The Intracellular Location of Yeast Heat-shock Protein 26 Varies With Metabolism

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Abstract. An antibody highly specific for heat-shock protein (hsp)26, the unique small hsp of yeast, and mutants carrying a deletion of the *HSP26* gene were used to examine the physical properties of the protein and to determine its intracellular distribution. The protein was found in complexes with a molecular mass of >500 kD. Thus, it has all of the characteristics, including sequence homology and induction patterns, of small hsps from other organisms. When log-phase cells growing in glucose were heat shocked, hsp26 concentrated in nuclei and continued to concentrate in nuclei when these cells were returned to normal temperatures for recovery. However, hsp26 did not concentrate in nuclei under a variety of other conditions. For example, in early stationary-phase cells hsp26 is induced at normal growth temperatures. This protein was generally distributed throughout the cells, even after heat shock. Similarly, in cells genetically engineered to synthesize hsp26 in the presence of galactose, hsp26 did not concentrate in nuclei, with or without a heat shock. To determine if the failure of hsp26 to concentrate in the nucleus of these cells was due to the fact that the protein had been produced at 25°C or to a difference in the physiological state of the cell, we investigated the distribution of the heat-induced protein in cells grown under several different conditions. In wild-type cells grown in galactose or acetate and in mitochondrial mutants grown in glucose or galactose, hsp26 also failed to concentrate in nuclei with a heat shock. We conclude that the intracellular location of hsp26 in yeast depends upon the physiological state of the cell and not simply upon the presence or absence of heat stress. Our findings may explain why previous investigations of the intracellular localization of small hsps in a variety of organisms have yielded seemingly contradictory results.

EXPOSING cells or whole organisms to temperatures that are 5–10°C above their normal growing temperature results in the synthesis of the heat-shock proteins (hsps).[1] Organisms as diverse as mammals, yeasts, higher plants, eubacteria, and even archaebacteria exhibit this response. Nearly all species synthesize hsps belonging to three different gene families, with molecular masses in the range of 80–90, 68–75, and 15–30 kD, respectively. Within these families, sequence homologies are observed in both protein coding and regulatory regions (Ingolia et al., 1980, 1982; Karch et al., 1981; Craig et al., 1982; Bienz, 1984; Hunt and Morimoto, 1985; Pelham, 1982; Holmgren et al., 1981; Cohen and Meselson, 1984; Schoffl et al., 1984). The most highly conserved protein is hsp70, so designated because its molecular mass is close to 70 kD. The predicted amino acid sequence for the human protein is 73% identical to the *Drosophila* protein, and 50% identical to the *Escherichia coli* homolog, dnaK. Hsp83 is also highly conserved. The predicted amino acid sequence of the human protein indicates an amino acid identity of 41% with the *Drosophila* protein (Hackett and Liss, 1983) and 42% with the *E. coli* protein (Bardwell and Craig, 1987).

The small hsps are much less conserved in size and sequence, but the proteins from all eukaryotes examined to date show a relationship to each other and to the vertebrate α-crystallins (Ingolia and Craig, 1982; Russnak and Candido, 1985; Schlesinger, 1984; Czarnecka et al., 1985; Nagao et al., 1985). The proteins have similar hydropathy profiles and small regions of amino acid identity. Their most invariant feature is the amino acid consensus sequence aa_1-gly-aa_2-leu-aa_3-aa_4-aa_5-aa_6-pro-aa_7 located near the carboxy terminus. (The amino acids designated by aa_{1-7} also show a high degree of homology but are not invariant.) The small hsps and the α-crystallins also share the property of forming highly polymeric structures, with sedimentation coefficients of 15–20 S (Arrigo et al., 1985; Schuldt and Kloetzel, 1985; Fransolet et al., 1979; Nover et al., 1983; Arrigo and Welch, 1987).

Another feature shared by the small hsps of various organisms is induction during normal growth, at particular stages in development. A common feature of these developmental inductions is that only a subset of the hsps are produced. For example, in *Drosophila*, mRNAs for several small hsps are

1. *Abbreviations used in this paper*: DAPI, 4',6-diamidino-2-phenylindole; hsp, heat-shock protein.

induced during late larval and early pupal stages (Sirotkin and Davidson, 1982; Cheney and Shearn, 1983; Ireland and Berger, 1982; Ayme and Tissieres, 1985) and during oogenesis (Zimmerman et al., 1983). A strikingly similar pattern of developmental expression occurs in *Saccharomyces*, in which hsp26 mRNAs accumulate during the transition from the log to the stationary phase of growth and during the course of sporulation (Kurtz et al., 1986). The small hsps are also induced in lilies during meiosis (Bouchard, 1987). The conservation of developmental inductions in such diverse organisms suggests that the small hsps may play a role in development as well as in the response to stress.

Since the induction of hsps generally coincides with the acquisition of tolerance to heat and other stresses, it has been postulated that hsps are an essential component of tolerance (see Lindquist, 1986, for review). Several studies have suggested that the small hsps, in particular, may be responsible for acquired thermotolerance. For example, a mutant strain of *Dictyostelium* that does not synthesize the small hsps also does not acquire thermotolerance (Loomis and Wheeler, 1982). In *Drosophila*, ecdysone induces the synthesis of small hsps (but not hsp70) and also induces tolerance to extreme temperatures (Berger and Woodward, 1983). Also, tomato cells, which constitutively synthesize hsp68 and hsp70, are thermosensitive but become thermotolerant when given a mild heat treatment that induces the small hsps (Nover and Scharf, 1984).

However, genetic experiments in *Saccharomyces cerevisiae* have failed to uncover a function for the unique small hsp of this organism, hsp26 (Petko and Lindquist, 1986). Deletion and disruption mutations have no detectable effect on growth at high temperatures, on the acquisition of thermotolerance, on spore development, nor on germination (Petko and Lindquist, 1986). It may be that another protein is able to substitute for hsp26. If so, it bears little homology to hsp26. No cross-reacting genes have been detected by low stringency DNA hybridization and no cross-reacting proteins have been observed with a polyclonal anti-hsp26 antibody (Petko and Lindquist, 1986; see below). An alternative view is that the phenotype of the mutation is more subtle than expected. Since we have been unable to determine the function of hsp26 through genetic analysis, we have initiated a characterization of the protein itself with the hope that such investigations might lead to testable hypotheses concerning its function.

Because of its small cell size and problems presented by cell wall removal, the yeast *S. cerevisiae* has not been a favored organism for protein localization studies. Nevertheless, in the case of the small hsps, *S. cerevisiae* presents distinct advantages. First, this organism has only one major small heat-shock protein, hsp26. Second, strains carrying deletions of the unique *HSP26* gene have been produced by site-directed mutagenesis, providing unambiguous verification of antibody specificity. Third, the effects of cellular physiology on protein localization can be determined by examining cells that (*a*) are in the log or the stationary phase of growth, (*b*) are in either respiratory or fermentative metabolism, (*c*) harbor mutations in mitochondrial DNA (respiration deficient), and (*d*) are engineered to produce the protein in the absence of heat. In this paper, we present the results of our investigations of hsp26 localization in yeast.

Materials and Methods

Yeast Strains and Culture

Strain LM-1, constructed from A364a and K210A, has the genotype:

$$\frac{a}{\alpha}\ \frac{adel}{+}\ \frac{ade2}{ade2}\ \frac{+}{ade5}\ \frac{CAN^s}{can^R}\ \frac{gall\text{-}4}{+}\ \frac{his7\text{-}1}{his7\text{-}2}\ \frac{+}{leu1\text{-}12}\ \frac{lys2\text{-}2}{lys2\text{-}1}\ \frac{+}{met13d}\ \frac{+}{trp5d}\ \frac{tyr1\text{-}2}{tyr1\text{-}1}\ \frac{ura1}{+}\ \frac{+}{ura3\text{-}13}.$$

All other strains used in this study were derived from strain W303 as described by Petko and Lindquist (1986).

Glucose-grown cells were cultured in YPDA (1% yeast extract, 2% Bactopeptone, 2% glucose, 10 μg/ml adenine sulfate) to log (5×10^6 cells/ml) or stationary phase (4 h after reaching plateau phase density of $1\text{–}2 \times 10^8$ cells/ml). Acetate- or galactose-grown cells were cultured in the same media, with 2% dextrose replaced by 2% acetate (YPA) or 2% galactose (YPG), respectively.

The *gall-HSP26* fusion was created by adding Bg III linkers to the Nru I site of the *HSP26* gene (Petko and Lindquist, 1986). The resulting Bg III fragment containing hsp26-coding sequences was inserted into the Bam HI site of pBM150 (Johnston and Davis, 1984). The fusion gene, containing the entire hsp26 open reading frame, 56 bp 5′ of the initiating ATG, and 31 bp 3′ of the terminating TAA, was transformed into *hsp26*$^-$ deletion mutants as described by Petko and Lindquist, 1986.

Antiserum Production

The immunogen was prepared from cells of strain LM-1 heat shocked at 39°C for 5 h. Cells were lysed by agitation on a vortex mixer in the presence of glass beads (0.1 mm diameter) and ice cold 100% ethanol, 1 mM PMSF. Proteins were collected by centrifugation and resuspended in sample buffer (65 mM Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) for separation on 10% SDS-polyacrylamide gels (Laemmli, 1970). Hsp26 was cut from the gels and electroeluted into dialysis tubing. Electroeluted proteins were precipitated in ice cold 100% ethanol, dried, and resuspended in PBS, pH 7.4. New Zealand White rabbits (>2 kg) were immunized both intramuscularly and subcutaneously with 50 μg of protein in complete Freund's adjuvant. For a period of 6 wk, rabbits were boosted with 25–50 μg of protein in incomplete Freund's adjuvant every 2 wk, and bled 1 wk after each boost.

Protein Labeling and Immunoprecipitation

Yeast cells (10 ml) were grown at 25°C to log phase ($0.5\text{–}1 \times 10^7$/ml) in minimal acetate medium (1% KOAc, 0.6% yeast nitrogen base, 5% phthalic acid, pH 5.5), supplemented with the appropriate substrates for auxotrophic growth. [^{3}H]isoleucine (400 μCi, 106 Ci/mM) was added immediately after a shift to 39°C and incubation continued for 2 h. Since normal protein synthesis is only partially repressed at 39°C and is fully restored within 2 h, both heat-shock and normal cellular proteins were labeled. Cells were collected by centrifugation, washed twice with water, and resuspended in 150 μl PBS containing 1 mM PMSF. Cells were transferred to 10 × 75-mm glass tubes containing 0.2 g of glass beads (0.1 mm diameter) and disrupted by agitation on a vortex mixer for 6 min.

Immunoprecipitation reactions were performed essentially as described by Julius et al. (1984) with minor modifications. To avoid nonspecific adsorption of proteins to *Staphylococcus aureus*, the lysates were pretreated as follows. Labeled lysate (10 μl) was added to 450 μl of 1% Triton X-100 in PBS in a 1.5-ml microfuge tube. 25 μl of IgGsorb, a 10% suspension of *S. aureus* cells (The Enzyme Center Inc., Malden, MA), was added followed by end-over-end mixing at 4°C for 30 min. The mixture was clarified by centrifugation in a microfuge (13,000 *g*) for 5 min and the supernatant transferred to a fresh tube. Preimmune rabbit serum (5 μl) was added, mixed, and the mixture was clarified by centrifugation as above. A fresh aliquot of IgGsorb (25 μl) was added, mixed, clarified by centrifugation, and the supernatant removed to a fresh tube.

For immunoprecipitation, anti-hsp26 rabbit serum (5 μl) or 1% Triton X-100, PBS (5 μl) was added to the pretreated lysate, and incubated with mixing at 4°C for 60 min. IgGsorb (25 μl) was then added and mixed end over end at 4°C for 30 min. Immune complexes were collected by centrifugation in a microfuge (13,000 *g*), and washed by resuspension and centrifugation in 1 ml each: 1% Triton X-100, 0.1% BSA in PBS; 1% Triton X-100, 1% BSA, 1% β-mercaptoethanol, 0.1% SDS in PBS. Pellets were resus-

pended in 40 μl sample buffer and heated for 3 min in boiling water. Samples were clarified by centrifugation for 5 min in a microfuge (13,000 *g*) and the supernatant was layered (35 μl) onto a 12.5% SDS-polyacrylamide gel (Laemmli, 1970). After electrophoresis, the gel was prepared for fluorography according to Laskey and Mills (1975) and exposed to preflashed XAR-5 film (Eastman Kodak Co., Rochester, NY) at −80°C.

Gel-filtration Chromatography

Drosophila S2 cells (1.5 × 10^8 cells/7.5 ml) were heat shocked at 37°C for 45 min, and returned to 25°C for 2 h with [^{3}H]leucine (1.875 mCi, 186 Ci/mM) added during the last hour. The cells were collected by centrifugation, rinsed in PBS, and resuspended in 500 μl column buffer (50 mM Tris, 100 mM NaCl, 1 mM PMSF, 5 mM EDTA, pH 7.5, 0.02% Na-azide). Cell lysates were prepared by sonication followed by agitation in the presence of glass beads (0.1 mm diameter) on a vortex mixer. Lysates were clarified by three rounds of centrifugation in a microfuge (15 min, 13,000 *g*) at 4°C, with the supernatant transferred to a fresh tube between spins. The entire sample was applied to a Biogel A 0.5-M (Bio-Rad Laboratories, Richmond, CA) gel-filtration column equilibrated in column buffer. (Identical results were obtained when samples were prepared in, and separated on, columns equilibrated in column buffer containing 300 mM NaCl or 5 mM $MgCl_2$ without added EDTA.) 1.5-ml fractions were collected and sample buffer (25 μl of a 4× solution) was added to 75 μl of each fraction for analysis on a 10% SDS-polyacrylamide gel (Laemmli, 1970). The labeled proteins were visualized by fluorography as described above.

Yeast cells (50 ml) grown at 25°C to 5.5 × 10^6/ml were labeled with [^{3}H]isoleucine (1 mCi, 106 Ci/mM) for 45 min at 39°C. Cells were collected by centrifugation, washed once with water, and resuspended in 500 μl column buffer. Cell lysates were prepared by sonication, clarified by centrifugation, and loaded onto the column as described above. Fractions were collected, and proteins from each fraction were electrophoretically separated as described above.

Papain Digestion of Antiserum

Mercuripapain dissolved in coupling buffer (0.1 M $NaHCO_3$ buffer, pH 8.3), was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) in a ratio of 5 mg mercuripapain/1 ml Sepharose gel according to the manufacturer's instructions.

Efficient cleavage was obtained by digesting 2 mg of antiserum with 4 mg of papain in a volume of 3 ml. The mercuripapain–Sepharose conjugate (800 μl) was washed three times with 1 mM EDTA, 5 mM cysteine, pH 6.6, to remove the mercury and reactivate the papain. Activated papain–Sepharose was resuspended in 1.7 ml of the same buffer and 2 mg (500 μl) of ammonium sulfate–precipitated antiserum was added. The mixture was incubated for 24 h (or until digestion was complete) by end-over-end mixing at room temperature. Complete digestion of antibody into Fab fragments was monitored on SDS–polyacrylamide gels using sample buffer without β-mercaptoethanol. Fc cleavage products and uncleaved antibodies were removed by adsorption to *S. aureus* cells (Calbiochem-Behring Corp., San Diego, CA). Digested antibody was mixed at room temperature with a 10–20% vol of *S. aureus* cells for ~30 min. *S. aureus* cells were removed by centrifugation.

Fixation, Immunofluorescence, and Sectioning

Cells were prepared according to the procedure of Kilmartin and Adams (1984) with some modifications. 1-3 × 10^8 cells were collected by centrifugation and fixed for 2 h at 25°C in 5 ml of buffer A (35 mM KPO_4, 0.5 mM $MgCl_2$, pH 9) plus 0.6 ml 37% formaldehyde. The cells were collected by centrifugation and rinsed three times with buffer B (35 mM KPO_4, 0.5 mM $MgCl_2$, 1.2 M sorbitol, pH 6.8). They were resuspended in 1 ml buffer B containing 50 μl freshly prepared zymolyase (20 mg/ml) (Kirin Breweries, Tokyo, Japan), and 10 μl β-mercaptoethanol for 15–30 min at room temperature. The resulting spheroplasts were collected by centrifugation, rinsed twice in buffer B, and three times in 0.1% BSA in PBS (BSA/PBS).

For whole mounts, cells were resuspended in 1 ml 1% BSA/PBS, and one drop of cell suspension was placed into one chamber of an eight-chamber slide (Miles Laboratories, Inc., Naperville, IL). Excess liquid was drawn off and the cells were allowed to dry. Before reaction with antibodies, cells were incubated for 10 min with 0.2% Triton X-100 in PBS, rinsed three times with PBS, and then incubated for 15 min in 1% BSA/PBS. Cells were then incubated for 1 h with rabbit anti-hsp26 Fab serum diluted 1:6 into 1% BSA/PBS, rinsed three times with PBS, and washed twice for 15 min with 1% BSA/PBS. After washing, cells were incubated for 1 h with fluorescein-conjugated F(ab')$_2$ goat anti-rabbit IgG F(ab')$_2$ (Jackson Immunoresearch Laboratories Inc., Avondale, PA) diluted 1:50 into 1% BSA/PBS, rinsed, washed as before, rinsed in PBS, dried, and mounted. To stain tubulins, cells were incubated with monoclonal antibody YOL/34, a rat anti-tubulin IgG, diluted 1:10 (Accurate Chemical & Scientific Corp., Westbury, NY); and then with F(ab')$_2$ goat anti-rat IgG diluted 1:300 (Cappel Laboratories, Cochranville, PA). All incubations were carried out in a moist chamber at room temperature.

For sectioning, cells were prepared for 10 min in 0.2% Triton X-100 in PBS with agitation, collected by centrifugation, rinsed twice in PBS, and washed for 15 min in 0.1% BSA/PBS. The cells were then transferred to 10 × 75-mm glass tubes and incubated in 300 μl of anti-hsp26 Fab rabbit serum for 1 h. Cells were collected by centrifugation, rinsed, and washed for 15 min in 1% BSA/PBS. This procedure was repeated with fluorescein-conjugated F(ab')$_2$ goat anti-rabbit F(ab')$_2$. Cells were then transferred to 1.5-ml microfuge tubes and collected by centrifugation (13,000 *g*).

Dehydration, infiltration, and embedding into JB-4 plastic were performed according to the manufacturer's instructions (Polysciences Inc., Warrington, PA) with the following modifications. Before infiltration, solution A was filtered through charcoal to minimize autofluorescence of the plastic. Embedded samples were polymerized at −20°C to prevent quenching of the fluorescence. After polymerization, blocks were cut into 1-μm sections and adhered directly onto glass slides. Sections were incubated for 10 min in 30 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS and then mounted.

Microscopy

All stained preparations were mounted in freshly prepared 70% glycerol in PBS containing 1 mg/ml *p*-phenylenediamine (Johnson and de C. Nogueira Araujo, 1981). Slides were examined with a Nikon Optiphot microscope fitted for phase-contrast imaging and epifluorescence. All photographs were taken on Tri-X pan film (ASA 400) (Eastman Kodak Co.), except those in Fig. 5 which were taken using Kodak Technical (2415) pan film.

Results

Preparation of Anti–Yeast Hsp26 Serum and Characterization of the Protein

Hsp26 is the only Coomassie Blue–stainable protein in the 26-kD region of SDS–polyacrylamide gels, as determined by comparing total cellular proteins from wild-type yeast cells with those from cells carrying a deficiency for the *HSP26* gene (Petko and Lindquist, 1986). To produce an immunogen, total cellular proteins were electrophoretically separated, the 26-kD band was excised from the gel, and the protein was eluted. Of two rabbits immunized with this material, both produced a highly specific, high titer antiserum. A single, immunoreactive band of 26 kD was observed when total proteins from heat-shocked cells were electrophoretically separated, transferred to nitrocellulose, reacted with the antiserum, and visualized with a second, enzyme-linked antibody (Western blot analysis). The band was not observed with proteins from wild-type cells in the log phase of growth at 25°C nor with proteins from heat-shocked *hsp26*$^-$ cells. The band was observed with proteins from wild-type cells grown into stationary phase or transferred to sporulation medium at 25°C (Kurtz and Lindquist, 1984; Kurtz et al., 1986; Petko and Lindquist, 1986). Thus, the previously described developmental induction of hsp26 mRNA in yeast cells (Kurtz et al., 1986) is accompanied by induction of the protein.

Since Western blot analysis involves protein denaturation, it does not provide sufficient evidence for the monospecificity of the antiserum in immunofluorescent analysis. There-

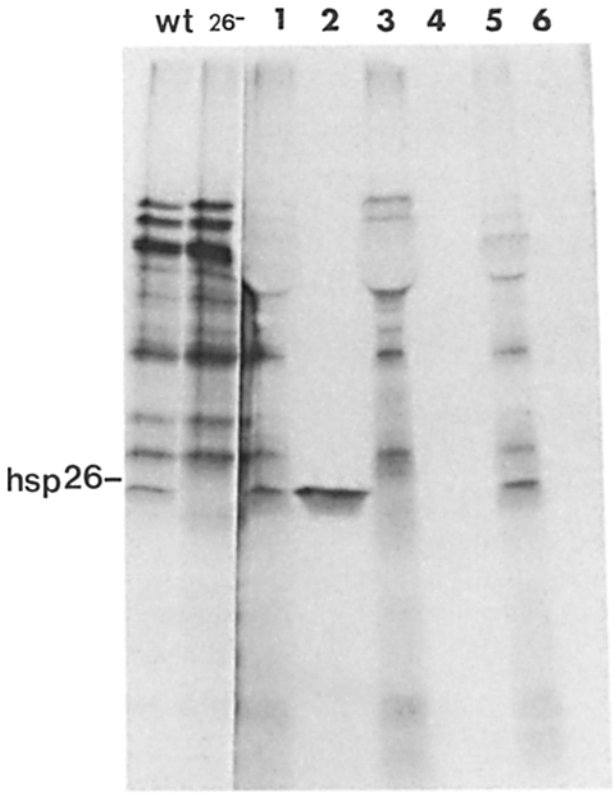


Figure 1. Immunoprecipitation of hsp26. Wild-type (*wt*) and *hsp26*⁻ yeast cells (*26*⁻) were labeled with [³H]isoleucine for 2 h at 39°C. Anti-hsp26 serum was coupled to *S. aureus* cells and used to precipitate labeled proteins from yeast cell lysates. Immune complexes were eluted with sample buffer, analyzed on 12.5% SDS-polyacrylamide gels, and visualized by fluorography. (*wt* and *26*⁻) Total lysate from wild-type and hsp26⁻ cells. (Lanes *1–6*) *S. aureus* precipitation in the presence of immune serum with unbound (lane *1*) and bound (lane *2*) wild-type lysate; and unbound (lane *3*) and bound (lane *4*) *hsp26*⁻ lysate; *S. aureus* precipitation with wild-type lysate in the absence of unbound (lane *5*) and bound (lane *6*) immune serum.

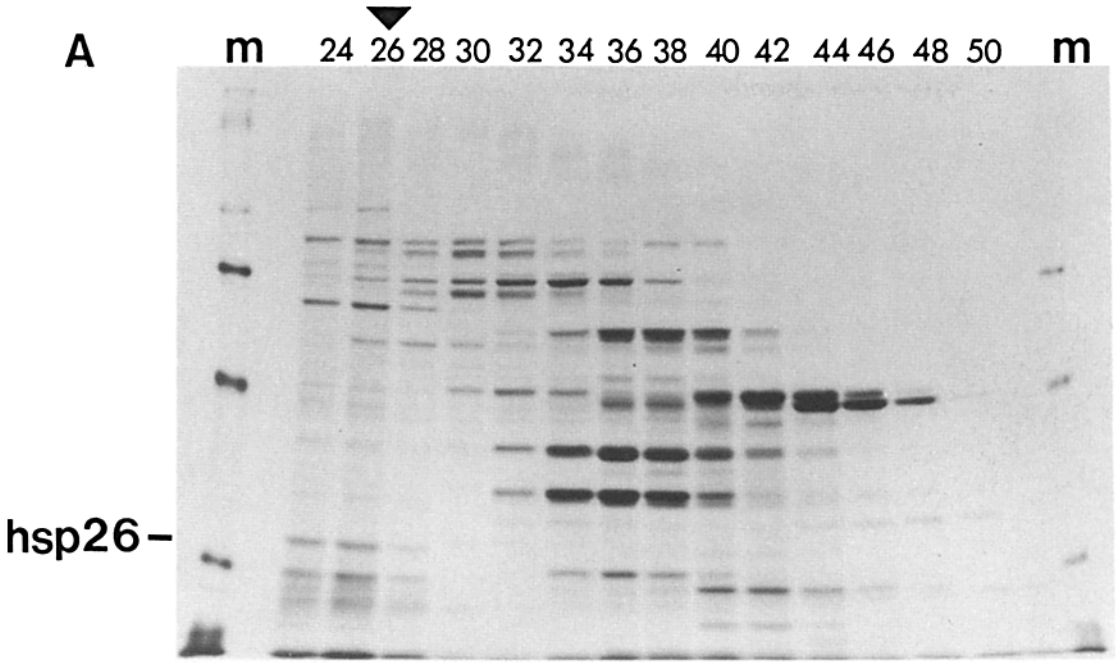
fore, we tested the specificity of the antiserum in immunoprecipitation reactions with proteins from native cell lysates. Isogenic *HSP26*⁺ and *hsp26*⁻ yeast cells were incubated with [³H]isoleucine for 2 h immediately after a shift to 39°C, to label both heat-induced and normal cellular proteins. The cells were lysed with glass beads in PBS, antiserum was added, and reactive proteins were precipitated with *S. aureus* cells. Only one labeled protein was recovered from the precipitate and it had an apparent molecular mass of 26 kD (Fig. 1, lane *2*). This protein was not recovered from *hsp26*⁻ cell lysates (Fig. 1, lane *4*) nor was it recovered from wild-type cell lysates incubated in the absence of anti-hsp26 serum (Fig. 1, lane *6*). Taken together, the results of the Western blot and immunoprecipitation reactions demonstrate that the antiserum is highly specific for hsp26. The immunoprecipitation reactions also suggest that no other proteins form stable complexes with hsp26 in substantial quantities.

Although experiments in *Dictyostelium*, *Drosophila*, and tomato suggest that the small hsps might play a role in thermotolerance, a deletion of the *HSP26* gene in yeast has no detectable effect on thermotolerance. Therefore, if immunolocalization studies in yeast are to have general significance it is important to establish that the yeast protein is a bona fide member of the small hsp family. DNA sequence analysis indicates that the protein contains the small hsp consensus sequence near its carboxy terminus (Susek, R., and S. Lindquist, unpublished observations; and see Introduction). Furthermore, the protein is regulated in a manner that is similar to other small hsps, being induced by heat shock, other forms of stress, and at particular stages in development (Kurtz et al., 1986). Perhaps the most distinctive feature of the small hsps in other organisms is their assembly into large polymeric granules (Arrigo et al., 1985; Schuldt and Kloetzel, 1985; Fransolet et al., 1979; Nover et al., 1983). Therefore, to provide an additional point of comparison, we examined the chromatographic behavior of yeast hsp26 and the *Drosophila* small hsps by gel filtration (Fig. 2). Both the four small hsps of *Drosophila* and hsp26 of *Saccharomyces* were exclusively found in the void volume (fractions 24–30) indicating that the proteins were in complexes with a molecular mass of >500 kD. (The identity of yeast hsp26 in column fractions was confirmed by Western blot analysis.) Identical results were obtained using 100 or 300 mM NaCl-column buffer with or without 5 mM $MgCl_2$. Thus, by every criterion available, the protein is a bona fide member of the small hsp family.

Specificity of Anti-Yeast Hsp26 Serum for Immunofluorescent Localization

To determine the intracellular distribution of hsp26, yeast cells were fixed in 3.7% formaldehyde, treated with zymolyase (25 μg/ml) to remove cell walls, and then permeabilized with 0.2% Triton X-100. The cells were incubated with anti-hsp26 rabbit serum, then goat anti-rabbit IgG conjugated to fluorescein. Surprisingly, nonheat-shocked wild-type cells and heat-shocked *hsp26*⁻ cells stained as brightly as heat-shocked wild-type cells. High background staining such as this is a common problem in indirect immunofluorescence analysis of yeast cells. A possible source of the artifact is the presence of glycoproteins in the cell wall, since such proteins commonly bind nonspecifically to the Fc portions of antibodies. Although the zymolyase treatment should remove most of these proteins, we reasoned that a sufficient residue might remain to produce nonspecific staining. To eliminate such binding, the rabbit antiserum was incubated with papain bound to an inert support in order to cleave the antibodies into Fab and Fc fragments. After removal of the papain, Fc fragments and uncleaved antibodies were depleted from the serum by incubation with *S. aureus* cells. This Fab-enriched antiserum was reacted with yeast cells and the Fab fragments were selectively visualized with fluorescein-labeled $F(ab')_2$ fragments of goat anti-rabbit $F(ab')_2$ IgG.

As demonstrated in Fig. 3, staining by this method was highly specific for hsp26. The antiserum stained heat-shocked, wild-type cells brightly (Fig. 3, *a* and *b*). Wild-type cells growing at 25°C and heat-shocked *hsp26*⁻ cells did not stain (Fig. 3, *c–f*). All the localization experiments presented here were performed by this method. In every experiment, nonheat-shocked *HSP26*⁺ cells or heat-shocked *hsp26*⁻ cells were included as controls and did not stain.



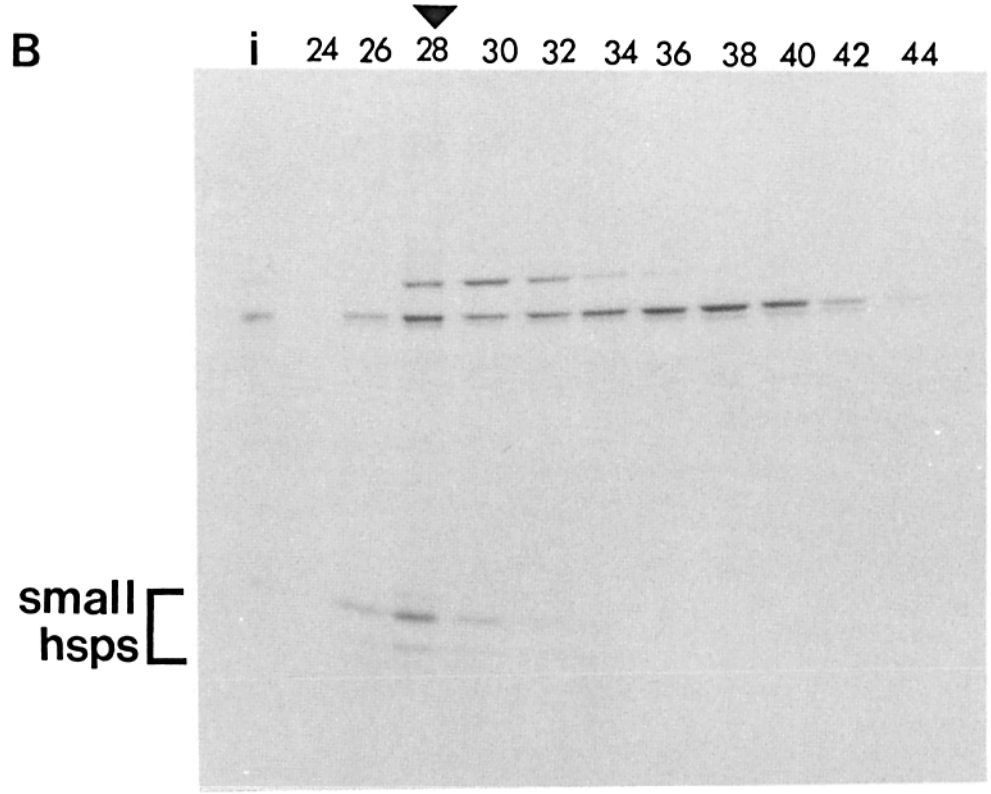


Figure 2. Gel-filtration chromatography of *Saccharomyces* and *Drosophila* heat-shocked cell lysates. (*A*) Yeast cells were labeled with [^{3}H]isoleucine for 45 min at 39°C. Nondenatured cell lysates were separated on a Bio Gel A 0.5-M, gel-filtration column. Proteins from fractions 24–50 were analyzed on 10% SDS-polyacrylamide gels and visualized by fluorography. The peak elution fraction for hsp26 is indicated by an arrowhead (▼). Molecular mass standard lanes (*m*), 200, 97.4, 68, 43, and 25.7 kD, respectively. (*B*) *Drosophila* cells were labeled with [^{3}H]leucine for 1 h after a 37°C heat shock. Lysates were prepared and analyzed as in *A*. Heat-shocked cell lysate before gel filtration (lane *i*).

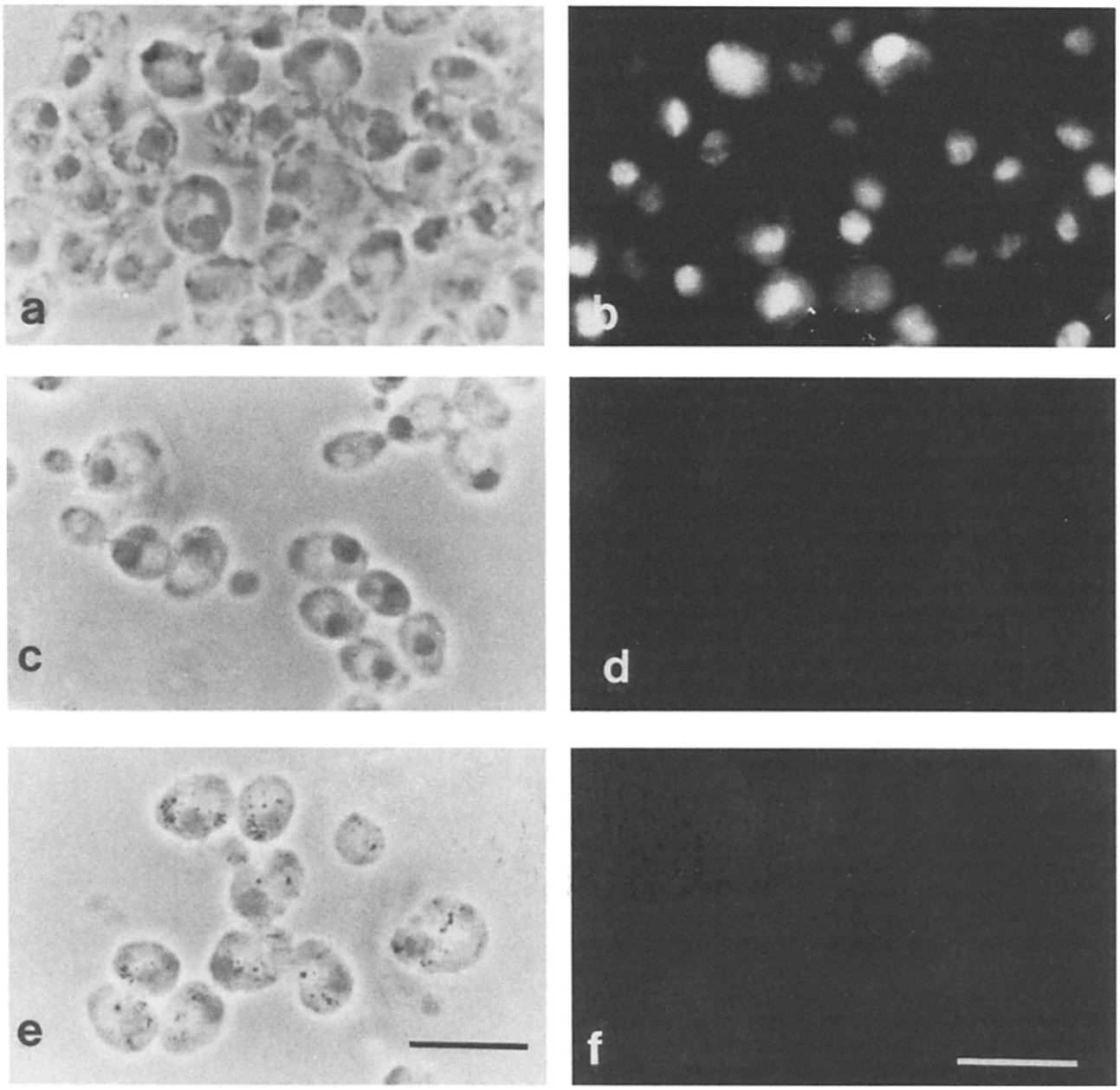


Figure 3. Specificity of anti-hsp26 serum for immunofluorescence localization. Log-phase cells (5×10^6/ml) growing in glucose were heat shocked at 39°C for 1 h or kept at 25°C. The cells were fixed in 3.7% formaldehyde, incubated with zymolyase and then Triton X-100, and incubated with anti-hsp26 Fab rabbit serum. Cells were stained with fluorescein-conjugated $F(ab')_2$ goat anti-rabbit IgG $F(ab')_2$. Phase-contrast (*a*, *c*, and *e*) and fluorescent photomicrographs (*b*, *d*, and *f*). Wild-type heat-shocked cells (*a* and *b*), *hsp26*$^-$ heat-shocked cells (*c* and *d*), and wild-type nonheat-shocked cells (*e* and *f*).

Hsp26 Concentrates in Nuclei During Heat Shock and Recovery in Log-phase Cells Growing in Glucose

The experiment of Fig. 3 was performed with log-phase cells (5×10^6/ml) growing at 25°C in a rich glucose medium. This medium supports growth of yeast cells at the highest possible rates. The cells were heat shocked at 39°C for 1 h, the optimal temperature for induction of hsps. This heat treatment does not measurably reduce cell viability. Hsp26 was present in the cytoplasm of heat-shocked cells, but was clearly concentrated within or around the nuclei (Fig. 3, *a* and *b*). Nuclear staining was not a peculiarity of individual strains. In fact, it was also observed in another species of *Saccharomyces*, *S. carlsbergensis* (data not shown). The intensity of staining over the nucleus varied somewhat from cell to cell and occasional cells showed no nuclear concentration. Some cells did not stain at all. Whether this is due to the absence of hsp26 in these cells or to a technical artifact is unclear.

To determine whether hsp26 is located within the nucleus or only peripherally associated with it, cells were heat shocked at 39°C for 1 h, stained by indirect immunofluorescence with anti-hsp26 Fabs, and embedded in plastic. Thin

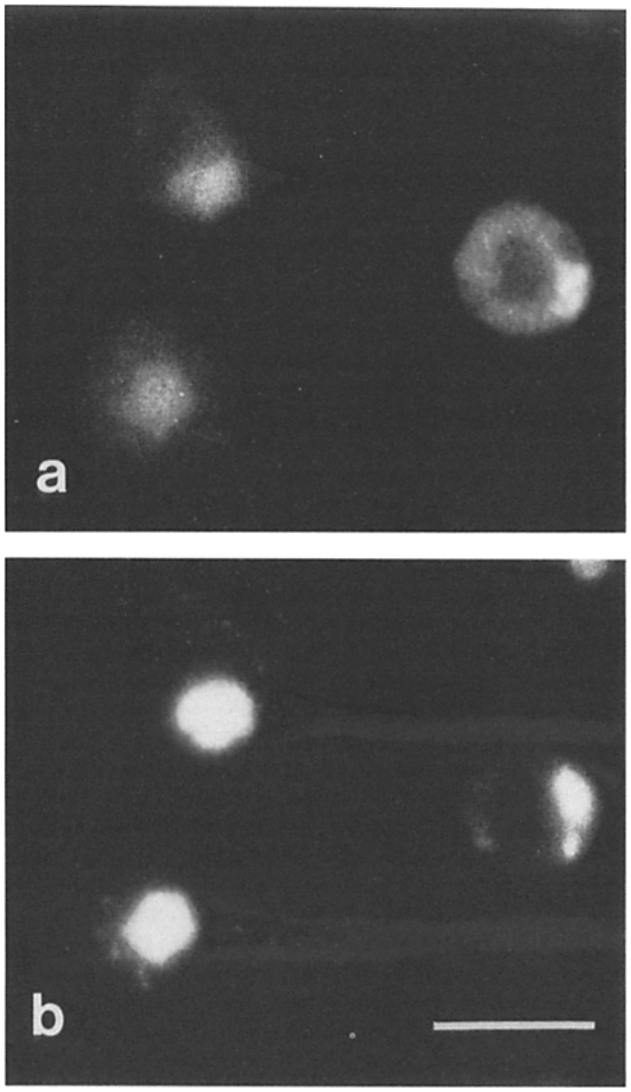


Figure 4. Immunofluorescent localization of hsp26 in sectioned cells. Log-phase (5×10^6/ml), heat-shocked cells growing in glucose were fixed and stained with anti-hsp26 Fab serum as for Fig. 3. The cells were embedded in JB-4 plastic and sectioned (1 μm) as described in Materials and Methods. Sections were also stained with DAPI. Immunofluorescence (*a*); DAPI fluorescence (*b*).

sections were stained with DAPI, a DNA-specific fluorescent dye, to visualize nuclei. As shown in Fig. 4, hsp26 is indeed located within the nucleus. In examining large numbers of cells by this method a doughnut-shaped staining pattern, indicative of peripheral localization, was never observed. Again, the degree of nuclear concentration varied somewhat from cell to cell. (The unstained, dark region within the cell corresponds to the vacuole.)

Next we investigated the intracellular localization of hsp26 during recovery from heat shock. Log-phase, glucose-grown cells that had been heat shocked at 39°C for 1 h were allowed to recover at 25°C for 2 or 4 h. As may be seen in Fig. 5, the protein continued to concentrate in or around nuclei during recovery. That these cells were truly recovered was demonstrated by the fact that cell densities increased from 4×10^6 to 3×10^7 per ml during this period. Note also that most cells were unbudded in the heat-shocked sample, but during recovery the cells were distributed among all stages of budding. Nuclear concentration of hsp26 was clearly visible in both mother cells and buds.

Yeast cells growing in glucose will also resume growth, after a transient arrest, with continuous culture at 39°C. Synthesis of normal cellular proteins is fully restored after ~2 h at 39°C and hsp synthesis is repressed, plateauing at a higher level than at 25°C (Lindquist et al., 1982). Surprisingly, in cells maintained at 39°C the protein did not continue to concentrate in the nucleus. Nuclear concentration was much less apparent after 2 h at 39°C (Fig. 6, *c* and *d*) and after 3 h, hsp26 was distributed throughout the cell (Fig. 6, *e* and *f*).

Hsp26 Does Not Concentrate in Nuclei in Stationary-phase Cells Growing in Glucose

In addition to its induction by heat, hsp26 is induced during yeast development, in the normal course of sporulation and as cells transit from log- to stationary-phase growth (Kurtz et al., 1986). Since spore walls are not penetrated by digestive enzymes, antibodies, or fixatives, immunofluorescent staining of spores is problematic. We therefore restricted our investigations of developmentally induced hsp26 to stationary-phase cells.

In cells grown to early stationary phase (2×10^8/ml for 4 h) in glucose media at 25°C, hsp26 staining was distributed throughout the cell. Some protein was located in or around nuclei but without the concentration that was observed in heat-shocked cells (Fig. 7, *a* and *b*). To determine whether nuclear staining was peripheral to or localized within nuclei, stationary-phase cells were stained with anti-hsp26 Fab serum and embedded in plastic. Plastic sections (1 μm) were also stained with DAPI to visualize nuclei. Although some protein was found within the nucleus, hsp26 was primarily distributed along the nuclear periphery and in the cytoplasm (data not shown).

Since stationary-phase cells are more resistant than log-phase cells to killing by heat, by cell wall–degrading enzymes, and by various chemicals (Schenberg-Frascino and Moustacchi, 1972; Deutch and Parry, 1974; Parry et al., 1976), they also might be less readily penetrated by antisera. To control for this possibility, a portion of the culture was stained with a monoclonal antibody specific for yeast tubulin. The pattern of nuclear staining we observed with this antibody in stationary-phase cells (Fig. 7, *e* and *f*) was consistent with previous reports (Kilmartin and Adams, 1984) and virtually every cell was stained. Since the cells were adequately penetrated by the undigested tubulin antibodies, they must be adequately penetrated by the anti-hsp26 Fab fragments.

To determine if the cytoplasmic protein would translocate to the nucleus upon heat shock, stationary-phase cells were heat shocked at 39°C for 1 h (Fig. 8, *c* and *d*). To inhibit protein synthesis, cycloheximide (1 μg/ml), was added to a portion of the culture just before heat shock, so that the redistribution of preexisting hsp26 could be assessed (Fig. 8, *e* and *f*). (Fig. 8, *g–j*, demonstrates that this concentration of the drug inhibited hsp26 synthesis in heat-shocked, log-phase cells.) The intracellular distribution of hsp26 in stationary-phase cells was not changed by heat shock, whether or not cycloheximide was added.

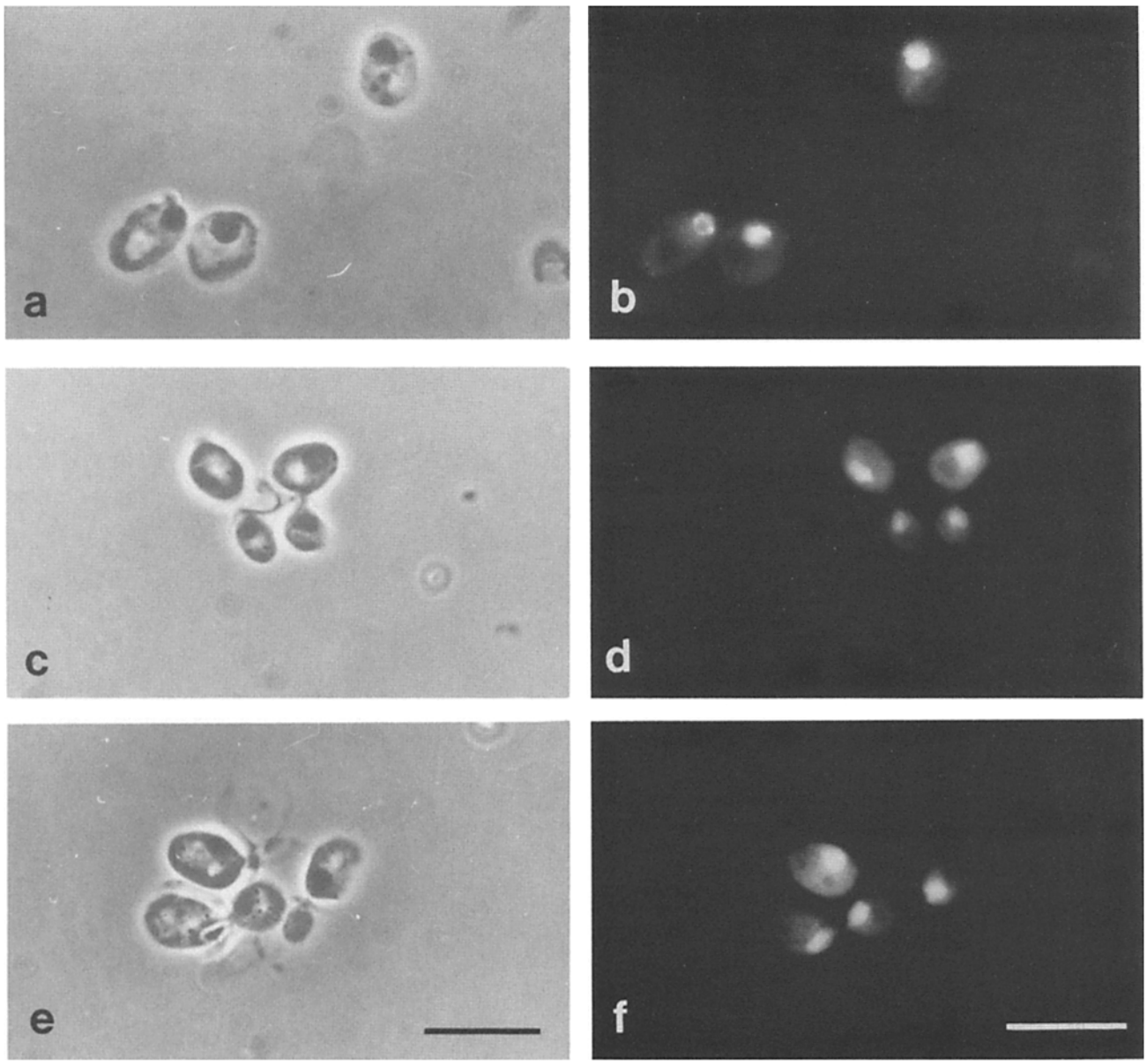


Figure 5. Immunofluorescent localization of hsp26 during recovery from heat shock. Log-phase (5×10^6/ml) cells growing in glucose were heat shocked at 39°C for 1 h and then returned to 25°C to recover for 2 or 4 h. Cells were fixed and stained with anti-hsp26 Fab serum as for Fig. 3. Phase-contrast (*a*, *c*, and *e*) and fluorescent (*b*, *d*, and *f*) photomicrographs. Heat-shocked cells (*a* and *b*). Cells recovered for 2 (*c* and *d*) or 4 h (*e* and *f*).

Hsp26 Does Not Concentrate in Nuclei of Log-phase Cells Growing in Galactose

Two hypotheses to explain the nonnuclear distribution of hsp26 in heat-shocked, stationary-phase cells come immediately to mind. First, stationary-phase cells may not perceive a 1-h heat shock at 39°C as stressful, since these cells are extremely resistant to heat. Second, the hsp26 protein that is produced at normal temperatures may be structurally different from the protein that is produced during heat shock and may therefore be incapable of translocating to nuclei. To investigate these possibilities we engineered yeast cells to produce hsp26 during log-phase growth at normal temperatures and asked whether the protein would concentrate in nuclei with or without a heat shock.

To produce hsp26 in log-phase cells at normal growth temperatures, cells were transformed with a recombinant *HSP26* gene. The *HSP26* protein-coding sequences and 54 nucleotides of sequence upstream of the initiating ATG were fused to the *GAL1* promoter (Johnston and Davis, 1984). With this recombinant gene, hsp26 should be induced by galactose not by heat. Since the recipient cells in the transformation were originally *hsp26*$^-$ the recombinant gene is the only source of hsp26 in the cell.

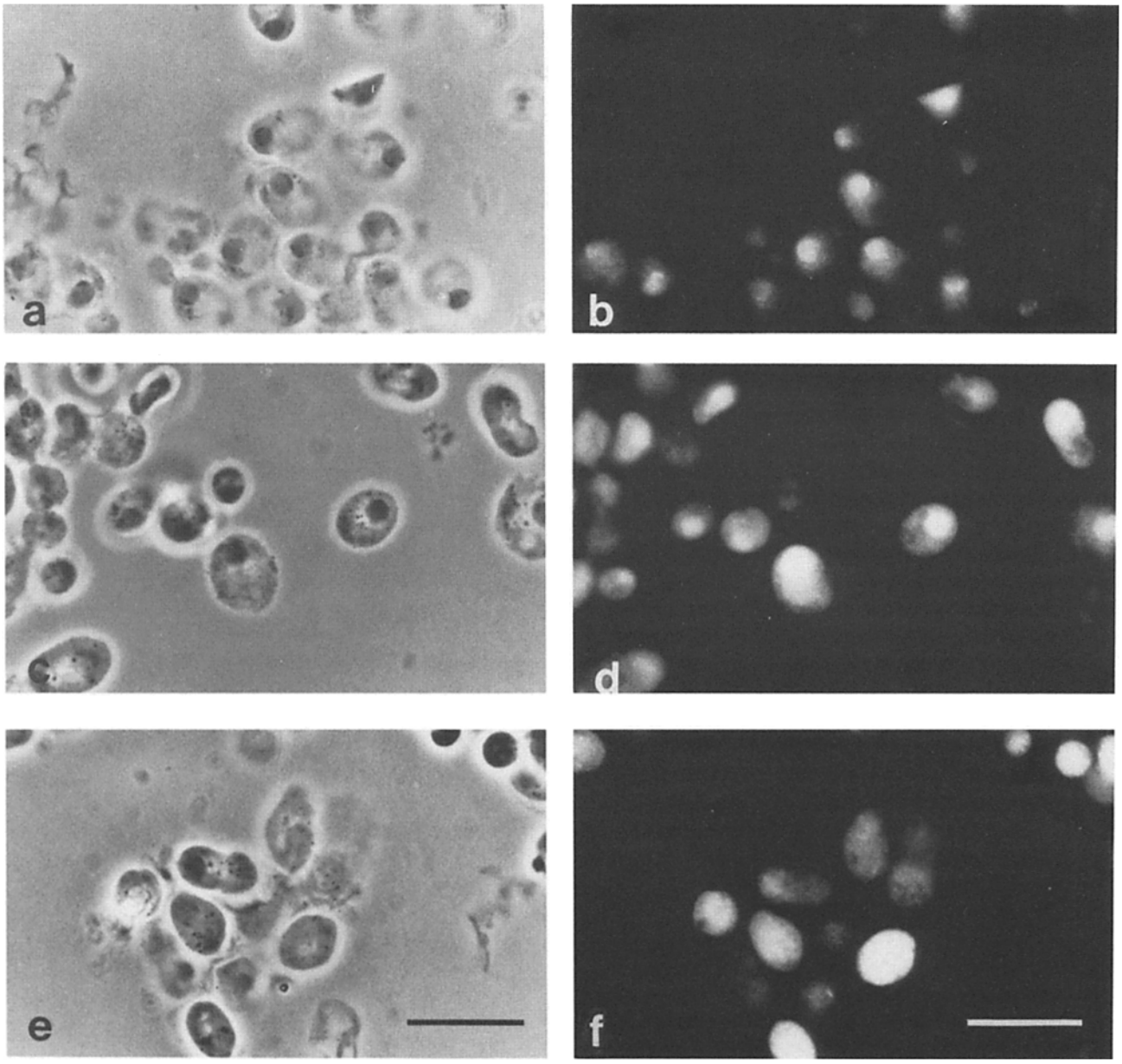


Figure 6. Immunofluorescent localization of hsp26 during a prolonged heat shock. Log-phase (5×10^6) cells growing in glucose were heat shocked at 39°C. Cells were fixed and stained with anti-hsp26 Fab serum as for Fig. 3. Phase-contrast (*a*, *c*, and *e*) and fluorescent (*b*, *d*, and *f*) photomicrographs. Cells heat shocked for 1 (*a* and *b*), 2 (*c* and *d*), or 3 h (*e* and *f*).

As expected, when the transformants were grown in glucose they did not stain with the anti-hsp26 Fab serum and hsp26 was not detected by Western blot analysis (data not shown). When the cells were grown in galactose at 25°C, hsp26 was strongly induced and was readily detected by both immunofluorescent staining (Fig. 9, *a* and *b*) and Western blot analysis (data not shown). The protein did not concentrate in the nuclei of these cells but had a diffuse cellular distribution. When galactose-grown transformants were exposed to heat shock the preexisting protein did not relocalize to nuclei (Fig. 9, *c* and *d*). (Note that the intensity of staining decreased in these cells. This was expected since heat shock represses transcription from nonheat-shock promoters.) To determine if heat-induced hsp26 would concentrate within nuclei of galactose-grown cells, wild-type cells were grown to log phase and heat shocked at 39°C. Surprisingly, the heat-induced protein also did not concentrate in nuclei (Fig. 9, *e* and *f*). Thus, neither of our initial hypotheses adequately explain the failure of hsp26 to enter the nucleus in stationary-phase cells. It cannot be that log-phase cells growing in galactose do not perceive 39°C as stressful, since they rapidly induce the full spectrum of hsps (data not shown), the hallmark of the stress response. Furthermore, it is not only the protein produced at 25°C that fails to enter the nucleus

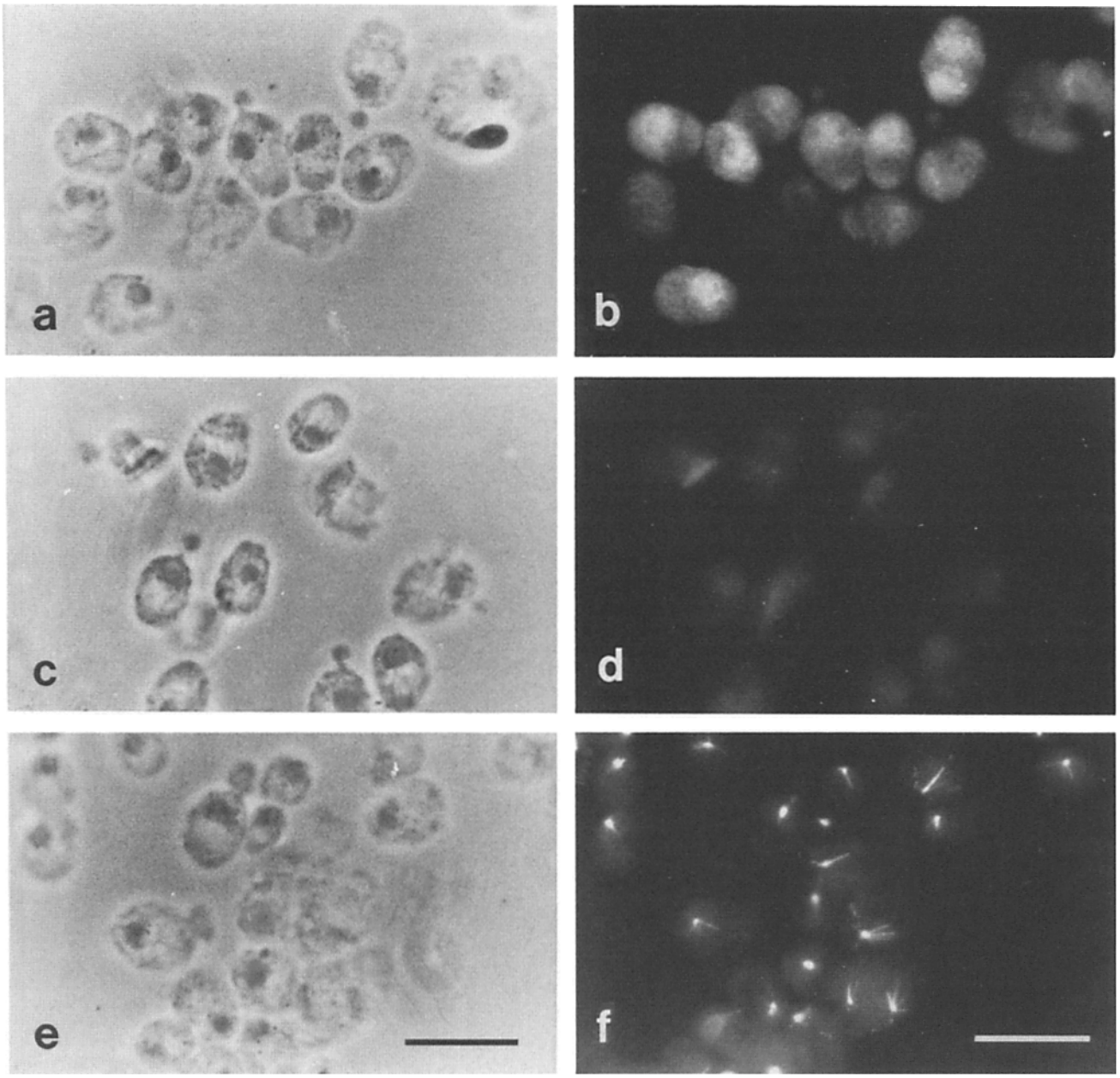


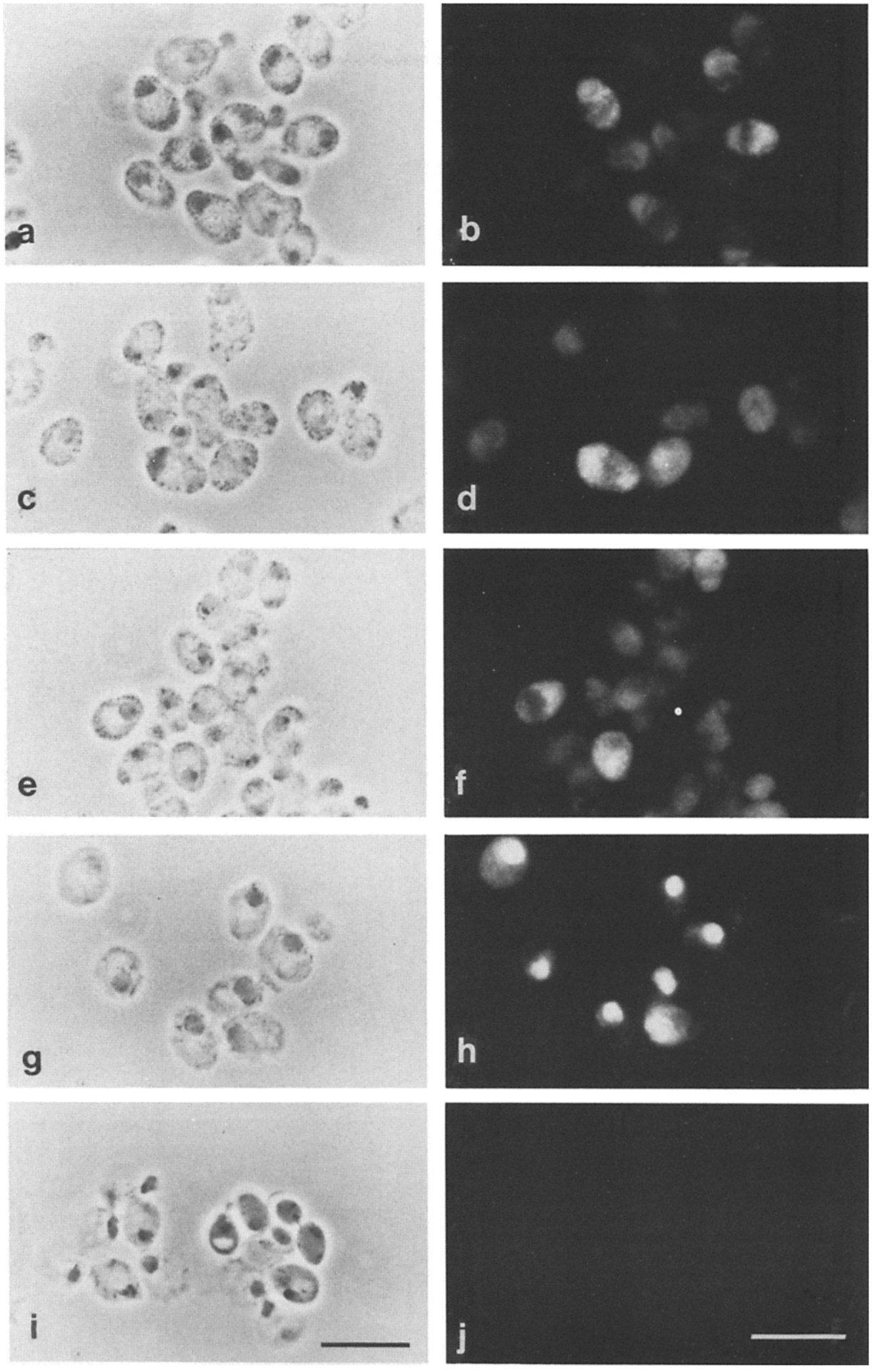
Figure 7. Immunofluorescent localization of hsp26 during stationary-phase growth. Wild-type or *hsp26*$^-$ stationary-phase cells (1–2 × 10^8 cells/ml for 4 h) growing in glucose were fixed and stained with anti-hsp26 Fab serum (*a–d*) or anti-yeast tubulin monoclonal antibody, YOL/34 (*e* and *f*) as described in Materials and Methods. Phase-contrast (*a*, *c*, and *e*) and fluorescence (*b*, *d*, and *f*) photomicrographs. Wild-type stationary-phase cells (*a* and *b*), *hsp26*$^-$ stationary-phase cells (*c* and *d*), wild-type stationary-phase cells (*e* and *f*).

with heat shock. The protein produced at 39°C in galactose also fails to enter the nucleus.

Hsp26 Does Not Concentrate in Nuclei When Grown on Carbon Sources Other than Glucose

We next investigated the relationship between hsp26 localization and respiratory metabolism. Galactose, like glucose, is a fermentable carbon source but, unlike glucose, it does not cause catabolite repression of respiration. Respiratory pathways are also derepressed in stationary-phase cells, when glucose is exhausted. Thus, the failure of hsp26 to enter nuclei during heat shock in log-phase, galactose-grown cells

Figure 8. Immunofluorescent localization of hsp26 in heat-shocked stationary-phase cells. Stationary-phase cells (1–2 × 10^8 cells/ml for 4 h) growing in glucose were kept at 25°C or heat shocked for 1 h at 39°C with or without the prior addition of cycloheximide (1 μg/ml). Cells were fixed and stained with anti-hsp26 Fab serum as for Fig. 3. Phase-contrast (*a*, *c*, *e*, *g*, and *i*) and fluorescent (*b*, *d*, *f*, *h*, and *j*) photomicrographs. Stationary-phase cells kept at 25°C (*a* and *b*), heat shocked at 39°C (*c* and *d*), or treated with cycloheximide before heat shock (*e* and *f*). Log-phase cells heat shocked at 39°C for 1 h (*g* and *h*) or treated with cycloheximide before heat shock (*i* and *j*).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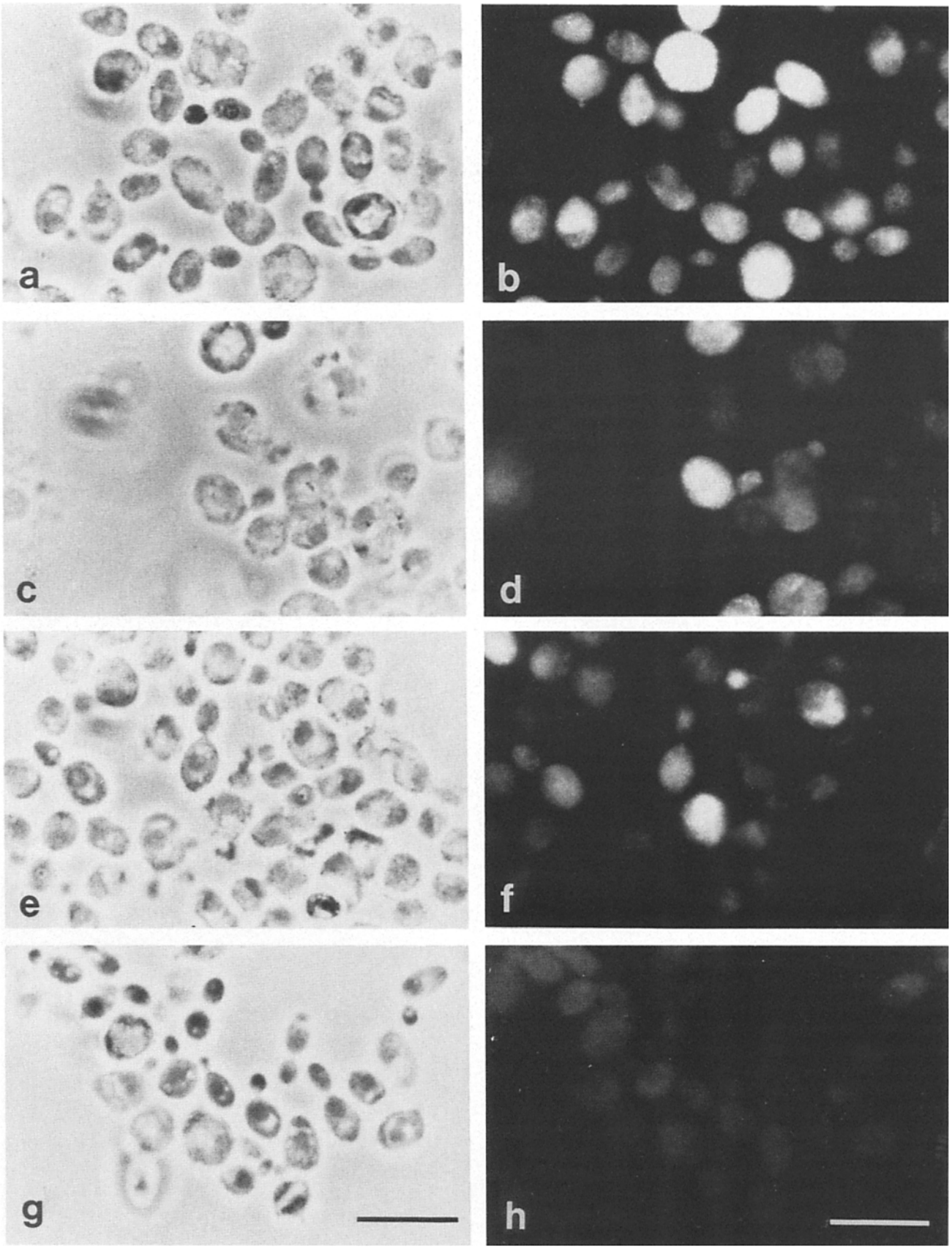

Figure 9. Immunofluorescent localization of hsp26 in galactose media. To produce hsp26 in log-phase cells at normal temperatures, a *gall–HSP26* fusion gene was transformed into *hsp26*$^-$ cells resulting in the synthesis of hsp26 in the presence of galactose. Wild-type and transformed cells were grown to log phase in galactose and maintained at 25°C or heat shocked at 39°C for 1 h. Cells were fixed and stained with anti–hsp26 Fab serum as in Fig. 3. Phase contrast (*a*, *c*, *e*, and *g*) and fluorescent (*b*, *d*, *f*, and *h*) photomicrographs. Transformants maintained at 25°C (*a* and *b*) or heat shocked (*c* and *d*). Wild-type cells heat shocked (*e* and *f*) or maintained at 25°C (*g* and *h*).

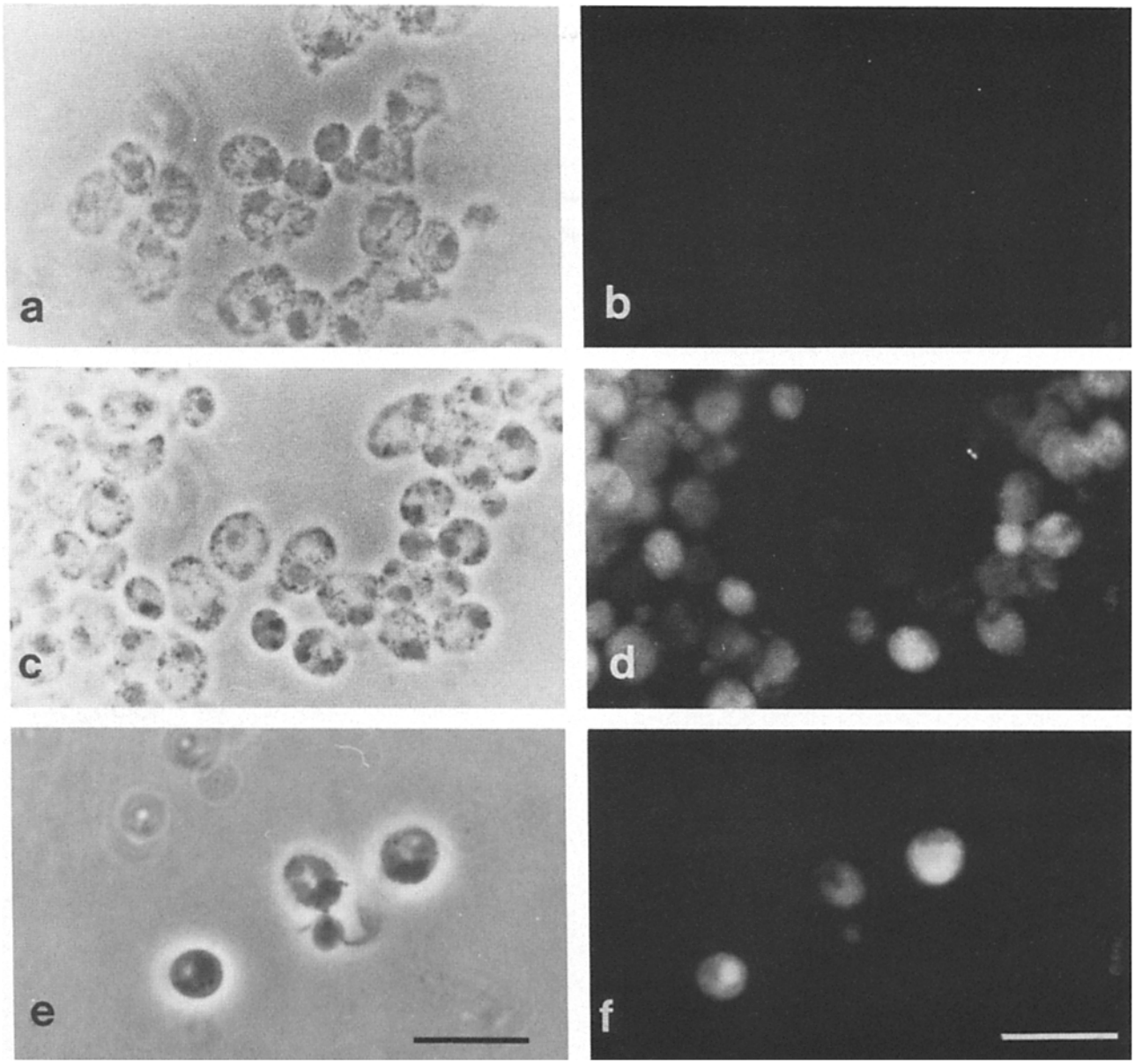

Figure 10. Immunofluorescent localization of hsp26 in wild-type cells grown in acetate media and ρ^o cells grown in glucose media. Log-phase (5×10^6/ml), wild-type cells growing in acetate were heat shocked at 39°C for 1 h or maintained at 25°C. ρ^o cells growing in glucose were heat shocked at 39°C for 1 h. The cells were fixed and stained with anti-hsp26 Fab serum as in Fig. 3. This figure shows one of the few cells with nuclear staining. Phase contrast (*a*, *c*, and *e*) and fluorescent (*b*, *d*, and *f*) photomicrographs. Wild-type cells growing in acetate at 25°C (*a* and *b*) or heat shocked (*c* and *d*). Heat-shocked ρ^o cells growing in glucose (*e* and *f*).

and in stationary-phase, glucose-grown cells might be related to the fact that both are respiring.

When wild-type cells grown in acetate (a medium which forces them into respiratory metabolism) were heat shocked at 39°C for 1 h, hsp26 was strongly induced. Again the protein was generally distributed with little or no concentration in nuclei (Fig. 10, *c* and *d*). Since yeast cells cannot grow in acetate media above 37°C, it might be argued that this temperature is simply too toxic to permit relocalization of hsp26 to nuclei. However, when acetate-grown cells were heat shocked at 37°C, the protein still failed to concentrate in nuclei (data not shown). Thus, in three different conditions in which respiration is active, (*a*) log-phase cells growing in acetate, (*b*) log-phase cells growing in galactose, and (*c*) early stationary-phase cells grown in glucose, hsp26 does not concentrate in nuclei. In the one case we have examined in which respiration is repressed, log-phase cells growing in glucose, hsp26 does concentrate in nuclei.

Is the repression of respiratory metabolism sufficient to determine cytoplasmic or nuclear localization of hsp26? If so, in cells that are deficient in respiration and restricted to fermentation, hsp26 should concentrate in nuclei, whether the cells are growing in glucose or galactose. Respiration deficient ρ^o cells grown in glucose or galactose media were heat shocked at 39°C for 1 h. When ρ^o cells were grown in glucose media, some cells showed slight nuclear concentration (Fig. 10, *e* and *f*). Most showed none at all. Hsp26 also showed little or no concentration in nuclei of ρ^o cells grown

in galactose media (data not shown). Thus, a block in respiration is not sufficient to produce nuclear localization of hsp26.

Discussion

The major conclusion of our experiments is that the intracellular localization of hsp26 is highly dependent upon the state of cellular metabolism. Hsp26 concentrates in nuclei when yeast cells growing in glucose are heat shocked in the log phase of growth. Surprisingly, hsp26 does not concentrate in nuclei under most other conditions. For example, the protein is induced at normal temperatures during the transition from the log phase to the stationary phase of growth. It does not concentrate in the nuclei of these cells, even if they are heat shocked. Since stationary-phase cells are more resistant to killing by heat than are log-phase cells (Schenberg-Frascino and Moustacchi, 1972; Deutch and Parry, 1974; Parry et al., 1976), it might be argued that hsp26 does not translocate to the nucleus because the heat shock is not perceived by stationary-phase cells as stressful. However, our other findings demonstrate that nuclear localization of hsp26 is not stress dependent. First, the protein does not concentrate in the nuclei of heat-shocked, log-phase cells when they are growing in acetate or in galactose. Since the heat treatments used in these experiments produce as strong an induction of hsps as in glucose-grown cells, they are certainly perceived as stressful (Lindquist et al., 1982 and unpublished data). Conversely, when cells growing in glucose are heat shocked and returned to normal temperatures, hsp26 continues to concentrate in nuclei for at least three generations. By this time the cells have fully recovered from the stress, having normal growth rates and normal patterns of protein synthesis (Lindquist et al., 1982).

In stationary-phase cells grown in glucose and log-phase cells grown in acetate or galactose, respiration is active (Lagunas, 1976) and in all three cases hsp26 does not concentrate in nuclei. In log-phase cells growing in glucose, respiration is repressed (Lagunas, 1976) and hsp26 does concentrate in nuclei. These results might suggest that the protein concentrates in nuclei when respiration is repressed. However, the protein also fails to concentrate in the nuclei of respiration-deficient (ρ^o), log-phase cells growing in galactose or glucose. Thus, we have observed nuclear localization of hsp26 only when cells are growing primarily by fermentation but are capable of respiration.

Interestingly, the results with ρ^o cells are in agreement with another curious result. As described above, when log-phase, glucose-grown cells are heat shocked and returned to 25°C, hsp26 continues to concentrate in nuclei as growth resumes. However, when these same cells are maintained at 39°C, nuclear localization of hsp26 dissipates as growth resumes. In the former case, respiration is repressed but not completely inactive. In the latter, as well as in ρ^o cells, it is completely inactive.

In other organisms, experiments investigating the intracellular distribution of the small hsps have yielded conflicting results. Several cell fractionation studies indicate that they are present in nuclei. Their subnuclear localization has variously been attributed to chromatin (Arrigo et al., 1980; Loomis and Wheeler, 1980), nucleoli (Arrigo et al., 1980), and the nuclear skeleton (Sinibaldi and Morris, 1981; Levinger and Varshavsky, 1981). Immunolocalizations have also yielded varying results. In tomato cells, the small hsps are found in large cytoplasmic granular aggregates, often in close proximity to the nucleus during heat shock, with nuclear staining observed in some cells during recovery (Neumann et al., 1987). In chicken cells, the proteins are also found in large cytoplasmic aggregates (Collier et al., 1988). In *Drosophila* the small hsps of salivary gland cells were found to be nuclear during heat shock and cytoplasmic during recovery (Arrigo and Ahmed-Zadeh, 1981). In another report using the same antibody, the proteins of *Drosophila* tissue-culture cells were found in cytoplasmic granules and nucleoli during heat shock and showed a more diffuse cytoplasmic staining during recovery (Duband et al., 1986). In a third case, using a different antibody, the staining pattern of the small hsps closely paralleled that of a vimentin-like intermediate filament protein which collapses around the nuclei of salivary gland cells during heat shock. The proteins were found in nuclei only with severe, lethal heat treatments, causing the authors to suggest that nuclear localization may be an artifact of impending cell death (Leicht et al., 1986).

The results presented in this paper demonstrate that nuclear localization is not an artifact of cell death. They also provide a plausible explanation for the conflicting findings in these previous studies. That is, different localization patterns may have been obtained simply because the cells used were in different metabolic states.

We find that the intracellular localization of hsp26 in yeast is more dependent upon cellular physiology than it is on heat stress per se. In this regard, it may be relevant that the small hsps are related to the α-crystallins. Although no enzymatic function has been assigned to the α-crystallins, the crystallins of both vertebrates and invertebrates are generally either enzymes or closely related to enzymes (Wistow and Piatigorsky, 1987). ϵ-Crystallin, found in the lens of crocodiles and many birds, is a functional lactate dehydrogenase (Wistow et al., 1987). δ-crystallin, the major protein of the embryonic lens in all birds and reptiles, has amino acid sequence similarity to both human and yeast arginosuccinate lyase (Beacham et al., 1984; O'Brien et al., 1986). And τ-crystallin, the major lens protein in some fish, reptiles, birds, and lampreys, shows strong amino acid sequence similarity with the sequences of human and yeast enolases (Giallongo et al., 1986; Chin et al., 1981). It may be that the α-crystallins and the related small hsps have important metabolic as well as structural roles.

Unfortunately, we cannot yet draw any conclusions about what specific features of metabolism control hsp26 localization and function, since the culture conditions and mutations we have examined affect a variety of metabolic pathways. Hopefully, the availability of specific mutations affecting many different metabolic processes in yeast will provide an avenue for identifying the features that control hsp26 localization. This in turn may provide a key for unlocking the function of these ubiquitous, poorly understood proteins.

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