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# Temperature Adaptation of House Keeping and Heat Shock Gene Expression in *Neurospora crassa*

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Mohsenzadeh, S., Saupe-Thies, W., Steier, G., Schroeder, T., Fracella, F., Ruoff, P., and Rensing, L. 1998. Temperature adaption of house keeping and heat shock gene expression in *Neurospora crassa*. *Fungal Genetics and Biology* 25, 31–43. Adaptation of house keeping and heat shock gene expression was determined in *Neurospora crassa* during continuous exposure to different temperatures. Steady-state values of total protein synthesis differed little after incubation for 24 h at temperatures between 15 and 42°C. Adaptation kinetics at 42°C showed an initial, transient inhibition of total protein synthesis. Similar kinetics were observed with actin synthesis and tubulin mRNA. A priming 1-h heat shock of 42°C 2 h prior to a second continuous exposure to 42°C abolished the inhibitory effect of the second treatment and resulted in "acquired translational tolerance." Steady-state values of HSP70 synthesis rates revealed increasing levels with increasing temperatures after incubation for 24 h at different temperatures. Adaptation kinetics of the synthesis rates of different HSPs *in vivo* revealed maximal rates after 2 h and then a decrease to the elevated steady-state levels. The total amount of the major constitutive and inducible HSP70 isoform as determined by Western blots reached a maximum 2 h after the beginning of 42°C exposure and only a slight decrease (25%) of the maximal value after 24 h. The inducible isoform of HSP70, in contrast, reached a maximum after 4–8 h and then decreased strongly after 24 h. HSP mRNAs reached maximal amounts 45–60 min after the beginning of 42°C exposure and then declined after 8 h as determined by *in vitro* translation. Northern blots revealed maximal mRNA amounts of the inducible HSP70 after 30 min and zero amounts after 4 h exposure to 42°C. After a shift to 42°C HSP70 isoforms were immediately translocated into the nucleus and reshuttled into the cytoplasm during the following 6 h. The nuclear content of HSP70 remained elevated during the adapted steady state at 24 h. It is concluded that the adapted state after 24 h is based on enhanced amounts of constitutive isoforms in the cytoplasm and in the nucleus, whereas the inducible isoforms of HSP70 show faster adaptation kinetics. 

*Index Descriptors:* protein synthesis; heat shock genes; HSP70; acquired translational thermotolerance; nuclear localization.

Cells exposed to elevated temperatures (heat shock) first activate the expression of heat shock genes and reduce the expression of "house-keeping" genes (Nover, 1991; Hendrick and Hartl, 1993). During continued exposure to elevated temperatures—at least within a tolerable range—cells adapt and return to a state of gene expression closer to normal. This is observed on both the transcriptional and the translational levels (Lindquist and Craig, 1988; Nover, 1991) and is apparently paralleled by a state called

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"acquired thermotolerance" (Mizzen and Welch, 1988; Parsell and Lindquist, 1993). This acquired thermotolerance is one of the important adaptive responses to changes in the ambient temperature and contributes to the cell's homeostasis under different conditions. Acquired translational thermotolerance may also be effective in the "temperature compensation" of the circadian period length (Rensing *et al.*, 1995, 1997).

In poikilothermic organisms the most conspicuous response of cells is observed during the first hours after exposure to higher temperatures or stress. The subsequent adaptive response, however, continues during longer exposure periods, an aspect which has rarely been analyzed. Many investigations, like the study on *Neurospora crassa* (Plesofsky-Vig and Brambl, 1985), focus on the immediate responses of heat shock and house keeping protein synthesis to a temperature elevation. Therefore, we extended the kinetic studies of the heat shock response to a 24-h exposure and analyzed the immediate changes as well as the steady-state values of house keeping and stress gene expression during the adapted state.

The mechanism of acquired thermotolerance is little understood. Various heat shock proteins (HSP)[2] such as HSP104 in yeast or HSP70 and HSP27 in *Drosophila* and mammalian cells (Li and Werb, 1982; Laslo, 1988; Landry *et al.*, 1989; Li *et al.*, 1992) seem to be involved, as deduced from the analysis of the adaptation kinetics or from studying mutational defects or overexpression of certain HSP species. In *Neurospora crassa*, a low-molecular-weight HSP was shown to be important for surviving the combined stress of heat shock and carbohydrate limitation (Plesofsky-Vig and Brambl, 1995). HSPs are apparently involved in the adaptation of splicing (Yost and Lindquist, 1986, 1991), of ribosome and polysome formation (Pelham, 1984; Li and Duncan, 1995), of protein synthesis (Nelson *et al.*, 1992; Panniers, 1994), as well as of protein folding and translocation—the basic "chaperone" functions (Becker and Craig, 1994; Plesofsky-Vig, 1996). From kinetic studies Li and Duncan (1995) concluded that the induced form of HSP70 may be decisive in providing transient translational thermotolerance after a priming heat treatment. In order to test this assumption we determined the kinetics of the amount of induced *hsp70*-mRNA and HSP70 after a priming HS and analyzed the adaptation of protein synthesis during a second HS period.

The results of the steady-state analysis showed a clear homeostasis of protein synthesis between 15 and 42°C and an increase from 15 to 35°C by a factor of 1.36 ($Q_{10}$ = 1.18). An adaptive response is particularly observed in kinetic studies during exposure to 37 and 42°C. The initial inhibition of house keeping proteins after a shift to 42°C can be prevented if cultures are primed by a previous heat treatment. This is compatible with a role of HSPs in translational thermotolerance. Inducible *hsp70*-mRNA as well as inducible HSP70 protein concentrations adapt quickly during continued exposure to 42°C, whereas the expression of the constitutive isoform of HSP70 remains elevated during the whole exposure and may, therefore, be more closely related to the adapted steady state of house keeping gene expression.

[2] Abbreviations used: HSP, heat shock protein; DTE, dithioerythriol; PBS, phosphate-buffered saline; IgG, immunoglobulin; SSC, standard saline citrate; HSF, heat shock factors.

## MATERIALS AND METHODS

### *Organism and Culture Conditions*

We used the *band* (*bd*) mutant of *Neurospora crassa* (*bd*-A No. 1858, Fungal Genetics Stock Center, Kansas City) showing a well defined circadian conidiation rhythm but otherwise very similar properties as the wild-type strain. Stock cultures were maintained on Horowitz slants (Davis and DeSerres, 1970). Conidia were harvested by using sterile distilled water (4 ml per vial) filtered through glass wool and a defined concentration transferred to the autoclaved growth medium (Vogel, 1956). The medium was supplemented with 2% sucrose. Starting with $10^6$ conidia/ml (at 25°C), the culture grew exponentially between 5 and 45 h after inoculation and entered the stationary phase after about 55 h, if the medium was not exchanged in a batch culture. In order to determine the adaptation and adaptation kinetics of total protein and HSP synthesis, discs were cut out from mycelial mats (after 30 h of growth) by a cork borer and transferred to fresh medium. These discs thus remained in the growth phase. In addition the medium was changed after 12 h.

### *Sample Preparation and Isolation of Nuclei*

For Northern blot experiments $2 \times 10^8$ conidia were added to 500 ml medium. The cultures were shaken continuously in a 1-L Fernbach flask kept in constant light at 25°C. After 20 h the culture was shifted to 42°C and probes were taken at the intervals indicated.

For the determination of overall protein and HSP synthesis, mycelia were ground in precooled mortars with a

defined amount of sea sand and 0.1 M Tris–HCl buffer (pH 7.2). The homogenate was then centrifuged (36,000*g*) for 1 h at 4°C in order to isolate the cytoplasmic components. The protein content of the postmitochondrial supernatant was determined according to Bradford (1976).

For isolation of cytoplasm and nuclei a modified method of Loros and Dunlap (1991) was used. All procedures were performed at 4°C. Mycelia were homogenized in a vibrogen mill containing 25 g glass pearls of 2 mm diameter. Glass pearls and mycelia were mixed with ice-cold nuclear buffer I (1 M sorbitol, 7% Ficoll 400 (w/v), 20% glycerol (v/v), 5 mM $MgCl_2$, 10 mM $CaCl_2$, 1% surfynol (v/v) and 3 mM dithioerythriol (DTE), pH 7.5), and mycelia homogenized by three 45-s pulses of vibrogen action. The homogenate was then centrifuged at 1500*g* for 10 min and the supernatant again centrifuged at 15,000*g* for 10 min.

The pellet contained the nuclei which were then washed by nuclear buffer II (5 mM Tris–HCl, 5 mM $MgCl_2$, 20% glycerol (v/v), 3 mM DTE, pH 7.5). The relative purity of the nuclear fraction was analyzed by fluorescence microscopy after staining with 4-diamidino-2-phenylindole dihydrochloride. Using polyacrylamide gel electrophoresis (PAGE) and Coomassie staining the nuclear fraction was further tested with respect to the presence of histones. In contrast to the cytoplasmic fraction, histones were present in the nuclear fraction in significant amounts. Electron microscopic inspection of the nuclear fraction, however, also revealed fragmented nuclei. The nuclear fraction was dissolved in Laemmli sample buffer (Laemmli, 1970: 2% sodium dodecyl sulfate (SDS, w/v), 10% glycerol (v/v), 10 mM 2-mercaptoethanol, 0.1 M Tris, pH 6.8), homogenized by means of sonication (10 pulses) and boiled for 2 min.

The supernatant was again centrifuged at 15000*g* for 1 h. The resulting supernatant, the cytoplasmic fraction, was dialysed against dialysis buffer (5 mM Tris–HCl, pH 7.5), lyophilized, and resuspendend in Laemmli sample buffer for electrophoretic protein analysis.

### *Elevated Temperature*

For elevated temperature treatment of mycelia, the culture flasks or Petri dishes were transferred to a water bath of the respective temperature.

### *Antibody Preparation*

Rabbits were immunized with primary injections of 30 µg of dialized and lyophilized isolated HSP70 from *N. crassa* (Fracella *et al.,* 1993) in 0.25 ml PBS in a 1:1 mixture with Freund's complete adjuvant (Calbiochem, Bad Soden) distributed to two subcutaneous sites on the back. Booster injections of 15 µg of HSP70 were given at 14-day intervals after primary injection in an identical manner except that incomplete Freund's adjuvant (Calbiochem) was used. For affinity purification the antiserum was applied to columns which contained agarose and immobilized HSP70. Specific antibodies were eluted with 0.1 M glycine (pH 2.7) and neutralized and diluted (1:1000) in TPBS (0.2% Tween20 in PBS, pH 7.5). The specificity of these antibody preparations was analyzed by means of Western blotting and ELISA.

### *Radioactive Labeling and Electrophoresis*

Mycelial discs were labeled with L-[$^{35}$S]methionine (370 kBq $ml^{-1}$, 29.6 MBq $mmol^{-1}$; Amersham) for the times indicated. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was conducted as described by Laemmli (1970). Fluorography of the gels was carried out according to the procedure described by Bonner and Laskey (1974).

### *Western Blot Analysis*

For Western blotting, the proteins were separated by one-dimensional SDS–PAGE and transferred to a nitrocellulose membrane (BA83, Schleicher & Schuell, Dassel) using a Trans Blot Cell (Bio-Rad, Muenchen) (Towbin *et al.,* 1979). The blots were blocked with TPBS for 1 h at 25°C. After removal of the blocking solution, primary antibody (affinity-purified polyclonal rabbit anti-HSP70 against the major *Neurospora* HSP70; Fracella *et al.,* 1993) diluted in TPBS (1:1000), was added and left for 1 h at 25°C. Subsequently, the blots were washed with TPBS. Secondary antibody (goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate, Bio-Rad, Muenchen) diluted in TPBS (1:5000) was added and after 1 h at 25°C the blots were washed with TPBS. The immune complex was detected by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim).

### *RNA Isolation*

For *in vitro* translation and Northern blot analysis, RNA was extracted from lyophilized mycelia. Mycelia were homogenized in an ice-cold mortar and the RNA extracted by a mixture of phenol, chloroform, and isoamyl alcohol (24:24:2 v/v). RNA was precipitated by 4 M sodium acetate

(pH 6.0, 0.01 M EDTA) on ice. After repeated precipitations and centrifugations the final pellet was washed in 70% ethanol, dried, and dissolved in water.

### *Isolation of Poly(A)$^+$-RNA*

Isolation was performed by the batch procedure. One to two grams of oligo(dT) cellulose (Biolabs) was transferred into centrifugation vials together with the binding buffer (overnight) and then centrifuged and resuspended with 2× binding buffer (50 mM Tris–HCl, 2 mM EDTA, 1 M NaCl, 0.05% (w/v) SDS, pH 7.5). Then 2× binding buffer was added to an equal volume of dissolved RNA (5–10 mg), heated to 65°C, and cooled again to room temperature. The RNA was then incubated with 0.2–0.4 g oligo(dT) cellulose for 15 min and then centrifuged. The supernatant was repeatedly incubated with the oligo(dT) matrix. After the last centrifugation 2 ml elution buffer (25 mM Tris–HCl, 1 mM EDTA, pH 7.5) was added to the matrix; the suspension was then vigorously shaken for a few seconds, mildly shaken for 20 min, and then centrifuged. This procedure was repeated 2× at 65°C and the RNA precipitated from the combined supernatants by means of a 3 M sodium acetate solution (0.1 vol, pH 5.2) and 2.5 vol of ice-cold 100% ethanol (at −20°C). The overnight precipitate was centrifuged and the pellet washed, dried, dissolved, and centrifuged three times at 17,000*g* (2 min) in order to eliminate the rest of the cellulose.

### *In Vitro Translation*

We mainly used a heterologous translation system (Boehringer Mannheim, type II), a nuclease-treated reticulocyte lysate under standard conditions (1.5 mM $Mg^{++}$, 100 mM $K^+$) which gave optimal results. Protein synthesis was completely dependent on added exogenous RNA; mostly 10 µg total RNA/25 µl or 1 µg poly(A)$^+$ RNA/25 µl was used. For labeling of the synthesized proteins, 925 kBq L-[$^{35}$S]methionine was added to 25 µl. The proteins were separated by SDS–PAGE.

### *Northern Blot Analysis*

RNA was separated on an agarose gel and blotted on nylon Hybond N$^+$ (Amersham) membrane. After incubation with a prehybridization solution (1 h, mild shaking), this solution was exchanged by a hybridization solution to which 100–400 ng freshly denatured, digoxigenin-labeled DNA was added. Hybridization occured at 37°C for 48 h under continuous motion. After incubation the membranes were washed with 2× SSC at room temperature, with 0.1× SSC at 60°C (10 min, 2×) and with 1× SSC at 45°C.

The nylon membranes were then treated with blocking reagent (according to the Boehringer firm manual) and incubated with the antidigoxigenin–alkaline phosphatase conjugate for 30 min. Membranes were then washed and equilibrated for 5 min in 50 ml 0.1 Tris–HCl, pH 9.5, + 50 mM $MgCl_2$. The staining occurred by adding 35 µl NBT and 45 µl X-phosphate to 10 ml equilibration solution for 60 min to 24 h. The staining was stopped by incubation with TE for 20 min.

### *Quantitative Determinations of Band and Blot Densities*

Fluorographs of gels or stained blots were analyzed as to the relative density of the bands by means of a video system (INTAS, Göttingen) and a computer program (CREAM) was used to determine the number of pixels.

## RESULTS

### *Protein Synthesis at Different Temperatures*

Mycelia were grown at 25°C and then shifted to different temperatures. Immediately after the shift, [$^{35}$S]methionine was added and its incorporation into proteins determined after 60 min incubation at the different temperatures. The resulting synthesis rates revealed an optimum curve, with little synthesis at 5 and 50°C and a maximum at 30–35°C (not shown). Relatively small differences in the synthesis rates were observed at the temperatures ranging between 20 and 40°C.

Protein synthesis rates 24 h after a shift from 25°C to different temperatures (15–42°C), i.e., during the adapted state, differed even less. In a series depicted in Fig. 1 the medium was exchanged during the 24-h period in order to prevent a drop in the medium pH which was maintained at 5.8, as well as a drop in nutrients. [$^{35}$S]methionine (370 kBq ml$^{-1}$) was added during the last hour of the 24-h period. The results show a slight increase by a factor of 1.36 in the synthesis rates between 15 and 35°C corresponding to a $Q_{10}$ of 1.18 and a slightly steeper decrease between 35 and 42°C corresponding to an inhibitory $Q_{10}$ value of 0.77. Similar experiments were performed without medium

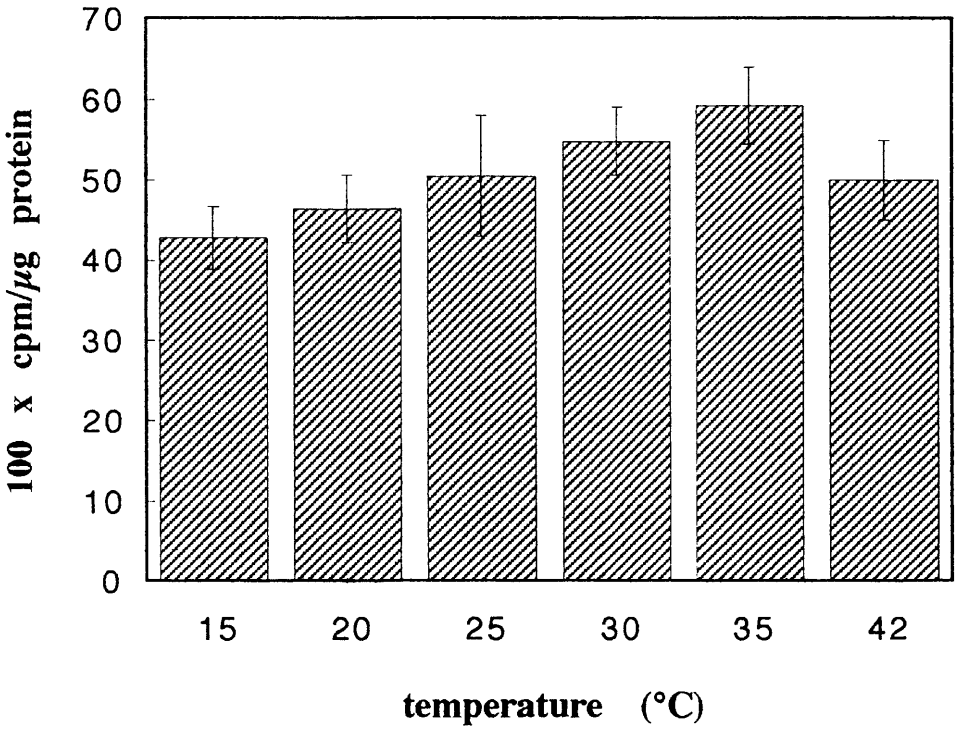


FIG. 1. Steady state (24 h) temperature adaptation of total protein synthesis. [$^{35}$S]Methionine incorporation into acid-precipitable protein (1 h incubation with the labeled amino acid) after 24 h exposure to different temperatures (abscissa). Ordinate: 100 × cpm/μg protein). Means of four independent experiments ± SD.

change (total of six series) with generally similar results except for slightly lower rates after 24 h. The results show the existence of adaptive mechanisms after both moderate (30–37°C and 20–15°C) or stressful (37–42°C) temperature shifts.

When the different proteins synthesized during the adapted state were analyzed by SDS–PAGE, most protein species showed an increased synthesis rate with increasing temperatures up to 35°C and then a decrease. The degree of this decrease at 42°C and hence temperature adaptation is apparently not identical among the different protein species. Heat shock proteins, on the other hand, showed an increase in their synthesis rate with increasing temperatures, but not at lower temperatures such as 15°C (not shown). The differing responses of different protein species reveal the overall adaptation curve of total protein synthesis as documented in Fig. 1.

## *Adaptation Kinetics*

More detailed information about the adaptation process of total and individual protein synthesis can be derived from determining the kinetics of total protein and of single-protein synthesis after exposure to elevated temperature.

In several series of experiments cultures were grown at 25°C and then shifted to either 37 or 42°C for a 24-h period during which the medium was changed once. The rate of [$^{35}$S]methionine incorporation into total protein was determined at different times (Fig. 2a). At 37°C, an immediate increase was observed until a maximum is reached after 5 h. Thereafter, a slight decrease toward a steady state level of 27% above the control was determined. At 42°C, an immediate drop below control level was followed by an increase and a later decrease of the synthesis rate slightly below control level.

Several other measurements of the adaptation kinetics of total protein synthesis showed similar results. When mycelia were kept at 42°C for 24 h the steady-state values of total protein synthesis always ranged slightly above or below control levels.

Under the same conditions as described above cultures were shifted from 25 to 42°C and the [$^{35}$S]methionine incorporation into actin was analyzed. The actin band on a

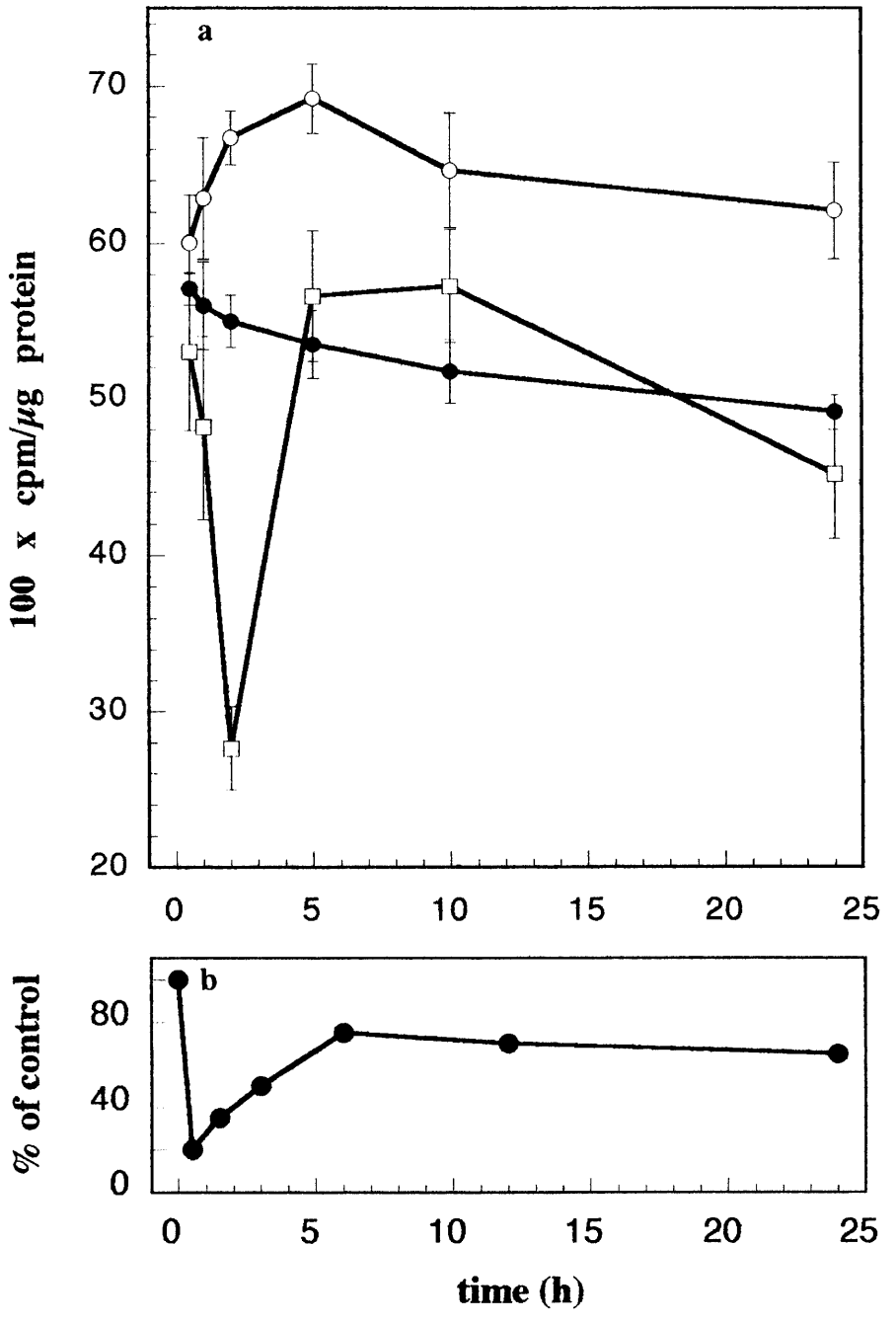


FIG. 2. Kinetics of temperature adaptation. (a) Total protein synthesis during exposure to 37°C (○) or 42°C (□). Controls (●) were kept at 25°C. Various times after the shift mycelia were incubated with [$^{35}$S]methionine for 1 h and the incorporation was determined. Means of three experiments ± SD. Ordinate: 100 × cpm/µg protein; abscissa: time in h after exposure to the different temperatures. (b) Actin synthesis during exposure to 42°C. Densitometric determination of the actin band after PAGE and subsequent fluorography. Ordinate: relative density of the actin band; abscissa: time in h after beginning of exposure to 42°C.

fluorograph taken after SDS–PAGE was determined densitometrically and identified by parallel Coomassie staining. The actin synthesis rate showed similar kinetics compared to total protein. After an initial strong decrease (0–30 min) adaptation to values closer to control values were observed during the following 6 h. One representative series is shown in Fig. 2b.

### *Acquired Translational Thermotolerance*

In order to test the effect of a "priming" heat shock given before the second continuous exposure on the adaptation kinetics one set of cultures was exposed for 1 h to 42°C and then allowed to recover at 25°C for 2 h before this set of cultures was exposed again to 42°C for 8 h. The second set of cultures was treated only with an exposure to 42°C for 8 h. The kinetics of both sets were significantly different (Fig. 3): the primed cultures did not show an initial inhibition of protein synthesis after the (second) shift to 42°C exposure, which is the case in the unprimed cultures. This acquired translational thermotolerance may be due to increased amounts of HSPs, particularly of the inducible HSP70 after the first priming heat shock (Li and Duncan, 1995), among other possible mechanisms (see Discussion).

### *HSP Synthesis at Different Temperatures*

HSP70 synthesis was analyzed by using a polyclonal antibody raised against the major member of the HSP70 family (Fracella *et al.*, 1993), which shows constitutive as well as heat shock-induced synthesis. Mycelia (aged 36 h) were exposed to the different temperatures for 1 h. Western blot analysis showed a maximum induction of HSP70 at about 43–45°C. This maximal response at about 43–45°C was also observed in other strains tested (not shown). The induction of HSP70 at 30, 37, and 40°C increased almost linearly, but comparatively little (by a factor of about 1.8–2.0) compared to the maximal induction at 43°C (about 5× control value). At higher temperatures (46, 50°C) the synthesis of HSP70 is drastically reduced and—at the highest temperature—inhibited below control levels (not shown).

When mycelia were allowed to adapt for 24 h to the different temperatures, the synthesis rate of HSP70 as determined by [$^{35}$S]methionine incorporation was slightly elevated at temperatures between 34 and 40°C with rates about 1.7–1.8 times higher than the controls (Fig. 4). A higher steady-state HSP70 synthesis is observed after 24-h adaptation to 42°C (approximately 2.5× higher than the control level). These results show that the adapted state of

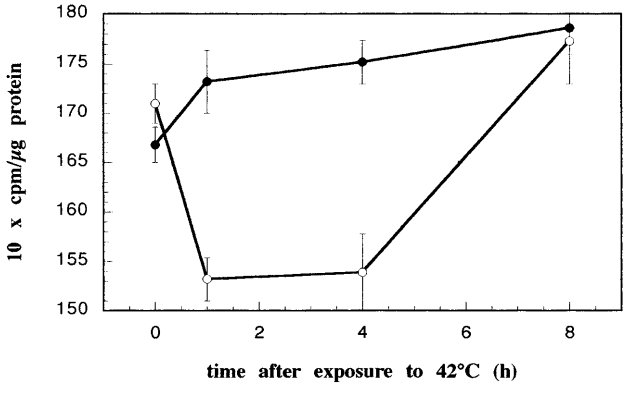


FIG. 3. Translational thermotolerance. Adaptation kinetics of total protein synthesis during exposure to 42°C. (●) Primed cultures initially exposed for 1 h at 42°C 2 h before the second exposure. The second exposure was identical to that of the control cultures which did not receive a priming treatment. (○) Control cultures treated as in Fig. 2a. Means of two experiments ± SD. Ordinate: 10× cpm/μg protein; abscissa: time in h after exposure to 42°C.

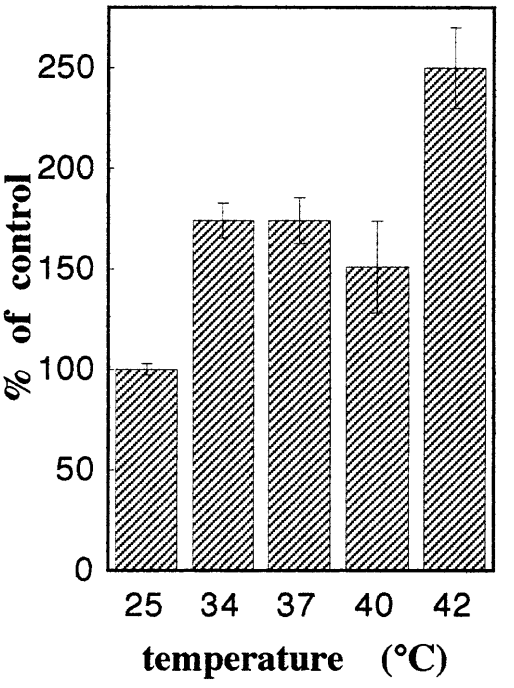


FIG. 4. Steady-state (24-h) temperature adaptation of HSP70 synthesis. [$^{35}$S]Methionine incorporation (1-h incubation) after 24-h exposure to different temperatures (abscissa). Fluorographs of proteins separated by one-dimensional PAGE were analyzed densitometrically with respect to the density of the HSP70 band, which was calculated as percentage of the control (25°C, ordinate). Means of three experiments ± SD.

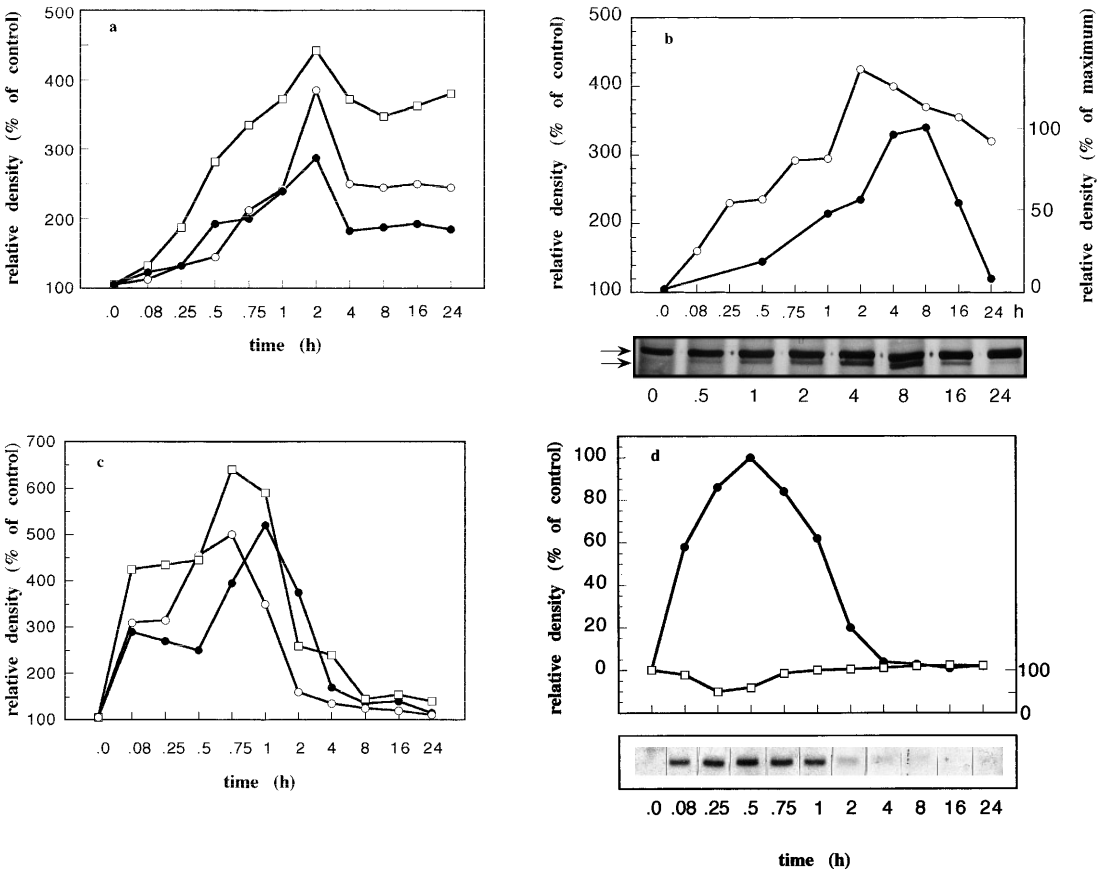


FIG. 5. Adaptation kinetics of heat shock gene expression during exposure to 42°C. (a) *In vivo* synthesis ([$^{35}$S]methionine labeling) of the three high-molecular-weight groups of HSPs (● HSP98, □ HSP84, ○ HSP70) at different times after exposure to 42°C (abscissa). Densitometric evaluation of fluorographs of one-dimensional PAGE. Mycelia were incubated for 30 min with [$^{35}$S]methionine. The shorter exposures to 42°C at the beginning of the kinetics were applied at the end of a 30-min incubation period. Ordinate: relative density of the fluorographic bands with respect to the control at 25°C (in percentage). (b) Amounts of HSP70 isoforms determined by Western blot. ○, total isoforms including constitutive and inducible forms expressed as percentage of controls (25°C, left ordinate); ●, inducible isoform expressed as percentage of maximal density (right ordinate). Below: Western blots showing the constitutive and inducible isoforms. Abscissae: times after beginning of exposure to 42°C in h. (c) Heat shock proteins synthesized in an *in-vitro* translation system. Poly(A)+ mRNAs were isolated at different times after exposure to 42°C and the[$^{35}$S]methionine incorporation into the different HSPs (● HSP98, □ HSP84, ○ HSP70) was determined. The incorporation rate was measured as in (a) and calculated in percentage of the controls (ordinate). Abscissa as in (a). (d) Amounts of inducible *hsp70* mRNA and tubulin mRNA determined by Northern blots with a heterologous probe (*Drosophila*) for *hsp70* and a homologous probe for tubulin mRNA, respectively. ●, *hsp70* mRNA expressed as density in percentage of the maximal value (left ordinate): □, tubulin mRNA expressed as percentage of controls (right ordinate). Abscissa as in (a). Below: Northern blots of *hsp70* mRNA.

house keeping gene expression after 24 h is accompanied by an increased synthesis rate of HSPs.

In order to arrive at a more detailed picture of the effect of elevated temperature on HSP synthesis and *hsp* gene expression, we also analyzed the kinetics of their adaptation to elevated temperature (42°C).

## *Adaptation Kinetics of HSP Synthesis*

When [$^{35}$S]methionine incorporation was determined by densitometric measurements of the different HSP bands during 24-h exposure to 42°C a transient increase of three high-molecular-weight HSP bands was observed, containing several members of the HSP70, HSP90, and HSP100 families (Fig. 5a). All HSPs reached maximal synthesis rates 2 h after the beginning of the 42°C exposure. During the adapted state the level of HSP synthesis remained elevated, in the case of HSP70 by a factor of about 2.5 higher than control level.

The amount of the major HSP70 isoform(s) (Kapoor *et al.*, 1995) during continued exposure to 42°C was determined by using a polyclonal antibody directed against

HSP70 (Fracella *et al.,* 1993). Maximal amounts of this species which is constitutively expressed and enhanced by heat shock, were observed after 2-h exposure. Thereafter, this particular band shows only a slow decline (Fig. 5b). This slow decline was consistently observed in three independent series of experiments (not shown).

The inducible isoforms of HSP70 (Fracella *et al.,* 1993) may be determined with the same antibody after extended gel runs which lead to a second band. This band appears only during heat shock and shows a slightly higher mobility compared to the major HSP70 species (Fig. 5b). This isoform showed no expression in the controls, but maximal amounts 4–8 h after the beginning of the 42°C treatment. Almost no inducible HSP70 was detected after 24 h. If this band, in fact, represents the inducible isoform of HSP70, the results strongly indicate a faster adaptation kinetics of this form.

### *Adaptation Kinetics of hsp Gene Expression*

In order to determine the kinetics in the amounts of different *hsp*-mRNA species we isolated poly(A)$^+$ RNA and translated the mRNA in a heterologous *in-vitro* translation system. The translated [$^{35}$S]methionine-labeled proteins were separated by SDS–PAGE and their synthesis rates determined by fluorography and densitometric evaluation. From the resultant protein synthesis of the three HSP families (HSP100, HSP84, and HSP70) a rapid increase of the respective *hsp*-mRNAs to maxima 45 min or 60 min after the beginning of exposure to 42°C and a rapid decline between 1 and 4 h after exposure can be deduced (Fig. 5c). The rate of [$^{35}$S]methionine incorporation into actin was taken as control. The rate remained rather constant except for a short initial decrease (not shown).

The induced and constitutive forms of HSP70 and their mRNAs may exhibit different kinetics, as concluded from Fig. 5b. Therefore, the amount of inducible *hsp70*-mRNA was determined by Northern-blot analysis, using an inducible *Drosophila hsp70*-gene (Karch *et al.*, 1981) as a probe. We applied this probe because some *Drosophila hsp* genes show about 70% homology to *Neurospora hsp* genes, for example the *grp 78* gene (D. Techel, unpubl. results). The inducible *hsp70*-gene from *Drosophila* hybridized with *Neurospora* mRNA only after heat shock, indicating that it is homologous to one (or more) inducible *hsp70*-genes of *Neurospora* (Häfker *et al.,* 1998). The blots revealed maximal amounts of inducible *hsp70*-mRNA 30 min after beginning of exposure to 42°C. Then a rapid decline is observed until 4 h of exposure when the amount of mRNA reaches control values again (Fig. 5d). This result shows that transcriptional inhibition of the inducible *hsp70*-gene already occurs after about 30 min of heat shock exposure. The tubulin mRNA level remained relatively constant except for a slight transient decrease.

### *Nuclear Localization of HSP70*

The amount of HSP70 in the nucleus and cytoplasm was measured after raising the temperature to 37°C or to 42°C in order to test possible relations between the nuclear localization of HSP70 and protein synthesis (Hayashi *et al.*, 1991). After a shift to 37°C an immediate and steep increase of nuclear HSP70 was observed, reaching a maximum after 2 h (Fig. 6a). The nuclear concentration of HSP70 then declines, but remains above the control level by about 300% during the whole exposure. After the temperature shift the cytoplasmic HSP70 concentration first declined and then increased after 4–8 h to elevated levels (about 200% of the control) and then decreased to about 150% until the end of the experiment (24 h).

After a shift to 42°C, a steeper and higher increase of nuclear HSP70 occurs up to a maximum of 800% after 2 h (Fig. 6b). Thereafter the nuclear HSP70 declines to a steady-state value of about 400%. The kinetics of the cytoplasmic HSP70 again showed an early decrease followed by an increase until about 16 h after the temperature shift (350% of the control) and then a slight decline to a steady-state level of about 300% above the control. Similar results were obtained when these experiments were repeated with another strain (not shown).

## DISCUSSION

The main aim of this investigation was to determine the extent of adaptation of protein and HSP synthesis during continued exposure to higher temperatures (37 and 42°C) and to analyse the adaptation kinetics of several potentially important variables such as nuclear localization of HSPs in *N. crassa.* Temperature adaptation appeared interesting because the homeostasis of gene expression at different temperatures—which also includes the degradation rates of proteins (Mohsenzadeh *et al.*, 1994)—has considerable implications for the homeostasis of cell metabolism and cell behavior under different stress conditions. Under

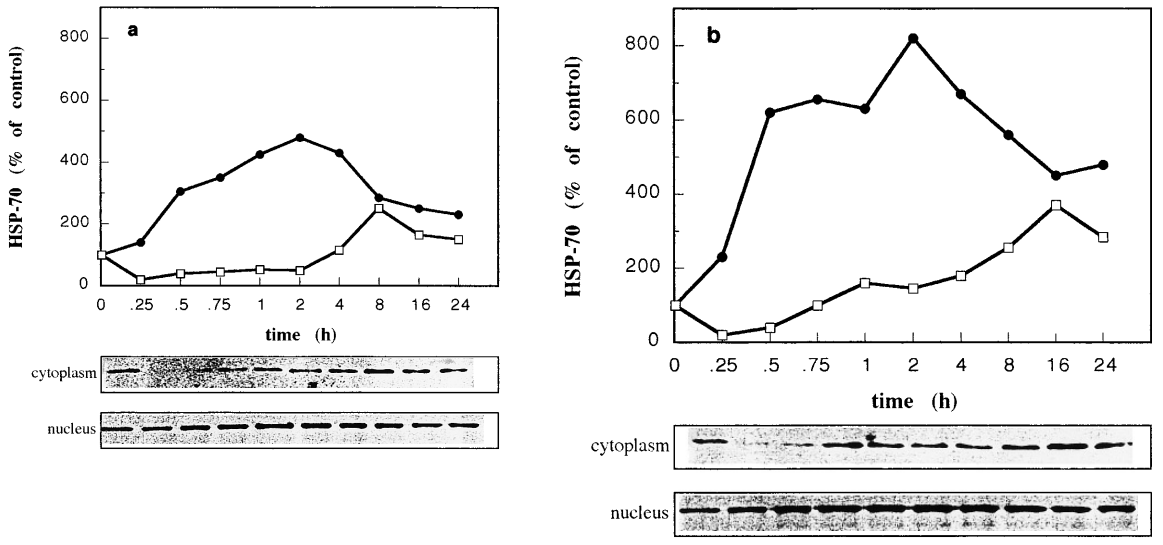


FIG. 6. Adaptation kinetics of the cytoplasmic and nuclear content of HSP70. □, cytoplasmic fraction; ●, nuclear fraction. (a) After exposure to 37°C. (b) After exposure to 42°C. Ordinates: density of the HSP70 band in a Western blot in percentage of the control. Abscissa: times after beginning of exposure to 37 or 42°C in h. Below: Western blots.

natural conditions this homeostasis may be advantageous with respect to the temperature changes occurring regularly throughout 24 h and may well reach differences of more than 15°C. It may also be advantageous with respect to seasonal changes of temperature and adaptation of species to different climates. Furthermore, a particular feature of circadian rhythms, the temperature compensation of its period length, may rely on several adaptive mechanisms, including homeostasis of protein turnover (Rensing *et al.*, 1997).

## *Heat Shock-Induced Inhibition and Subsequent Adaptation of House Keeping Gene Expression*

Heat shock apparently inhibits all parts of the expression of genes and associated regulatory processes: transcription; processing of mRNA, particularly splicing; import of transcription factors and export of RNA through the nuclear pore complex; initiation and elongation of protein synthesis and the functional state of the synthetic apparatus (ribosomes and their associated proteins, aminoacyl tRNAs); as well as competition among mRNAs and other compounds, mRNA-binding to stabilizing proteins, amino acid uptake and availability of ATP, and folding and transport of proteins as well as posttranslational modifications (review: Nover, 1991). Only some of these processes have been analyzed with respect to adaptation.

Transcription of house keeping genes such as tubulin or actin has been used as reference during heat shock treatments (review: Nover, 1991) and was shown to adapt quickly also in *Neurospora* (see Fig. 5d).

A major target of heat shock is the translational apparatus. As shown in *Neurospora* and in many other organisms (review: Nover, 1991), this leads to an immediate inhibition of the overall synthesis rate caused probably by interference with several translational processes, but particularly with its initiation. This general inhibitory action of HS was also observed when the synthesis rate of a single protein species, such as actin, was determined (Fig. 2b).

A particularly interesting adaptive mechanism may be based on the cooperation of a HSP70 species (SSB70 of yeast) with translating ribosomes (Nelson *et al.*, 1992), which may relate to the association of *Neurospora* HSPs with ribosomes as reported by Vassilev *et al.* (1992). HSPs seem to play a role in aiding the passage of a newly synthesized protein through the ribosome by associating with the nascent polypeptide. Mutants of *ssb70* can be reactivated by increased copy numbers of a gene encoding the EF-1α-like protein. In reticulocyte lysates, the efficiency of protein synthesis was positively correlated with the amounts of HSPs and its constitutive isoforms (Panniers, 1994).

During continuous heat stress, the adaptive increase of protein synthesis in normal rat kidney cells was paralleled by a reshuttling of HSP70 from the nucleus into the

cytoplasm (Hayashi *et al.,* 1991; Liu *et al.,* 1992), a correlation also found in this study. In *Neurospora,* however, a continued elevated level of HSP70 remained in the nucleus even in the adapted state (Fig. 6), indicating a continued stabilizing role in mRNA processing or ribosomal assembly.

Generally, a higher content of HSPs is believed to promote "acquired thermotolerance" (Parsell and Lindquist, 1993) which is also assumed for translational thermotolerance (DeMaio *et al.,* 1993). In *Neurospora,* the adapted steady state after 24-h exposure to different temperatures always showed elevated synthesis rates and amounts of HSPs, indicating a role in translational adaptation. We cannot distinguish between protective effects of the different HSP70 isoforms as suggested by Li and Duncan (1995), who postulated a particular role for the inducible HSP70 in this respect. HSP98 of *Neurospora* (a protease) is equally enhanced after the priming heat shock and may also contribute to thermotolerance, because it is homologous to the yeast HSP104 (Vassilev *et al.,* 1992), known to play an important role in temperature adaptation (Sanchez and Lindquist, 1990). A marked thermotolerance was observed when mycelia were exposed to 42°C 2 h after a priming 42°C treatment (Fig. 3).

In *Neurospora,* adaptation of total protein synthesis also occurred at lower temperatures (15°C) without additional HSP synthesis. Furthermore, exposures to 30 or 37°C led to only a limited increase and subsequent adaptation of house keeping protein synthesis. Again, this adaptation occurred with only small changes in the HSP content. These results and several findings in other organisms indicate that there exist homeostatic mechanisms of protein synthesis independent of the amount of HSPs (Hahn and Li, 1990; Hallberg, 1986).

It is interesting to note that in contrast to protein synthesis, other variables such as growth rate did not adapt as much: the increase in dry weight per 48 h revealed differences of a factor of 3 between 15 and 35°C (i.e., a $Q_{10}$ of 1.5, not shown). This may be due to a higher temperature dependence of the catalytic activity of enzymes and of the corresponding accumulation of their products such as the cell walls.

## *Heat Shock-Induction and Subsequent Adaptation of hsp-Gene Expression*

The immediate initiation of transcription of heat-inducible *hsp*-genes is mainly due to activation of one (or two) heat shock factor(s) (HSF; Wu, 1995). Binding of HSP70 to the HSF is proposed to be an important regulatory mechanism to prevent activation under normal conditions (Mosser *et al.,* 1993). After stress, HSP70 is assumed to bind preferentially to malfolded proteins and thus release the monomeric HSF which then forms a trimer. The subsequent adaptive deactivation of HSF during continued stress can be explained by the increased amounts of newly synthesized (free) HSP70 (Craig and Gross, 1991). Trimerization of HSF might also occur as a direct response to heat exposure, but aided by HSC70 (Schlesinger and Ray, 1993). It is, however, not clear whether these changes play an important role, especially in organisms such as yeast and *Neurospora,* which show a continuous DNA-binding of HSF (U. Meyer, unpubl. results).

*Hsp*-mRNA is stabilized during stress (Dellavalle *et al.,* 1994); however, during continued heat stress, poly$(A)^+$-*hsp*-mRNA of *Drosophila* was shown to be completely deadenylated, which led to lower translational capacity (Sachs, 1990). Since we isolated poly$(A^+)$ -RNA for *in vitro* translation, this deadenylation of *hsp*-mRNA may explain the drastic decrease observed after 8 h (Fig. 5b) in contrast to the HSP synthesis *in vivo* (Fig. 5a). A similar complete disappearance of *hsp70* mRNA was observed by Kapoor *et al.* (1995) for the major *hsp70* gene after 3-h exposure to 48°C. This treatment, however, might have been unphysiologically high and therefore detrimental to further *hsp70* expression.

*Hsp*-mRNA is translated preferentially under stress conditions. Most of the *hsp*-mRNAs show long (150–250 nucleotides) leader sequences with high adenine content (45–49%, Hultmark *et al.,* 1986), which are apparently needed for translation at higher temperatures. Probably, *hsp*-RNAs do not develop secondary structures in their 5′ untranslated regions and thus do not need the cap-binding protein complex (Sierra and Zapata, 1994). They may thus compete successfully with house keeping mRNAs and interfere with their translation as long as they are present in higher amounts.

We conclude from our data that the observed temperature adaptation of total house keeping proteins and of tubulin and actin synthesis after 24 h of exposure to 42°C may be due to enhanced amounts of the major constitutive HSP70 and other HSPs. The adaptation kinetics of the inducible isoform of *hsp70* genes and inducible HSP70 protein is considerably faster and reaches control values already 4 and 24 h, respectively, after the beginning of the HS treatment. With respect to the daily temperature changes, the constitutive HSPs may support a long-term adaptation, whereas the inducible isoforms may respond to the daily rise in environmental temperature.

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