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Heat Shock Response of *Neurospora crassa*: Protein Synthesis and Induced Thermotolerance

NORA PLESOFSKY-VIG* AND ROBERT BRAMBL

Department of Plant Pathology, University of Minnesota, Saint Paul, Minnesota 55108

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At elevated temperatures, germinating conidiospores of *Neurospora crassa* discontinue synthesis of most proteins and initiate synthesis of three dominant heat shock proteins of 98,000, 83,000, and 67,000 M_r and one minor heat shock protein of 30,000 M_r. Postemergent spores produce, in addition to these, a fourth major heat shock protein of 38,000 M_r and a minor heat shock protein of 34,000 M_r. The three heat shock proteins of lower molecular weight are associated with mitochondria. This exclusive synthesis of heat shock proteins is transient, and after 60 min of exposure to high temperatures, restoration of the normal pattern of protein synthesis is initiated. Despite the transiency of the heat shock response, spores incubated continuously at 45°C germinate very slowly and do not grow beyond the formation of a germ tube. The temperature optimum for heat shock protein synthesis is 45°C, but spores incubated at other temperatures from 40 through 47°C synthesize heat shock proteins at lower rates. Survival was high for germinating spores exposed to temperatures up to 47°C, but viability declined markedly at higher temperatures. Germinating spores survived exposure to the lethal temperature of 50°C when they had been preexposed to 45°C; this thermal protection depends on the synthesis of heat shock proteins, since protection was abolished by cycloheximide. During the heat shock response mitochondria also discontinue normal protein synthesis; synthesis of the mitochondria-encoded subunits of cytochrome *c* oxidase was as depressed as that of the nucleus-encoded subunits.

Widely divergent species of animals, plants, fungi, and bacteria respond to exposure to supraoptimal temperatures by suppressing the synthesis of many normal proteins and synthesizing large quantities of a few distinctive proteins (2, 3, 6, 10, 15, 33, 35, 49), the most prominent of which have been shown to be remarkably conserved throughout evolution (19). This response to high temperatures is reversible, with recovery involving the restoration of the normal pattern of protein synthesis (11, 33). These heat shock proteins, whose synthesis is induced by elevated temperatures, apparently help protect the cells against otherwise lethal effects of exposure to still higher temperatures (24, 32). In *Drosophila melanogaster* (12) and *Saccharomyces cerevisiae* (31), heat shock protein synthesis also is known to be required for the resumption of normal protein synthesis during recovery.

The heat shock response is an experimental system that is providing useful information about cellular mechanisms for regulating gene expression at both the transcriptional and posttranscriptional levels. Exposure to high temperatures causes a rapid and dramatic change in the pattern of gene expression in all organisms examined through mechanisms that include the cessation of ongoing transcription and the initiation of new transcription (2). In addition, the translation of normal mRNAs is curtailed during heat shock. In *Drosophila* (4, 27, 39) and HeLa cells (16), these normal mRNAs are preserved in a relatively inactive form during heat shock and utilized efficiently again for normal protein synthesis during recovery. In contrast, preexisting mRNAs of *S. cerevisiae* cells are degraded at an accelerated rate, and recovery requires new transcription of normal mRNAs (31, 35).

We are examining the response of *Neurospora crassa* to high temperatures to determine how this organism may shift patterns of gene expression in response to a defined stimulus, and we are especially interested in posttranscriptional mechanisms for this alteration of gene expression. The life cycle of *N. crassa* includes a dormant cell, the asexual conidiospore, with preserved mRNA that is thought to be translated into protein when spores are activated for germination (7). Therefore, this organism normally exercises translational control over gene expression during at least this stage of development, but the range of expression patterns available to the germinating spore remains undefined. It is possible, for example, that the population of preserved mRNA contains alternate candidates for translation and that the spore may selectively express those mRNAs that encode the proteins appropriate for specific nutritional or environmental circumstances. We have begun to study the effect of heat shock on gene expression in *N. crassa* (i) to determine whether alternate patterns for mRNA translation may exist in the germinating spores as well as in postemergent cells and (ii) eventually to help characterize the molecular mechanisms employed by the cell in discriminating among these translatable mRNAs.

In the present study, we have characterized the response of germinating spores to supraoptimal temperatures and show that the intensity of this response depends on the specific temperature and the duration of exposure. We established that these cells produce three major heat shock proteins of 98,000, 83,000, and 67,000 M_r and that protein synthesis during heat shock is required to protect the cells against a higher, lethal temperature. In addition, we have found that three heat shock proteins of lower molecular weight are associated with mitochondria. This response to heat shock, the exclusive synthesis of a limited set of proteins, is transient and apparently highly regulated, since its initiation is extremely rapid and its duration is limited, despite the continued stimulus of high temperatures.

MATERIALS AND METHODS

Cell culture. Conidiospores of *N. crassa* 74-OR23-1A (Fungal Genetics Stock Center, Arcata, Calif.), produced and

* Corresponding author.

TABLE 1. Spore survival under different temperature regimens

Experimental treatment[a]	Mean no. of viable spores ± SD[b]	% of control
Effects of exposure to a single temperature		
30°C (normal growth temperature)	129 ± 7.9	100
45°C	117 ± 10.8	91
50°C	23 ± 8.1	18
Effects of exposure to high temperature following incubation at 30°C		
30°C → 46°C	119 ± 9.3	92
30°C → 47°C	105 ± 5.2	81
30°C → 48°C	16 ± 3.8	12
30°C → 49°C	6 ± 4.2	5
30°C → 50°C	1 ± 0.9	1
30°C → 45°C (1 h) → 50°C	82 ± 5.6	64
Duration of induced protection after shiftdown to 30°C		
45°C → 30°C (1 h) → 50°C	84 ± 4.0	65
45°C → 30°C (2 h) → 50°C	33 ± 5.0	26
45°C → 30°C (3 h) → 50°C	14 ± 3.3	11
Requirement for protein synthesis[c]		
30°C → 45°C (1 h, CHI) → 50°C	3 ± 0.6	2

[a] Treatment at each temperature was for 2 h unless otherwise noted.

[b] The number of spores surviving each treatment was determined by diluting the spore suspension 5,000-fold, spreading 150 μl onto sorbose-containing agar nutrient medium, and counting the discrete colonies that formed.

[c] To test the requirement for protein synthesis, 50 μg/ml of cycloheximide (CHI) was added to the culture 10 min before shift to 45°C.

harvested under nonactivating conditions as previously described (7), were suspended in a sucrose (2% [wt/vol]) minimal medium (46) at a concentration of 1 mg/ml and incubated in a liquid shake culture (180 rpm) at the temperatures indicated. Cellular proteins were radioactively labeled by the addition of 24 μCi of [^{35}S]methionine (1,200 to 1,450 Ci/mmol) or [^{3}H]leucine (146 Ci/mmol) to 30 ml of spore suspension. After 30 min of incubation, 0.5 μmol of unlabeled methionine or 1 μmol of unlabeled leucine was added, and the cells, after being cooled on ice, were rapidly collected by filtration. Cell cultures reached 44°C within 2 min and 45°C by 4 min after transfer from a 30°C water bath to a 45°C water bath.

Cell survival. A sample of spores was removed from the cell suspension and diluted 5,000-fold; 150 μl of the spore dilution was plated onto agar medium containing sorbose (9), and discrete colonies were counted after 4 to 6 days. The assays yielded average values (from at least six trials) that are within a 90% confidence interval for the control treatment. The result of each experimental treatment is expressed as the percentage of the control value. Cycloheximide was added at 50 μg/ml of spore suspension.

Germination rate and pulse-labeling. A minimum of 300 spores per sample was counted under a light microscope to determine the percentage of spores that had developed germ tubes longer than one spore diameter. At 30-min intervals 10 ml of spore suspension was incubated for 15 min with 0.5 μCi of [^{14}C]leucine or [^{14}C]uracil to measure rates of protein synthesis or RNA synthesis, respectively (7). Acid-precipitable radioactivity was counted by liquid scintillation spectrometry. All radiolabeled precursors were purchased from New England Nuclear Corp. (Boston, Mass.) or Amersham Corp. (Arlington Heights, Ill.).

Protein extraction. Cells were filtered, washed, and suspended in buffer containing 62.5 mM Tris-hydrochloride (pH 6.7), 2% sodium dodecyl sulfate (SDS), 0.75 mM phenylmethylsulfonyl fluoride, and 7.5% 2-mercaptoethanol. Proteins were extracted from the spores by homogenization for 60 s with glass beads in a Braun MSK homogenizer (4×10^3 rpm), accompanied by limited CO_2 cooling. The extracts were heated in a boiling water bath for 3 min and then centrifuged at 12,000 × *g* for 10 min. The supernatant fluids were frozen at −80°C. Before electrophoresis, the thawed cell extracts were heated for 3 min in a boiling water bath and centrifuged for 5 min.

Isolation of mitochondria. The mitochondrial fraction from radiolabeled cells was isolated by differential centrifugation, and highly purified mitochondria were prepared by the flotation gradient method (22). The mitochondria, collected from the interface of 44 and 55% sucrose, were pelleted and solubilized in SDS sample buffer. Before electrophoresis the solubilized mitochondria were heated for 3 min in a boiling water bath.

Gel electrophoresis of extracts. Proteins in the cell or mitochondrial extracts were separated by one-dimensional SDS electrophoresis (30) in 10% polyacrylamide cylindrical gels with constant voltage of 100 V or in 0.75-mm slab gels with constant voltage of 90 V. The cylindrical gels were frozen with CO_2 and sliced into 1-mm slices from which the radioactive proteins were solubilized (50). A toluene-based scintillation fluid was added for the quantification of radioactivity in a Packard 460 CD spectrometer. Proteins separated on the slab gels were fixed with trichloroacetic acid and autoradiographed with Kodak XAR-5 film for 20 h at −80°C. Apparent molecular weights were determined by reference to Coomassie blue-stained protein standards.

Analysis of subunits of cytochrome *c* oxidase. The mitochondrial fraction from radiolabeled cells was isolated, lysed with Triton X-100, and reacted with an antiserum prepared against the dissociated subunits of cytochrome *c* oxidase (8). The immunoprecipitates were solubilized in SDS sample buffer, and the peptide subunits were separated by electrophoresis in SDS–15% polyacrylamide gels (30). The radioactivity of gel slices was determined by liquid scintillation spectrometry.

RESULTS

Effect of temperature on spore germination and cell survival. *N. crassa* spores incubated in nutrient medium at 30, 35, and 40°C had similar kinetics of germination, reaching 95% germination by 6 h. In contrast, only 6.5% of the spores incubated at 45°C had germinated by 6 h, and they attained a maximum of 35% germination by 11 h. Spores that germinated at 45°C developed short germ tubes, ca. 5 to 10 spore diameters long, which did not elongate further or branch at the high temperature.

Spores that had initiated germination at 30°C for 2 h were subsequently subjected to elevated temperatures for an additional 2 h. Their survival was quantified by a plating assay and expressed (Table 1) as a percentage of the control spores surviving incubation at 30°C, the normal growth temperature. Germinating spores incubated at 45 and 46°C survived nearly as well as the control, and the survival of spores incubated at 47°C was only slightly lower (81% of the control). In contrast, the exposure of spores to higher temperatures for 2 h drastically reduced viability, resulting in only 12% spore survival at 48°C, 5% at 49°C, and less than 1% survival at 50°C. The lethality of exposure to 50°C was greatly reduced when spores were exposed to 45°C for 1 h before their transfer to 50°C; 64% of these spores survived the 50°C treatment. This protective effect of exposure to 45°C lasted for 1 h at 30°C but diminished progressively by 60%/h thereafter, resulting in 26 and 11% survival at 50°C

after 2 and 3 h of incubation, respectively, at 30°C. To test whether protein synthesis was required for thermal protection, we inhibited protein synthesis with cycloheximide during the 45 and 50°C incubations and assayed cell survival; this drug blocks the incorporation of radiolabeled amino acid into all of the heat shock proteins described below. Under these conditions of inhibition, only 2% of the spores survived incubation at 50°C, despite their preexposure to 45°C. This reduced viability was not due to a long-term toxic effect of cycloheximide, since cells grown continuously at 30°C for 5 h and treated with cycloheximide during the last 3 h of incubation had a survival rate that was similar to the control. Furthermore, the presence of cycloheximide at 50°C was not toxic to the cells, since the addition of cycloheximide 50 min after the spores were exposed to 45°C and 10 min before their transfer to 50°C did not reduce cell survival.

The thermal resistance that germinating spores derived from experiencing a nonlethal heat shock was greater than the protection conferred on spores by dormancy, a stage-specific cellular organization that protects ungerminated conidiospores against extremes of cold, heat, and desiccation (42). Dormant spores that were inoculated directly into 50°C medium and incubated at that temperature for 2 h had a survival rate that was 18% of that of the control, which is higher than the 1% survival of spores that were incubated at 30°C for 2 h before their exposure to 50°C. However, this improved survival is considerably lower than the 64% survival rate conferred on germinating spores by a prior heat shock. Heat resistance due to heat shock, therefore, appears to be derived from a mechanism that is distinct from heat resistance due to dormancy. This conclusion is suported by our observation that dormant spores labeled during spore formation with [^{35}S]methionine did not contain detectable quantities of the major heat shock proteins described below.

Effect of temperature on patterns of protein synthesis. Cells of *N. crassa* were radiolabeled with [^{35}S]methionine, and proteins in the cellular extracts were separated by one-dimensional SDS-polyacrylamide gel electrophoresis. Radiolabeled proteins were visualized by autoradiography of slab gels. To compare more quantitatively the relative amounts of different proteins synthesized by cells at different temperatures and at different times of exposure, cylindrical gels were sliced, and the radioactivity of proteins in each slice was determined by scintillation spectrometry.

N. crassa cultures exposed to 45°C synthesized predominantly three proteins (Fig. 1) whose molecular weights are 98,000, 83,000, and 67,000, relative to protein standards. These proteins were synthesized by spores that initiated germination at 45°C, by spores incubated at 30°C for 2 h before exposure to 45°C, and by postemergent or mycelial cells after 7 h of growth (Fig. 2). The mycelial cells also produced a fourth dominant heat shock protein of 38,000 M_r and a minor heat shock protein of 34,000 M_r that were synthesized in lower quantities by younger cells. Another minor heat shock protein of 30,000 M_r, which apparently contains little or no methionine, was synthesized by cells of all ages upon exposure to high temperatures (see Fig. 6A) and was detected by radiolabeling with [^{3}H]leucine.

During the maximal expression of the heat shock proteins, the synthesis of normal proteins was severely depressed. We labeled different spore cultures for 30-min intervals throughout 150 min of exposure to high temperatures, added excess unlabeled methionine at the end of each 30-min period, and rapidly harvested the cells. We found that the heat shock pattern of protein synthesis persists only through the first 90 min of exposure to 45°C. After 60 min, there was a progressive increase in the synthesis of normal proteins, typified by actin, and a decline in heat shock protein synthesis (Fig. 3A). Cells grown continuously at 45°C and radiolabeled between 300 and 330 min of incubation had a near normal pattern of protein synthesis, although apparently accompanied by low levels of heat shock proteins. This change, after 90 min at 45°C, from heat shock protein synthesis to synthesis of normal proteins was also shown by germinating spores that had been preincubated for 2 h at 30°C and by mycelial cultures after 7 h of growth.

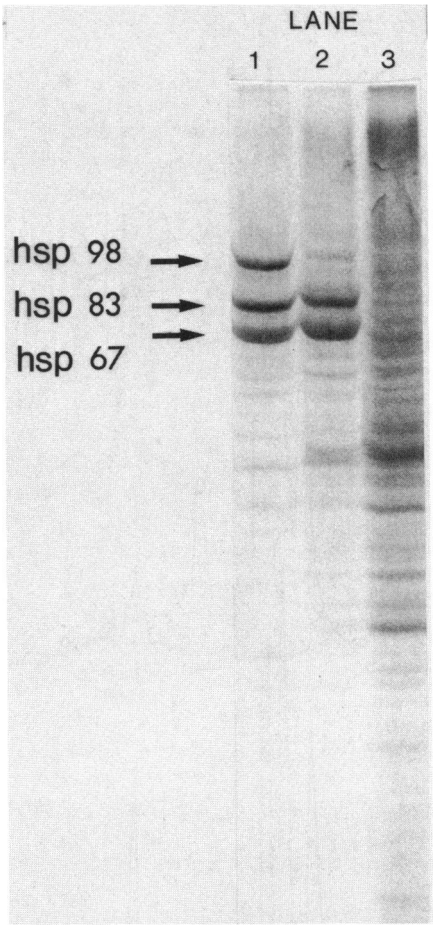


FIG. 1. Autoradiogram of [^{35}S]methionine-labeled heat shock proteins separated in an SDS–10% polyacrylamide gel. Proteins were extracted from spores that were incubated at 45°C in distilled water (lane 1) and at 45°C (lane 2) and 30°C (lane 3) in nutrient medium. Spores were radiolabeled between 30 and 60 min after culture inoculation. Twice as much protein was applied to lane 1 as to the other lanes. The arrows designate the three major heat shock proteins of 98,000, 83,000, and 67,000 M_r.

The recovery of the normal pattern of protein synthesis was accelerated when cells that had initiated the heat shock response during 30 min at 45°C were shifted down to 30°C; within the first 30 min after temperature shiftdown, the rate of heat shock protein synthesis diminished and normal protein synthesis began to increase (Fig. 3B). The protein synthesis pattern returned to normal during the second 30 min after shiftdown.

This response to lowered temperature is slow, however, in comparison with the rapid initial response to temperature shiftup. Spores incubated at 30°C for 2 h and shifted to 45°C were labeled with [^{35}S]methionine for 5-min intervals begin-

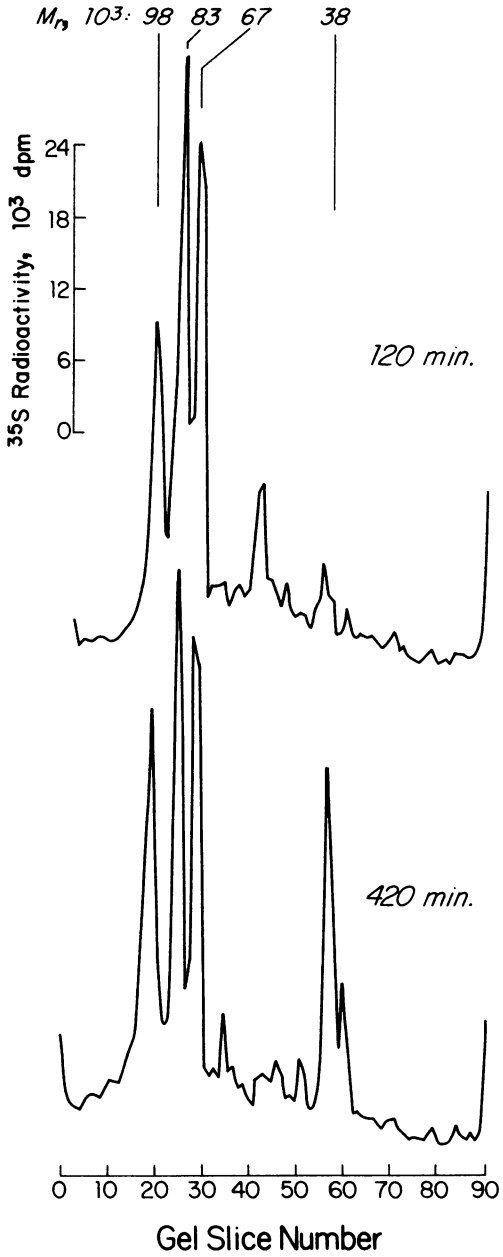


FIG. 2. Electrophoretic pattern of [^{35}S]methionine-labeled heat shock proteins synthesized by cells of two ages. Spores were incubated at 30°C for 2 h (top) or for 7 h (bottom) before being transferred to 45°C. Cultures were radiolabeled between 30 and 60 min after the shift to 45°C.

ning immediately after the shift to high temperature. The synthesis of heat shock proteins was evident within the first 5 min after the shift, and they increased dramatically in the subsequent 5-min intervals of the first 20 min of exposure to heat shock (Fig. 4). Normal protein synthesis began to decline only after 10 min of incubation at 45°C (Fig. 4), which indicates that although both responses are rapid, there is not an obligatory coupling between the initiation of heat shock protein synthesis and the decline of normal protein synthesis.

The three dominant heat shock proteins showed different kinetics of synthesis in the labeling experiments. The 98,000-M_r protein was most highly expressed early in the heat shock response, and its synthesis declined sooner than that of the other proteins (Fig. 3 and 4). Synthesis of the 67,000-M_r heat shock protein is uniquely resistant to temperature shiftdown, and it continued to be synthesized at its previous level for 30 min after the cells were shifted from 45 to 30°C, when the synthesis of other heat shock proteins had decreased (Fig. 3B). The synthesis of this protein also appeared to be less diminished during cellular recovery at 45°C than that of the other two proteins.

We compared the patterns and quantities of proteins synthesized during 30 to 60 min of spore incubation at different temperatures (Fig. 5) and found that whereas the 35°C pattern was indistinguishable from the 30°C pattern, at 40°C there was a low level of heat shock protein synthesis. Between 40 and 44°C, there was a progressive increase in heat shock protein synthesis and a decline in the synthesis of normal proteins. The expression of heat shock proteins was maximal in *N. crassa* at 45°C, and normal protein synthesis was severely reduced at this temperature. At higher temperatures, no normal protein synthesis was observed; heat shock protein synthesis was also reduced, relative to the 45°C pattern, by about 60% at 46°C and by more than 95% at 47°C, based on the incorporation of radiolabel into the three proteins. No heat shock protein synthesis was observable at 48°C. At all of these temperatures, a surplus of unincorporated [^{35}S]methionine was found in the spores at the end of the labeling interval. Proteins synthesized during 90 to 120 min of exposure to temperatures above 45°C were also examined (data not shown). We found that cells exposed to 46°C, like those incubated at 45°C, begin recovering their normal pattern of protein synthesis after 90 min at high temperature. In contrast, cells exposed to 47°C increased their synthesis of heat shock proteins in the 90- to 120-min

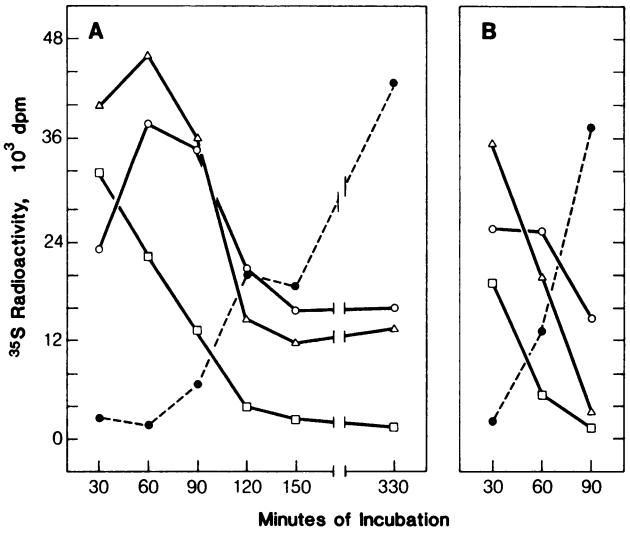
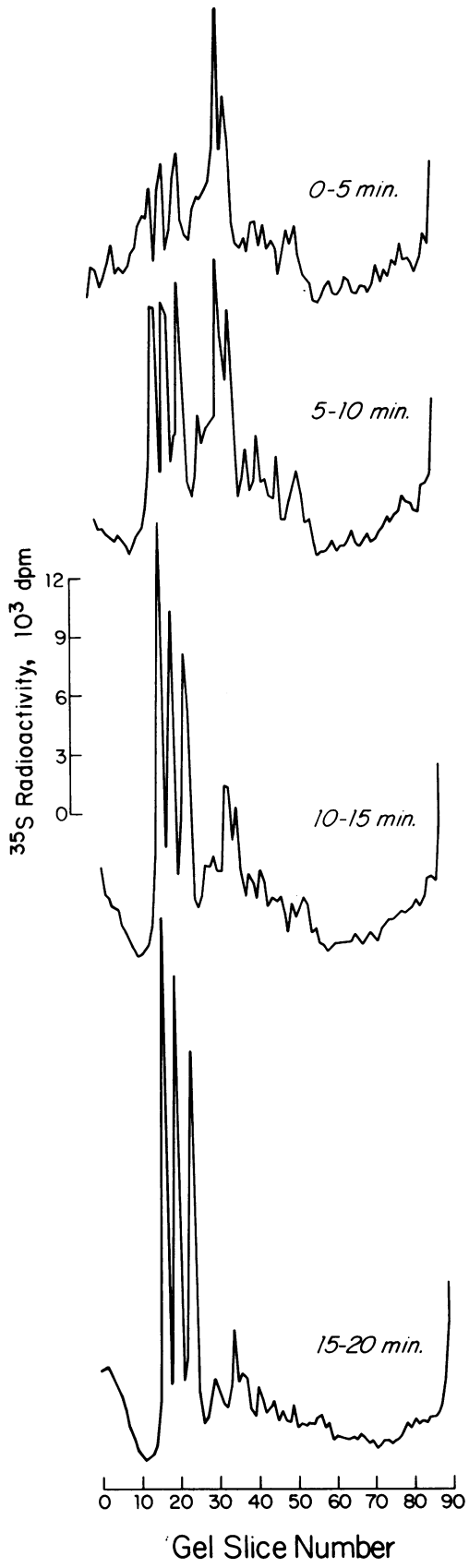


FIG. 3. Kinetics of synthesis of three heat shock proteins and one normal protein (immunologically identified as actin) during continuous spore incubation at 45°C (A) and during 30 min of incubation at 45°C and subsequent shiftdown to 30°C (B). Spores were radiolabeled with [^{35}S]methionine for consecutive 30-min intervals ending at the times indicated. Extracted proteins were separated electrophoretically in SDS-polyacrylamide gels that were sliced and counted. The radioactivity within individual peaks was integrated and plotted for each labeling interval. Synthesis of the 98,000-M_r heat shock protein (□), the 83,000-M_r heat shock protein (△), and the 67,000-M_r heat shock protein (○) decreases, whereas actin (●) production increases.



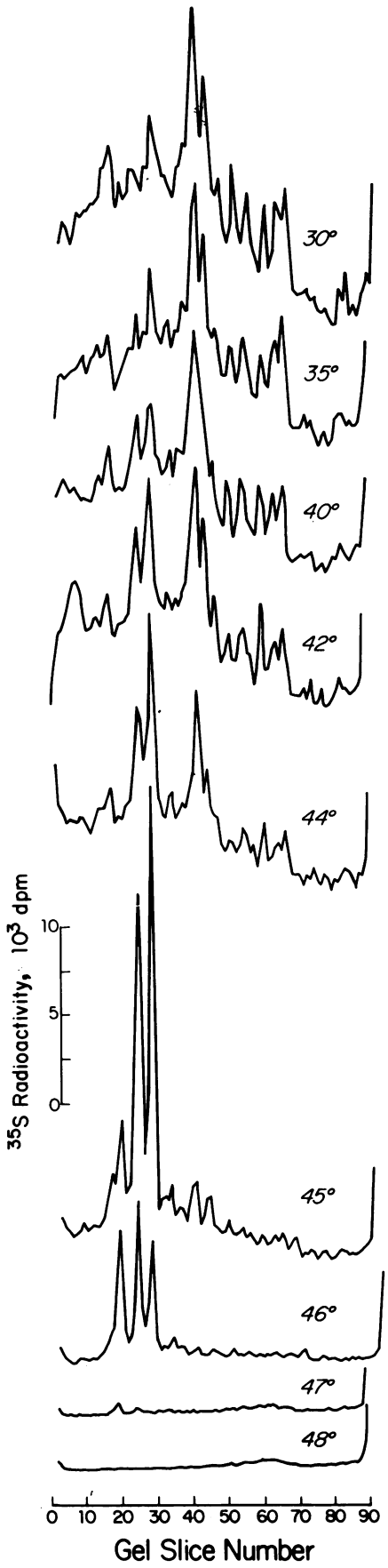
interval of exposure from the low level synthesized in the 30- to 60-min period.

The temperature optimum for synthesis of the major heat shock proteins, as a group, is 45°C. However, the synthesis of individual heat shock proteins between 30 and 60 min of exposure is affected differently by temperature variation. The 98,000-M_r protein was expressed in relatively low amounts at 45°C and below; more of this protein was synthesized at 46°C during this time interval, and at 47°C it became the dominant heat shock protein (as seen more clearly when the ordinate scale of Fig. 5 is expanded [data not shown]). The 98,000-M_r protein, therefore, appears to be maximally induced by temperatures higher than those which induce the other two major heat shock proteins. Even when its initial induction was high, however, the synthesis of this protein decreased rapidly during continued cellular exposure to high temperatures. Temperatures above 45°C diminished the synthesis of the 67,000-M_r heat shock protein more than that of the other two proteins.

We wished to determine whether, in addition to the repression of normal cytoplasmic protein synthesis, protein synthesis by *N. crassa* mitochondria is disrupted during heat shock. Therefore, we measured the synthesis of an enzyme complex, cytochrome *c* oxidase, that is composed of three subunits synthesized on mitochondrial ribosomes and four subunits that are encoded in nuclear DNA and synthesized on cytoplasmic ribosomes (8, 40). Spores that had germinated for 4 h at 30°C were transferred to 45°C, pulsed with [^{35}S]methionine at 15 min after the temperature shift, chased at 30 min with unlabeled methionine, and harvested at 45 min; other 4-h cells were kept at 30°C and radiolabeled similarly. The mitochondrial fraction from these two groups of cells was isolated, and a portion was reacted with antibody to the dissociated subunits of cytochrome *c* oxidase (8); subunits of the precipitated enzyme were separated by electrophoresis in SDS-polyacrylamide gels. During incubation at 45°C, the spores synthesized both mitochondrial and cytoplasmic subunits of cytochrome *c* oxidase at severely depressed levels; they incorporated into these peptides only 3% of the radioactive methionine that was incorporated by the spores kept at 30°C. These results indicate that during heat shock there is a severe depression of normal mitochondrial protein synthesis that parallels the depression of normal cytoplasmic protein synthesis.

Certain heat shock proteins appear to be associated with *N. crassa* mitochondria. Cells incubated at 30°C for 7 h were transferred to 45°C and labeled with [^{3}H]leucine between 15 and 30 min of exposure, as described above, or they were radiolabeled at 30°C. The isolated mitochondrial fractions were purified by centrifugation through sucrose gradients, solubilized in SDS, and subjected to electrophoresis. The mitochondria from cells labeled at 45°C were enriched in three proteins (Fig. 6B) that comigrate with the three minor heat shock proteins of 38,000, 34,000, and 30,000 M_r that are especially evident in whole cell extracts of 7-h cells (Fig. 6A); these proteins are not present in the mitochondria from cells labeled during growth at 30°C (Fig. 6C).

FIG. 4. Electrophoretic pattern of [^{35}S]methionine-labeled proteins synthesized during the first 20 min of heat shock, showing an increase in the synthesis of heat shock proteins and a decrease in normal protein synthesis. Spores were incubated for 2 h at 30°C before being transferred to 45°C. They were radiolabeled during consecutive 5-min intervals, beginning immediately after temperature shift.



When *N. crassa* spores were suspended directly in nutrient medium at 45°C, they synthesized the major heat shock proteins within 30 min (Fig. 3); however, they did not incorporate detectable [^{14}C]uracil into RNA until 60 min of incubation at this temperature. Heat shock protein synthesis also occurs under conditions that do not support germination. Spores suspended in distilled water, a medium in which they do not form germ tubes, strongly synthesized the three dominant heat shock proteins during 30 to 60 min of incubation at high temperatures (Fig. 1). Nevertheless, they incorporated no detectable [^{14}C]uracil into RNA throughout 180 min of incubation at 45°C. This suggests that spores that are initiating germination do not depend on new transcription for the synthesis of heat shock proteins and may be utilizing preserved mRNA (7) for translation into these proteins under the stimulus of a heat shock. Labeled precursor uptake experiments showed that a surplus of labeled uracil was present in the water-incubated spores, thus reducing the likelihood that precursor uptake was limiting the incorporation of radiolabel.

DISCUSSION

In response to exposure to high temperatures, *N. crassa* displays a dramatically altered pattern of protein synthesis. At 45°C three dominant high-molecular-weight proteins of 98,000, 83,000, and 67,000 *M*, are synthesized by germinating conidiospores and by postemergent, mycelial cells. These proteins are similar in size to evolutionarily conserved heat shock proteins of ca. 70,000 and 80,000 to 90,000 M_r that are synthesized by *Drosophila* (2), HeLa (21, 47), chicken (19), and yeast cells (13, 17) as well as many other organisms. In addition, postemergent *N. crassa* cells produce a fourth major heat shock protein of 38,000 M_r and a minor heat shock protein of 34,000 M_r. Another minor heat shock protein of 30,000 M_r is produced both by germinating conidiospores and by postemergent cells. Low-molecular-weight heat shock proteins are a common component of the heat shock response in other organisms. However, conservation among these proteins has not been noted, and antibody to the 24,000-M_r heat shock protein of chicken cells did not cross-react with similar heat shock proteins in other species, unlike antibodies to the two high-molecular-weight heat shock proteins (19). This synthesis of heat shock proteins in *N. crassa* is accompanied by severely depressed synthesis of normal cellular proteins. Kapoor (18) has reported that 12-h mycelial cells of *N. crassa* exposed to elevated temperatures synthesize five major heat shock proteins, three of which were intensely radiolabeled with [^{35}S]methionine; Grange et al. (14) also have observed that heat-treated conidiospores synthesize three heat shock proteins.

The response of *N. crassa* cells to high temperature is transient; after 60 min of exposure to 45°C, the synthesis of normal proteins resumes and heat shock protein synthesis decreases. Other microorganisms, including *S. cerevisiae* and *Escherichia coli*, also have a transient biosynthetic response to high temperatures, with normal protein synthe-

FIG. 5. Comparison of the electrophoretic patterns of proteins synthesized by spores incubated at different temperatures ranging from 30° to 48°C, showing a gradual increase in the synthesis of heat shock proteins and a decrease in the synthesis of normal proteins as the temperature of incubation is increased. Spores were radiolabeled with [^{35}S]methionine between 30 and 60 min after culture inoculation at the temperatures indicated.

sis restored by 120 min (28, 31, 33, 35) and 30 min (48) of exposure, respectively. However, the transiency of this response in yeast cells apparently depends on the availability of a fermentable carbon source, such as glucose. *S. cerevisiae* grown on the nonfermentable substrate acetate continued to synthesize heat shock proteins indefinitely at the high temperature (28). Michéa-Hamzehpour et al. (34) have reported that heat-treated *N. crassa* conidia utilize an alternate pathway of respiration that is inhibited by salicylhydroxamic acid but not by cyanide (23), and we have confirmed this in our laboratory (data not shown). It is possible that the ability of *N. crassa* to switch from the normal cyanide-sensitive pathway of respiration to the alternate, noncytochrome-mediated pathway is related to the transiency of its heat shock response. Despite their recovery of the normal pattern of protein synthesis, activated *N. crassa* conidia, unlike glucose-grown yeast cells, cannot develop normally at high temperatures; they are merely able to sustain a slow growth that is restricted to germ tube emergence. Development resumes when spore cultures are transferred to normal growth temperatures.

The temperatures that induce the synthesis of heat shock proteins in *N. crassa* range from 40 to 47°C, but the response is maximal at 45°C. Spores synthesize low amounts of heat shock proteins at 40°C despite their normal rate of germination at that temperature. A variation of only 1°C produces a large difference in the amount of proteins synthesized and in the pattern of synthesis. This difference in synthesis is not reflected, however, in the level of cell survival at different temperatures. Heat shock protein synthesis at 46°C is only ca. 40% the level at 45°C, but cell survival is the same at the two temperatures (92 and 91% of the control, respectively). Cell survival at 47°C is only slightly lower (81% of the control), despite a rate of heat shock protein synthesis that is less than 5% of that at 45°C; however, heat shock protein synthesis continues for a longer period of time at 47°C, rather than declining after 60 min of exposure. There is a correlation between the sharp drop in cell survival at 48°C and the lack of observable heat shock protein synthesis at that temperature. These results suggest that even low levels of heat shock proteins may be sufficient to contribute to the thermal resistance of cells.

Resistance to damage from exposure to 50°C was conferred on *N. crassa* cells by prior incubation at 45°C, but this protective effect was abolished when protein synthesis was inhibited by cycloheximide, a drug which we directly determined to block the incorporation of labeled amino acids into the heat shock proteins. This indicates that at least some of the proteins synthesized during heat shock are required to protect cells against thermal injury. Thermotolerance frequently has been found to develop in concert with heat shock protein synthesis in, for example, Chinese hamster fibroblasts (24), cultured *Drosophila* cells and tissues (36), and *S. cerevisiae* (32). Furthermore, preexposure to a mild heat shock extended the upper temperature limit at which *Drosophila* cells and larvae could synthesize heat shock proteins (26, 38). It has also been demonstrated that inhibitors of protein synthesis administered to yeast cells during a mild heat shock interfered with the development of tolerance for still higher temperatures (32). Furthermore, this requirement for protein synthesis is specific for proteins encoded in mRNA induced during heat shock, since a temperature-sensitive RNA transport mutant of yeast cells subjected to a mild heat shock required a shift to the permissive temperature before developing thermotolerance (32).

In addition to protecting cells from thermal damage, some

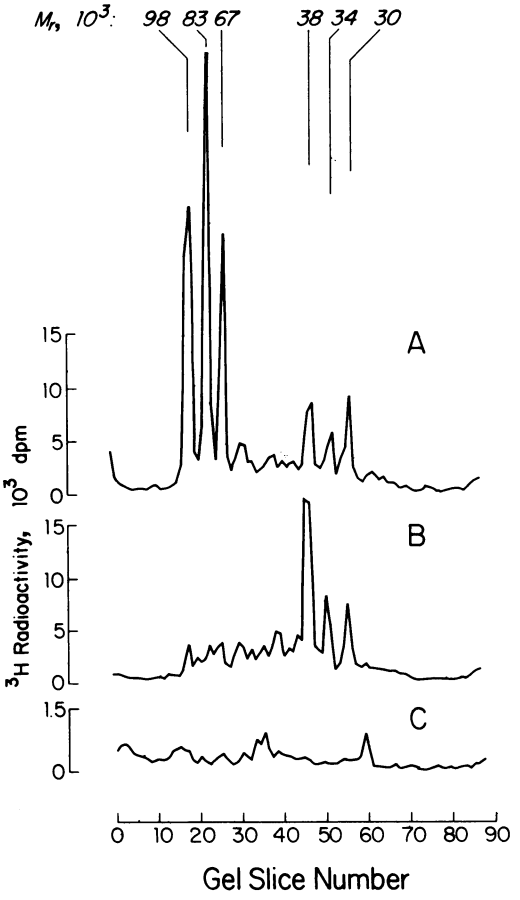


FIG. 6. Radiolabeled proteins from purified mitochondria of 7-h cells that were shifted to 45°C (B) or kept at 30°C (C). Cells were labeled with [^{3}H]leucine at 15 min after temperature shift (or after 7 h and 15 min of growth), chased with unlabeled leucine at 30 min, and harvested at 45 min. The proteins were separated by electrophoresis in SDS–10% polyacrylamide gels. The electrophoretic pattern of whole cell extracts from 7-h cells that were shifted to 45°C (A) is shown for comparison, with the relative molecular weights of the six heat shock proteins designated. The cells were radiolabeled with [^{3}H]leucine during 30 to 60 min after the shift to 45°C.

of these proteins may have a regulatory role in gene expression. Whereas protein synthesis in *Drosophila* (12) and yeast (31) cells is not required for the changed pattern of transcription during the initiation of the heat shock response, heat shock protein synthesis is required for the resumption of normal gene expression by these cells during recovery. When protein synthesis has been blocked or protein function has been interrupted in heat-shocked *Drosophila* cells by cycloheximide or amino acid analogs, respectively, there is a prolonged synthesis of heat shock mRNA and proteins after the shiftdown to normal temperature (12). DiDomenico et al. have suggested (11), on the basis of the kinetics of heat shock protein synthesis in *Drosophila* cells, that the 70,000-M_r heat shock protein may play an important role in the negative regulation of heat shock gene expression during recovery while positively regulating the expression of normal cellular genes. This is supported by the discovery that *E. coli* cells that are mutant in the *dnaK* gene, which encodes a heat shock protein that is 48% homologous with the 70,000-M_r heat shock protein of *Drosophila* cells (5), fail to recover their normal pattern of protein synthesis at high tempera-

tures (43). The accumulation of one or more heat shock proteins in *N. crassa* cells may be required for the recovery that occurs in this organism at both high and low temperatures, and this requirement may account for the prolonged synthesis of heat shock proteins that occurs at 47°C, a temperature at which they are synthesized at lower rates.

The localization of heat shock proteins within the cell may eventually help to reveal their functions. The 70,000-M_r heat shock proteins of *Drosophila* (1, 45) and mammalian (47) cells have been found in nuclei as well as in the cytoplasms, and their associations with nucleoli (1, 37, 47) and with heterogenous RNA (20) during heat shock have been reported. Several low-molecular-weight heat shock proteins (22,000 to 32,000 M_r) of *Dictyostelium* (29) and *Drosophila* (1) cells are also localized in nuclei during heat shock. The heat shock protein of 80,000 to 90,000 M_r appears to be restricted to a cytoplasmic location in these organisms (1, 21, 29). Studies of heat shock protein localization in *Drosophila* cells indicated that very little radiolabeled protein was associated with mitochondria (1, 44). We have found, however, that in *N. crassa* three heat shock proteins of low molecular weight are associated with mitochondria, and minor heat shock proteins have also been reported to be associated with the mitochondria of soybean seedlings (25).

Although the altered pattern of protein synthesis displayed by heat-shocked cells is apparently caused by a parallel change in gene transcription in most organisms, posttranscriptional regulation has also been found to be responsible for suppressing normal mRNA expression during the heat shock response (16, 27, 31, 35, 41) and for blocking the expression of heat shock mRNAs during recovery (11). Posttranscriptional mechanisms may also regulate the induction of heat shock protein synthesis in certain cases. *Xenopus laevis* oocytes appeared to synthesize a heat shock protein from preserved mRNA, since synthesis occurred in enucleated oocytes and in oocytes whose transcription was inhibited by α-amanitin (6). It is possible that dormant conidiospores of *N. crassa* similarly contain mRNAs for heat shock proteins that are selectively activated for translation early in germination at high temperatures. Spores that were incubated in water at 45°C, conditions under which they do not incorporate radiolabeled precursor into RNA, nevertheless strongly synthesized the three dominant heat shock proteins. We have recently found that the RNA from dormant spores contains sequences that hybridize with a cloned cDNA that we have isolated for one of the major heat shock proteins of *N. crassa* (unpublished data). We intend to quantify this mRNA and to examine the basis for its normal exclusion from translation or its selective recruitment for translation at high temperatures. It is likely that this implied posttranscriptional mechanism for the induction of heat shock protein synthesis applies only to germinating spores that would be unable to transcribe new mRNA rapidly.

We do not yet know the fate of normal mRNAs of *N. crassa* when mRNAs for heat shock proteins are being exclusively translated. They may be preserved during the heat shock response for expression later during recovery, as in *Drosophila* cells, or they may be rapidly destroyed, as occurs with *S. cerevisiae*. It has been reported that mitochondrial RNAs continue to be synthesized by *Drosophila* cells during heat shock (39). We found here, however, that *N. crassa* mitochondria do not synthesize normal proteins during heat shock. If mitochondrial RNAs are also synthesized by *N. crassa* during exposure to elevated temperatures, their lack of translation into protein would implicate translational control of mitochondrial gene expression during heat shock. Analyses of the metabolism of specific mRNAs for both heat shock and normal proteins will help us evaluate the contribution of posttranscriptional regulation to the heat shock response in *N. crassa* and to discriminate between such possible mechanisms of regulation as accelerated degradation and selective inactivation of mRNA.

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