frozen in liquid nitrogen 7 days after infection. Before freezing, the virus stock contained 85,000 cpm per milliliter of reverse transcriptase activity measured as described previously [M. D. Daniel *et al.*, *J. Virol.* **62**, 4123 (1988)]. Portions of this stock virus (0.25 ml) were inoculated intravenously into each of the four rhesus monkeys. One rhesus monkey from this group, 452-87, has died to date. Six rhesus monkeys were inoculated with virus produced by the lambda clone from human T cell lines. Virus contained within the supernatant of HuT 78 cells after transfection was used to infect CEMx174 cells; 65 days after infection of CEMx174 cells, portions of the cell-free supernatant containing virus were frozen in liquid nitrogen. Before freezing, the cell-free supernatant contained 10^6 cpm per milliliter of reverse transcriptase activity. This stock (2 ml) was inoculated intravenously into each of the six rhesus monkeys. Three of these rhesus monkeys, 19788, 17504, and 18955, have died to date. Animals were euthanized when near death.

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24 January 1990; accepted 12 March 1990

HSP104 Required for Induced Thermotolerance

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A heat shock protein gene, *HSP104*, was isolated from *Saccharomyces cerevisiae* and a deletion mutation was introduced into yeast cells. Mutant cells grew at the same rate as wild-type cells and died at the same rate when exposed directly to high temperatures. However, when given a mild pre-heat treatment, the mutant cells did not acquire tolerance to heat, as did wild-type cells. Transformation with the wild-type gene rescued the defect of mutant cells. The results demonstrate that a particular heat shock protein plays a critical role in cell survival at extreme temperatures.

CERTAIN TYPES OF STRESS, INCLUDing mild heat, condition cells and organisms to survive severe heat treatments that would otherwise be lethal. This phenomenon is called induced thermotolerance. Since virtually all treatments that induce thermotolerance also induce synthesis of heat shock proteins (hsps), it is commonly assumed that these proteins participate in protecting cells from extreme temperatures. Although accumulated circumstantial evidence supports this assumption, contradictions exist. In certain cases, thermotolerance develops in the absence of hsp synthesis, while in others, synthesis of hsps is insufficient to produce thermotolerance (*1*).

Techniques allowing production of mutations by site-directed mutagenesis in yeast have enhanced our understanding of the functions of hsps. For example, yeast cells carrying homozygous mutations in both the *HSC82* and *HSP82* genes do not grow at any temperature, while cells homozygous for mutations in either of the two genes alone can grow only at temperatures below 37°C (*2*). These proteins are apparently required for growth at all temperatures, but are required in higher concentrations for growth at higher temperatures. Cells carrying mutations in two members of the yeast *HSP70* gene family, *SSA1* and *SSA2*, are temperature-sensitive for growth at 37°C (*3*), while triple mutations in *SSA1*, *SSA2*, and *SSA4* are lethal (*4*). Cells that carry mutations in the heat-inducible polyubiquitin gene are hypersensitive to long exposure at 38.5°C, just above their maximum growth temperature (*5*).

These experiments demonstrate that certain hsps help cells to cope with temperatures at the upper end of their natural growth range or to survive long exposures to temperatures that are just beyond their growth range. However, none of these mutations compromise tolerance to extreme temperatures. In fact, the *SSA1* and *SSA2* double mutants, as well as the polyubiquitin mutant, are more tolerant than wild-type cells to extreme temperature thermotolerance. In *SSA1* and *SSA2* double mutants, cells show greater than normal expression of other hsps (*3, 6*), which might explain their increased thermotolerance. Mutations in genes unrelated to the hsp genes, such as *ard1*, *bcy1*, and *ras2*$^{VAL\ 19}$, have been observed to block thermotolerance (*7–9*). However, these mutations appear to affect thermotolerance indirectly by blocking the entry of cells into stationary phase.

Here we report the isolation of the *HSP104* gene of yeast and the construction and analysis of strains carrying mutations in this gene. Hsp104 is the largest, heat-inducible protein in yeast cells. It is not detectable at normal growth temperatures, but becomes a major product of protein synthesis shortly after a shift to high temperatures (Fig. 1A). It is also induced during the transition to stationary phase growth and early in sporulation (*10*).

Hsp104 protein that had been purified by ion exchange chromatography and SDS–polyacrylamide gel electrophoresis was used to produce in rabbits a highly specific polyclonal antiserum (*10*). The antiserum (Fig. 1B) was used to screen an expression library of yeast genomic DNA fragments in λgt11 (*11*). Of 3×10^5 recombinant phages screened, seven gave positive signals after purification. Restriction endonuclease mapping showed that these clones share overlapping fragments. We chose to characterize the clone containing the biggest insert (3.6 kb, YS-121) (Fig. 2A).

To localize heat-induced sequences within this clone, various restriction fragments were hybridized with total RNA isolated from (i) log phase cells grown at normal temperature (25°C), (ii) log phase cells subjected to heat shock at 39°C for 30 min, and (iii) stationary phase cells grown at 25°C. Three fragments hybridized with a strongly inducible 2.7-kb mRNA in cells subjected to heat shock and stationary phase cells. The size of this RNA is in accordance with the estimated molecular size of hsp104 (104 kD). The fragments used and their relative levels of hybridization are depicted in Fig. 2B. A typical Northern blot obtained with the Bgl II–Hind III fragment is shown in Fig. 1C. The direction of transcription was determined by hybridization with RNA probes generated from the Eco RI–Hind III fragment, subcloned in both orientations into a vector that contains the T7 promoter (pVZ1) (*12*). Southern (DNA) blot analyses, conducted at both low and high stringencies, indicated that this gene is unique in the yeast genome (*13*).

To create a mutation in sequences encoding the 2.7-kb heat-inducible RNA, a 1.2-kb fragment (Apa I to Bgl II) (Fig. 2A) was

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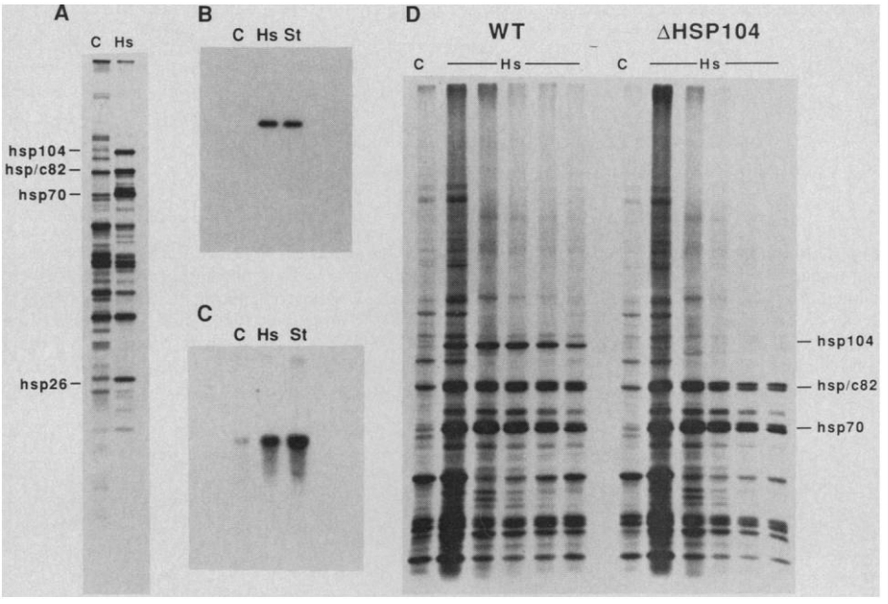

Fig. 1. Induction of hsp104 by heat or stationary phase arrest. (**A**) To measure protein synthesis in wild-type cells, mid-log phase cells grown at 25°C were maintained at 25°C and labeled with [^{3}H]-isoleucine for 30 min (C), or shifted to 39°C for 30 min (Hs) and then labeled for 30 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography (*24*). (**B**) Total cellular proteins from log phase cells growing at 25°C (C), log phase cells subjected to heat shock at 39°C for 30 min (Hs), or stationary phase cells grown at 25°C (St) were separated by SDS-PAGE, transferred to a nylon membrane, and reacted with a rabbit antiserum specific for hsp104; immune complexes were visualized by reaction with ^{125}I-labeled–protein A. (**C**) Total cellular RNAs (4 μg of RNA per lane) isolated from cells incubated at 25°C (C), cells subjected to heat shock at 39°C for 60 min (Hs), or cells in the stationary phase of growth (St) were denatured, electrophoretically separated, and hybridized to the nick-translated ^{32}P-labeled 0.6-kb Bgl II–Hind III fragment shown in Fig. 2B (*25*). (**D**) Protein synthesis was compared in the wild-type (aW303) and the mutant strain (aW303Δhsp104) (*26*). For the first lane in each panel, cells were labeled with [^{3}H]isoleucine for 15 min at 25°C. For remaining lanes, cells were shifted to 39°C and labeled for 15 min.

removed and replaced with the *LEU2* gene. Linear DNA containing this mutation (the Pvu I–Hind III fragment) (Fig. 2C) was transformed into haploid and diploid yeast cells. Gene conversion events, which replaced the wild-type gene with the mutation, were obtained by selecting for leucine prototrophy (*14*). The mutants were screened for the presence or absence of hsp104 by reacting electrophoretically separated proteins with antiserum to hsp104 protein (anti-hsp104). No cross-reacting material was detected in the mutant strains. Haploid transformants lacking hsp104 were obtained as readily as diploid transformants, indicating that the mutation is not lethal.

Since mutations in other yeast heat shock genes have pleiotropic effects on the synthesis of other hsps (*3, 6*), we examined the effect of the *hsp104* deletion mutation on the expression of other proteins. Wild-type and mutant cells were short term–labeled with [^{3}H]isoleucine at 25°C and at 39°C. Except for the absence of hsp104 in the mutant, the protein synthetic profiles of mutant and wild-type cells appeared to be identical (Fig. 1D). There was no obvious difference in the basal expression of major hsps at 25°C, nor was there any difference in the rate of their induction or synthesis during heat shock. We observed no detectable differences in the synthesis of other cellular proteins except, perhaps, for a very slight reduction in total protein synthesis with extended heat shock. Electrophoretic conditions for the gel in Fig. 1D were chosen to obtain maximum resolution of hsp104 from other proteins with similar molecular sizes. Gels optimized for the analysis of smaller proteins also showed no differences between mutant and wild-type cells.

We next analyzed the behavior of *hsp104* mutants with respect to growth at different temperatures. Growth rates at 25°C were identical for wild-type cells and cells carrying the mutation (Fig. 3A). When log phase cells were transferred from 25°C to 37.5°C, mutant cells continued growing at approximately the same rate as wild-type cells and reached similar stationary phase densities (Fig. 3A). As previously described (*2*), an *hsp83* deletion strain showed a reduced growth rate when shifted to 37.5°C.

To determine whether mutant cells were hypersensitive to long-term heat stress at 38.5°C, (as are the polyubiquitin mutants) cells were plated on rich dextrose medium. After incubation at 38.5°C for the times indicated (Fig. 3B), the plates were transferred to 25°C to allow surviving cells to form colonies. Under these conditions we observed no appreciable difference in viability between wild-type and mutant cells.

To measure thermotolerance, cells were incubated in liquid culture at 50°C for various lengths of time and plated to determine colony forming ability (Fig. 4). For basal

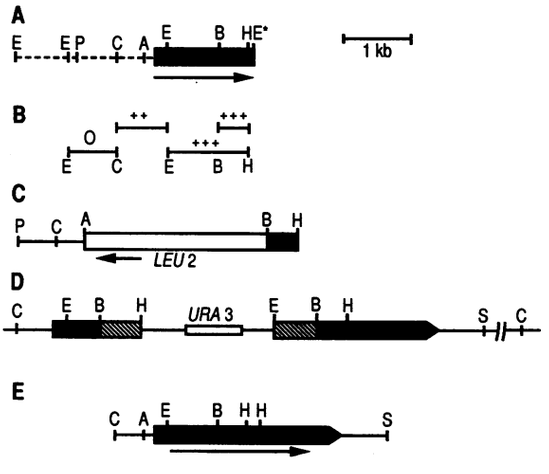

Fig. 2. Map of *HSP104* clones and constructs. (**A**) Restriction map of YS-121 includes the coding region (black box) and the direction of transcription (arrow). The Eco RI site (E*) was created during the construction of the λgt11 library, from which this clone was isolated. (**B**) Four restriction fragments were used as hybridization probes to localize the heat-inducible sequences in YS-121: strong (+++), moderate (++), and no (0) hybridization with the 2.7-kb heat-inducible RNA. (**C**) Map of the null *hsp104* deletion mutant allele, *hsp104::LEU2*. The *LEU2* gene replaced the Apa I–Bgl II fragment of *HSP104*. (**D**) Map of the integrative transformant created to rescue the 3′ end of the *HSP104* gene. The 1.2-kb Eco RI–Hind III fragment of YS-121 (hatched box) was subcloned into the integrative vector YIp5 (*27*), linearized, and integrated into the chromosome (black box) by homologous recombination. Total genomic DNA from this transformant was digested with Cla I and the plasmid was rescued in *Escherichia coli*. The Eco RI–Sph I fragment of this plasmid was ligated with the Cla I–Eco RI fragment of YS-121 and the Bam HI–Cla I fragment of the centromeric vector pRS316. After Sph I and Bam HI cleavage, blunt ends were created before ligation. (**E**) Restriction map of pYS104. Apa I (A), Bgl II (B), Cla I (C), Eco RI (E), Hind III (H), Pvu I (P), and Sph I (Sp).

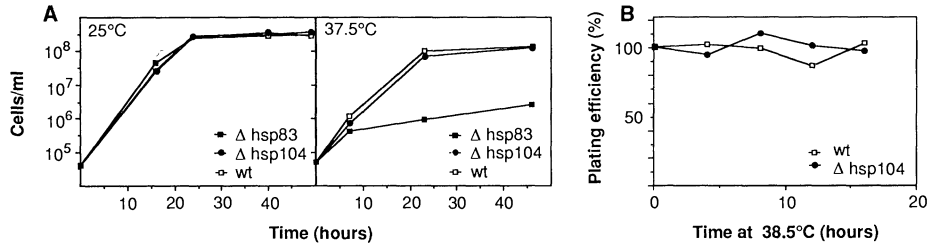

Fig. 3. Mutation of the *HSP104* gene does not affect growth at 25°C or at 37.5°C, nor long-term survival at 38.5°C. (**A**) Growth at 25°C and 37.5°C. Cells in the mid-log phase of growth (25°C) were diluted in YPDA liquid (1% yeast extract, 2% bactopeptone, 2% dextrose, 40 mg of adenine per liter) at 25°C or at 37.5°C (4×10^5 cells per milliliter) and counted at the indicated times with a hemacytometer. (**B**) Survival after chronic heat stress at 38.5°C. Mid-log phase cells (25°C) from a YPDA liquid culture were diluted, plated on YPDA plates (200 cells per plate) and incubated at 38.5°C. At the times indicated, the plates were returned to 25°C to allow colony formation by viable cells.

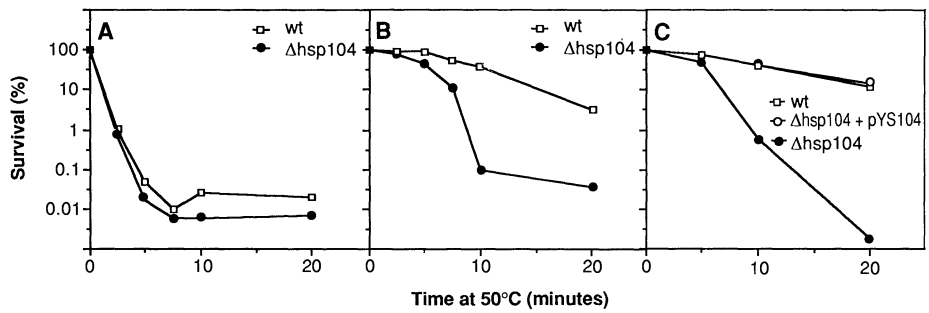

Fig. 4. Mutation of the *HSP104* gene affects the induction of thermotolerance. Cells were grown at 25°C to mid-log phase (6×10^6 cells per milliliter) in YPDA liquid (**A** and **B**) or in minimal media plus amino acids in order to maintain the pYS104 plasmid (**C**). Prior to the 50°C heat treatment, matched cultures were either maintained at 25°C (A) or preincubated at 37°C for 30 min (B and C). Following heat treatment at 50°C, cells were transferred to ice, diluted in ice-cold YPDA, and immediately plated on YPDA.

thermotolerance, log phase cells were shifted directly from 25° to 50°C. For induced thermotolerance, cells were incubated for 30 min at 37°C before being transferred to 50°C. The mutant cells died at nearly the same rate as wild-type cells when shifted directly to 50°C. However, when incubated initially at 37°C, the mutant cells showed a 100-fold reduction in survival compared to wild-type cells after 20 min at 50°C in rich media. In minimal media, the difference in killing rates was greater than 1000-fold. When wild-type and mutant cells were incubated at 37°C prior to heating at 50°C, the difference in survival was not uniform at all time points. After brief exposure to 50°C, pretreated mutant cells showed nearly the same degree of thermotolerance as wild-type cells.

If the defect in induced thermotolerance is due to the absence of the *HSP104* gene product, then reintroduction of the *HSP104* gene should rescue the defect. To determine if the mutant phenotype could be rescued by the *HSP104* gene, the *hsp104* deletion strain was transformed with the plasmid pYS104 (*15*). This plasmid contained the intact *HSP104* gene, as well as yeast centromeric sequences that maintained the plasmid at approximately one copy per cell (*16*). The transformed cells produced a 104-kD, heat-inducible protein, in approximately the same quantity as wild-type cells, that cross-reacted with anti-hsp104 antibody. The *HSP104* gene completely rescued the thermotolerance defect in the deletion strain (Fig. 4C). We thus concluded that this thermotolerance defect was due to the absence of *HSP104* gene product.

Our results demonstrate that a heat shock protein, hsp104, is required for induced thermotolerance. Why, then, do so many contradictory reports exist regarding the participation of hsps in thermotolerance? For example, in yeast as well as in other organisms, some studies show that inhibitors of protein synthesis block the induction of tolerance (*17, 18*). Our results are consistent with these studies because the inhibitors would block induction of hsp104. However, others have reported that inhibition of protein synthesis does not block thermotolerance (*19–21*). Some such reports may be dismissed because the effectiveness of the inhibitors was never demonstrated or because the gels employed to characterize hsp induction did not adequately resolve them from normal cellular proteins. But these problems do not apply in every case. Our work has uncovered two additional explanations: (i) hsp104 and other hsps are induced a few cell divisions before the stationary phase plateau (in YPDA medium, at 5×10^6 to 1×10^7) (*13*). Hence, small differences in the growth phase can yield large differences in basal hsp expression and thermotolerance; and (ii) choosing a single time point at which to measure thermotolerance carries the risk that the participation of a heat shock protein, like hsp104, would be missed. That is, at early points in the killing curve (Fig. 4, B and C), both mutant and wild-type cells show thermotolerance after pretreatment at 37°C. If only these points were considered, hsp104 would not appear to play a critical role in induced thermotolerance.

In effect, the *hsp104* mutation uncovers at least one additional thermotolerance factor that functions in the early stages of exposure to high temperatures. Whether or not this factor is another heat shock protein is as yet unclear.

With respect to evolutionary conservation of hsp104, most organisms have heat shock proteins in the size range of hsp104; with the exception of the mammalian protein, they have not been characterized. Mammalian hsp110 protein is concentrated in the nucleolus, where it appears to bind to RNA or to a complex of RNA binding proteins (*22, 23*). The gene encoding hsp110 has not been cloned, and its relation to hsp104 is unknown. However, electron microscopy of *hsp104* mutants subjected to heat shock (in contrast with wild-type cells) shows a dramatic condensation of electron-opaque material, arranged in a crescent-like shape underlying the nuclear membrane, which may be associated with the nucleolus (*13, 28*). Finally, *Drosophila* is one of the few eukaryotic organisms that does not have a heat-inducible protein in this size range, yet it displays inducible thermotolerance (*1*). It may be that *Drosophila* cells rely upon distinct thermotolerance factors, such as those uncovered by our mutation in yeast. Alternatively, the hsp104 analog in *Drosophila* cells might be a constitutively synthesized protein.

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30 January 1990; accepted 2 April 1990

Cell Interactions in the Sea Urchin Embryo Studied by Fluorescence Photoablation

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In many organisms, interactions between cells play a critical role in the specification of cell fates. In the sea urchin embryo, primary mesenchyme cells (PMCs) regulate the developmental program of a subpopulation of secondary mesenchyme cells (SMCs). The timing of this cell interaction was analyzed by means of a fluorescence photoablation technique, which was used to specifically ablate PMCs at various stages of development. In addition, the PMCs were microinjected into PMC-depleted recipient embryos at different developmental stages and their effect on SMC fate was examined. The critical interaction between PMCs and SMCs was brief and took place late in gastrulation. Before that time, SMCs were insensitive to the suppressive signals transmitted by the PMCs.

INTERACTIONS BETWEEN EMBRYONIC cells are an important mechanism by which cell fates are specified during development. In some organisms, including amphibians, sea urchins, and mammals, cell interactions play an especially significant role in the commitment process (*1*). Even in multicellular animals in which cell diversification is believed to be primarily under the control of cytoplasmic determinants segregated to different blastomeres during cleavage (for example, ascidians and nematodes), cell interactions are critical for the differentiation of certain cell types (*2*).

The sea urchin embryo has been a model for studying the specification of cell fate by cellular interactions. Isolated blastomeres can form complete larvae, providing evidence of regulative cell interactions that determine cell fates (*3, 4*). Cellular interactions are associated with the selection of cell fates by mesenchymal cell lineages in the sea urchin embryo. During normal ontogeny the skeletal structures of the larva are synthesized by primary mesenchyme cells (PMCs) (*5*). Late in gastrulation a second population of mesenchyme cells arises, the secondary mesenchyme cells (SMCs), which give rise to muscle cells, pigment cells, and portions of the coelomic sacs, but do not normally contribute to the larval skeleton (*3, 6*). These two cell lineages are segregated early in development, at the fourth cleavage division (*7*). PMC-depletion experiments indicate that an interaction takes place between the two cell populations during gastrulation (*8*). In the absence of the PMCs, 65 to 75 SMCs convert to the PMC phenotype and synthesize a complete larval skeleton. Therefore, SMCs can form skeletal structures, but this pathway of differentiation is normally suppressed by the PMCs. The suppression of SMC skeletogenesis is quantitatively dependent on the number of PMCs in the blastocoel. If at least 50 PMCs are present, SMC conversion is completely blocked. If fewer than 50 PMCs are present, the number of SMCs that express a skeletogenic phenotype is inversely proportional to the number of PMCs in the blastocoel (*9*). I now show that the critical interaction between the PMCs and SMCs is brief and occurs late in gastrulation, at the time the SMCs enter the blastocoel and begin to migrate.

The timing of the interaction between PMCs and SMCs was determined by means of a fluorescence photoablation technique that was used to specifically eliminate the PMCs at different stages of development. In previous experiments, microsurgical methods were used to remove PMCs from embryos immediately after their formation (*9*). At later stages of embryo development, this approach is impractical because the PMCs disperse within the blastocoel and adhere firmly to the basal surfaces of overlying epithelial cells. Therefore to ablate PMCs at later stages, endogenous populations of PMCs were removed microsurgically and replaced with equal numbers of PMCs that had been covalently labeled with rhodamine B isothiocyanate (RITC) (*10*). The fluorescently tagged PMCs were then selectively ablated at different developmental stages by irradiating the embryos with green light (λ_{max} = 550 nm). Previous studies have shown that rhodamine-labeled PMCs microinjected into the blastocoel of recipient embryos undergo normal migration and spiculogenesis (*11*). Such transplanted PMCs completely suppress the conversion of SMCs to a skeletogenic phenotype when microinjected into PMC-depleted embryos, provided that at least 50 cells are transplanted (*9*).

Other studies have used fluorescence methods to ablate embryonic cells by mi-

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