes and stained with Coomassie Blue to ensure equal loading. The blots were then reacted with an hsp104 or an hsp82 rabbit antiserum and immune complexes were visualized by reaction with ^{125}I-labeled protein A.

Tolerance to heat and other 'stresses'

Cells were grown at 25°C to mid-log phase ($2-6 \times 10^6$ cells/ml) in YPDA, minimal glucose, minimal galactose or minimal acetate media. For basal thermotolerance, cultures were sonicated briefly to disaggregate the cells and equal portions of the culture were transferred to glass tubes and exposed to 50°C for various lengths of time as previously described (Petko and Lindquist, 1986; Borkovich *et al.*, 1989; Sanchez and Lindquist, 1990).

For induced thermotolerance experiments, cells were pre-incubated at 37°C in a shaking water bath for 30 min, sonicated briefly and shifted to 50°C as for basal thermotolerance. After heating, cells were transferred to ice, diluted in ice-cold YPDA and plated onto YPDA medium. For other stress inductions, cells were pretreated at 25°C with vigorous aeration with 6% ethanol, 0.75 mM sodium arsenite, 5 mM cupric sulfate or 1.5 mM cadmium chloride for the times indicated in the text. To measure tolerance to other stresses, cells were treated with ethanol (20%), sodium arsenite (100 mM), copper sulfate (11 mM) or cadmium chloride (25 mM) at 25°C in large volumes (25–50 ml) with vigorous agitation or in small volumes (0.1–3 ml) without agitation. At various times, samples were removed and cells were washed in fresh YPDA before being plated to measure colony forming capacity. In all cases plates were incubated at 30°C for 3 days before colonies were counted. In all experiments each point is the average of three platings. In most cases, the variance between replicates was less than the width of the symbol. All experiments were repeated a minimum of three times.

Acknowledgements

We thank Irena Roizman for expert assistance and Dawn Parsell, Laura Arwood, Juliana Feder, Michael Welte, Jodi Vogel and Robert Petersen for helpful comments on the manuscript. This work was supported by a grant from the National Institutes of Health and by the Howard Hughes Medical Institute. Y.S. was supported by a post-doctoral fellowship from the Juan March Foundation.

References

Aujame,L. and Firko,H. (1988) *Mol. Cell. Biol.*, **8**, 5486–5494.
Aust,S.D., Morehouse,L.A. and Thomas,C.E. (1985) *J. Free Radic. Biol. Med.*, **1**, 3–25.
Barnes,C.A., Johnston,G.G. and Singer,R.A. (1990) *J. Bacteriol.*, **172**, 4352–4358.
Borkovich,K.A., Farrelly,F.W., Finkelstein,D.B., Taulien,J. and Lindquist,S. (1989) *Mol. Cell. Biol.*, **9**, 3919–3930.
Carper,S.W., Duffy,J.J. and Gerner,E.W. (1987) *Cancer Res.*, **47**, 5249–5255.
Chang,E.C., Kosman,D.J. and Willsky,G.R. (1989) *J. Bacteriol.*, **171**, 6349–6352.
Craig,E.A. and Jacobsen,K. (1984) *Cell*, **38**, 841–849.
Esposito,R.E., Dresser,M. and Breitenbach,M. (1991) *Methods Enzymol.*, **194**, 110–131.
Goldstein,S. and Czapski,G.J. (1986) *J. Free Radic. Biol. Med.*, **2**, 3–11.
Gottesman,S. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3513–3517.
Gross,C. and Craig,E.A. (1991) *Trends Biochem. Sci.*, **16**, 135–40.
Guy,C.L., Plesofsky,V.N. and Brambi,R. (1986) *J. Bacteriol.*, **167**, 124–129.
Hahn,G.M. and Li,G.C. (1982) *Radiat. Res.*, **92**, 452–457.
Hahn,G.M. and Li,G.C. (1990) In Morimoto,R.I., Tissieres,A. and Georgopoulos,C. (eds), *Stress Proteins in Biology and Medicine*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 79–100.
Hall,B.G. (1983) *J. Bacteriol.*, **156**, 1363–1365.
Hallberg,R.L., Kraus,K.W. and Hallberg,E.M. (1985) *Mol. Cell. Biol.*, **5**, 2061–2069.
Hightower,L.E. (1980) *J. Cell. Physiol.*, **102**, 407–427.
Kappeli,O. (1986) *Adv. Microb. Physiol.*, **28**, 181–209.
Kurtz,S., Rossi,J., Petko,L. and Lindquist,S. (1986) *Science*, **231**, 1154–1157.
Lagunas,R. (1986) *Yeast*, **2**, 221–228.
Laszlo,A. (1988) *Int. J. Hyperthermia*, **4**, 513–526.
Li,G.C. and Laszlo,A. (1985) In Atchinson,B.J. and Walden,D.B. (eds), *Changes in Eukaryotic Gene Expression in Response to Environmental Stress*. Academic Press, New York, p. 227.
Li,G.C., Li,L., Liu,Y.K., Mak,J.Y., Chen,L. and Lee,W.M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 1681–1685.
Lindquist,S. (1986) *Annu. Rev. Biochem.*, **55**, 1151–1191.
Lindquist,S. and Craig,E.A. (1988) *Annu. Rev. Genet.*, **22**, 631–677.
Morimoto,R.I., Tissieres,A. and Georgopoulos,C. Eds (1990) *Stress Proteins in Biology and Medicine*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Nover,L. Ed. (1991) *Heat Shock Response*. CRC Press, Boca Raton.
Parsell,D.A., Sanchez,Y., Stitzel,J.D. and Lindquist,S. (1991) *Nature*, **353**, 270–273.
Pelham,H.R. (1986) *Cell*, **46**, 959–961.
Petko,L. and Lindquist,S. (1986) *Cell*, **45**, 885–894.
Plesset,J., Palm,C. and McLaughlin,C.S. (1982) *Biochem. Biophys. Res. Commun.*, **108**, 1340–1345.
Plesset,J., Ludwig,J.R., Cox,B.S. and McLaughlin,C.S. (1987) *J. Bacteriol.*, **169**, 779–784.
Riabowol,K.T., Mizzen,L.A. and Welch,W.J. (1988) *Science*, **242**, 433–436.
Rothman,J.E. (1989) *Cell*, **59**, 591–601.
Sanchez,Y. and Lindquist,S.L. (1990) *Science*, **248**, 1112–1115.
Schenberg-Frascino,A. and Moustacchi,E. (1972) *Mol. Gen. Genet.*, **115**, 243–257.
Skowyra,D., Georgopoulos,C. and Zylicz,M. (1990) *Cell*, **62**, 939–944.
Solomon,J.M., Rossi,J.M., Golic,K., McGarry,T. and Lindquist,S. (1991) *The New Biologist*, **3**, 1106–1120.
Squires,C.L., Pedersen,S., Ross,B.M. and Squires,C. (1991) *J. Bacteriol.*, **173**, 4254–4262.
Stadtman,E.R. (1990) *Free Radic. Biol. Med.*, **9**, 315–325.
van Wijk,R. and Boon-Niermeijer,E.K. (1986) *Biona-Report*, **4**, 85–92.
Watson,K. (1990) *Adv. Microb. Physiol.*, **31**, 183–223.
Watson,K. and Cavicchioli,R. (1983) *Biotechnol. Lett.*, **5**, 683–688.
Watson,K., Dunlop,G. and Cavicchioli,R. (1984) *Febs Lett.*, **172**, 299–302.
Werner-Washburne,M., Becker,J., Kosic,S.J. and Craig,E.A. (1989) *J. Bacteriol.*, **171**, 2680–2688.
Widelitz,R.B., Magun,B.E. and Gerner,E.W. (1986) *Mol. Cell. Biol.*, **6**, 1088–1094.
Yost,H.J. and Lindquist,S. (1991) *Mol. Cell. Biol.*, **11**, 1062–1068.

Received on February 4, 1992