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Two Developmental Stages of *Neurospora crassa* Utilize Similar Mechanisms for Responding to Heat Shock but Contrasting Mechanisms for Recovery

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At the heat shock temperature of 45°C, there is a transient induction of the synthesis of heat shock proteins and repression of normal protein synthesis in cells of *Neurospora crassa*. Both conidiospores and mycelial cells resume normal protein synthesis after 60 min at high temperature. At the RNA level, however, these two developmental stages responded with different kinetics to elevated temperature. Heat shock RNAs (for hsp30 and hsp83) accumulated and declined more rapidly in spores than in mycelia, and during recovery spores accumulated mRNA that encoded a normal protein (the proteolipid subunit of the mitochondrial ATPase), whereas mycelia showed no increase in this normal RNA (for at least 120 min). Therefore, the resumption of normal protein synthesis in spores may depend upon accumulation of new mRNAs. In contrast, mycelial cells appeared to change their translational preference during continued incubation at elevated temperature, from a discrimination against normal mRNAs to a resumption of their translation into normal cellular proteins, exemplified by the ATPase proteolipid subunit whose synthesis was measured in the heat-shocked cells.

Exposure of cells to supraoptimal temperatures dramatically alters the patterns of gene expression in all organisms examined (1, 30, 32, 35). During exposure to high temperature, a distinctive group of proteins, the heat shock proteins, are synthesized from mRNAs that are usually newly transcribed in response to heat shock. At the same time, the synthesis of normal cellular proteins is sharply curtailed because of repressed transcription of their genes and due to posttranscriptional mechanisms, such as degradation (25) or inactivation of preexisting mRNAs (15, 22, 39).

During studies of germinating *Neurospora crassa* conidiospores, or asexual spores, we observed that the growth of this organism was arrested by exposure to 45°C and that growth resumed only after the cultures were shifted down to 30°C (the normal incubation temperature). We found that at 45°C *N. crassa* synthesizes large amounts of six heat shock proteins (with M_rs of 98,000, 83,000, 67,000, 38,000, 34,000, and 30,000) and normal protein synthesis is repressed (31). This changed pattern of synthesis occurs both in activated spores and in growing mycelial cells, and in both types of cells it persists only for the first 60 min of exposure to high temperature (31). During continued incubation at 45°C, *N. crassa* resumes the synthesis of normal cellular proteins and discontinues heat shock protein synthesis. A similarly transient response to high temperature has been documented for other organisms including *Saccharomyces cerevisiae* (26, 28), but these organisms adapt physiologically to the inducing temperature, at which they resume growth. In contrast, the temperatures that strongly induce synthesis of heat shock proteins in *Drosophila melanogaster* do not support growth in that organism (29), nor do they allow a return to normal protein synthesis (39) such as occurs in *N. crassa*. *N. crassa*, therefore, exhibits some features of both these two general patterns of response to heat shock. After initial repression, this mycelial fungus can resume normal protein synthesis at a heat shock temperature, but it cannot grow or develop at that temperature.

The basis for this resumption of normal protein synthesis during prolonged heat shock is not yet known. We have asked in our study whether *N. crassa* cells might retain normal mRNAs, untranslated early in the heat shock response, or whether, as in *S. cerevisiae* (22, 25), RNA levels alone determine what proteins are synthesized. The possibility of translational regulation in *N. crassa* holds particular interest, since there are relatively few well-documented cases of translational control by eucaryotic cells, with a prominent example being the selective discrimination by *D. melanogaster* against translation of normal mRNAs during heat shock (22, 39).

In the present study we measured accumulation and decline of specific RNAs in cells exposed to heat shock by using a cloned cDNA for a normal cellular protein (the proteolipid subunit of mitochondrial ATPase) and cloned cDNAs, whose isolation we describe here, for two heat shock proteins of *N. crassa* (hsp83 and hsp30). We found that both activated conidiospores and mycelial cells synthesize new heat shock transcripts in response to high temperature, but these two developmental stages accumulate heat shock RNAs and, subsequently, a normal RNA with sharply different kinetics. In addition, mycelial cells apparently retain normal mRNAs that are inefficiently translated during the first 60 min of heat shock, and they resume normal protein synthesis by a change in translational efficiency. On the other hand, spores do not require such translational control, since their recovery at high temperature includes accumulation of new transcripts for normal proteins.

MATERIALS AND METHODS

Strain. Conidiospores of *N. crassa* 74-OR23-1A (Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City) were produced and harvested under nonactivating conditions (4).

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RNA purification. *N. crassa* cells were disrupted (5) in 8 M guanidine hydrochloride, RNA was isolated by repeated precipitations from 6 M guanidine hydrochloride with a 0.5 volume of ethanol, and solubilization with Tris buffer (7, 8, 10) followed. For lipid-rich spore material, the supernatant was extracted with water-saturated chloroform-butanol (4:1 [vol/vol]) before precipitation. The RNA was stored at −80°C or was fractionated by oligo(dT) cellulose chromatography (2).

Preparation of cDNA library. Poly(A)$^+$ RNA isolated from mycelial cells that had been exposed to 45°C for 45 min was used to synthesize cDNA. The procedures of Gubler and Hoffman (14) were used for first-strand cDNA synthesis with AMV reverse transcriptase (Life Sciences, Inc.), to degrade poly(A)$^+$ RNA with *Escherichia coli* RNase H (Bethesda Research Laboratories, Inc.), and for second-strand cDNA synthesis with DNA polymerase I (Boehringer Mannheim Biochemicals). The double-stranded cDNA was treated (23) with S1 nuclease (Sigma Chemical Co.), tailed (11) with dCTP by terminal deoxynucleotidyl transferase (P-L Biochemicals, Inc.), and annealed (23) with dG-tailed, *Pst*I-cleaved pBR322 (Bethesda Research Laboratories) in a ratio of 1:25 (wt/wt, cDNA to plasmid). The hybrid plasmids were used to transform the DH1 strain of *E. coli* (provided by J. Messing), which was prepared for transformation by overnight incubation in calcium chloride (9). Transformed colonies (520 from 1.5 ng of cDNA) were selected by tetracycline resistance and ampicillin sensitivity.

Screening of cDNA library by differential hybridization. Ninety-five plasmids were isolated by the boiling method of Holmes and Quigly (16) and were screened by differential hybridization. The plasmids were denatured and applied (21) to nitrocellulose filters (BA85; Schleicher & Schuell, Inc.) in duplicate by means of an S&S Minifold apparatus. The filters, according to the directions of the vendor, were dried under vacuum and were prehybridized and hybridized at 42°C in 50% formamide to ^{32}P-labeled cDNA made either from poly(A)$^+$ RNA of *N. crassa* cells grown at 30°C or from poly(A)$^+$ RNA of cells heat shocked at 45°C for 45 min. The filters were washed under stringent conditions (6) and autoradiographed with intensifying screens (Cronex Lightning-Plus, Du Pont Co.) and X-Omat AR-5 film (Eastman Kodak Co.).

Screening of cDNAs by Northern blot hybridization. Twelve plasmids that displayed a clear difference in hybridization were isolated by the alkaline lysis procedure of Birnboim and Doly (3) and then purified through NACS mini-columns (Bethesda Research Laboratories), and the sizes of their inserts were determined by electrophoresis through a 1.5% agarose gel. Ten of the plasmids were individually labeled by nick translation (24) with [^{32}P]dCTP (3,500 Ci/mmol; New England Nuclear Corp.) for use as hybridization probes of Northern blots (38), containing poly(A)$^+$ RNA (1.6 μg) from cells grown at 30°C and from cells exposed to 45°C that had been separated electrophoretically in formaldehyde–1.3% agarose gels (33). Two plasmids were selected containing the largest inserts that hybridized preferentially to distinct 45°C mRNAs.

Quantification of the kinetics of RNA accumulation. Total RNA was extracted from approximately 1 g (wet weight) of cells after successive intervals of cell incubation at 45°C. For isolation of RNA from activated conidiospores, the spores were suspended in liquid nutrient medium (41) at a concentration of 10 mg/ml; at harvest the culture flasks were rapidly (within 2 min) cooled to 0°C by swirling them in granulated dry ice and the spores were collected by filtration at 4°C. For isolation of RNA from mycelial cultures, spores were suspended in 75 ml of liquid medium at an initial spore density of 1 mg/ml and allowed to grow for 7 h before experimental treatment. Cell cultures that were shifted to a 45°C water bath reached 44°C within 2 min and 45°C by 4 min after transfer. After the cellular material was homogenized in buffered 8 M guanidine hydrochloride, 5 μg of purified rabbit globin mRNA (Bethesda Research Laboratories) was added to the extract to allow later estimation of the efficiency of cellular RNA purification and the original cellular content of RNA.

By use of an S&S Minifold, 2.5 and 10 μg of each RNA preparation in 15× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) were applied under light vacuum to nitrocellulose filters (13) immediately after the binding buffer was drawn through each well to enhance uniformity of RNA binding (36). Each filter was dried, prehybridized, and hybridized to 500 ng of plasmid that had been nick translated with [^{32}P]dCTP (7,000 Ci/mmol; Amersham Corp.). Nicked pBR322 was added to the hybridization solution (2 to 2.5 ml) at a concentration of 25 μg/ml. After three 15-min washes at room temperature in 2 × SSC–0.2% sodium dodecyl sulfate (SDS), the filters were washed four times at 55°C with 0.1 × SSC–0.2% SDS. To measure proteolipid RNA levels in heat-shocked spores, we increased the sensitivity of detection by applying 10 and 20 μg of RNA to a filter and used as hybridization probe the isolated cDNA insert (40 ng) that had been radiolabeled to a high specific activity.

Radiolabeling and immunoprecipitation of the proteolipid subunit of the mitochondrial ATPase from cellular and mitochondrial extracts. For preparation of cellular extracts, individual flasks of nutrient medium containing 30 mg of *N. crassa* spores (1 mg/ml) were incubated at 30°C for 7 h, after which they were transferred to 45°C and radiolabeled with 75 μCi (2.5 μCi/ml) of [^{35}S]methionine (1,185 Ci/mmol; Amersham Corp.) for consecutive 30-min intervals. The radiolabeled methionine was chased with 1 μmol of unlabeled methionine immediately before the cells were collected by filtration. The cells were disrupted (5) in 3 ml of cold 0.1 M sodium phosphate (pH 8.0), and cellular debris was separated by centrifugation at 1,500 × *g* for 5 min. For mitochondrial isolation, 300 mg of spores was treated as described for cellular extracts, except that they were radiolabeled every other 30-min interval with 300 μCi (1 μCi/ml) of [^{35}S]methionine, chased with 5 μmol of unlabeled methionine, and homogenized in 10 ml of cold STE buffer (0.25 M sucrose, 1 mM EDTA, 50 mM Tris [pH 7.5]). The mitochondria, isolated by differential centrifugation (5), were suspended in 0.1 M sodium phosphate (pH 8.0) buffer.

A sample (7 mg of cellular protein or 1 mg of mitochondrial protein) in 1% Triton X-100 was reacted with 100 μl of proteolipid antiserum (42) and 50 μl of hydrated protein A-Sepharose (Pharmacia Inc.). After washes with 2% Triton X-100 and phosphate buffer, proteins that were bound to protein A-Sepharose were solubilized in electrophoresis sample buffer (20) and were separated by electrophoresis at 100 V through cylindrical SDS–12.5% polyacrylamide gels; cytochrome *c* was used as a tracking dye. The gels were frozen and sliced into 1-mm slices which, after solubilization (43), were counted by liquid scintillation spectrometry.

RESULTS

Selection of cloned cDNAs. We made a cDNA library from poly(A)$^+$ RNA isolated from heat-shocked mycelial cells to develop specific probes for mRNAs encoding heat shock

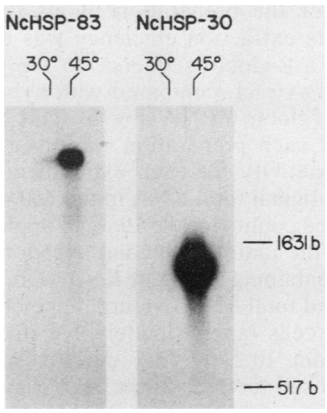

FIG. 1. Differential hybridization of two cloned cDNAs to distinct mRNAs from heat-shocked cells versus non-heat-shocked cells. A Northern blot of poly(A)$^+$ RNA (1.6 μg) from mycelial cells either kept at 30°C or transferred to 45°C for 45 min was probed with nick-translated plasmids containing *N. crassa* cDNA inserts. The activity of pNcHsp-83 was 6×10^7 dpm/250 ng, and hybridization was in 3.6 ml; pNcHsp-30 had 9×10^7 dpm/250 ng, and hybridization was in 6.5 ml. The XAR-5 film was exposed with two intensifying screens at −80°C for 20 h. Restriction fragments from pBR322 digested with *Hin*fI were the molecular weight markers. b, Bases.

proteins of *N. crassa*. The library was initially screened (95 clones from 520) by hybridization to cDNA prepared from heat shock (45°C) mRNA in preference to cDNA prepared from normal (30°C) mRNA. Ten plasmids that displayed strong differential hybridization were then radiolabeled and screened by hybridization to Northern blots of poly(A)$^+$ RNA from cells incubated at 45 and 30°C. Two of the plasmids were complementary to an mRNA of approximately 3,400 bases that was expressed at both temperatures but that was strongly induced by heat shock (Fig. 1). One of these plasmids (pNcHsp-83), which contains a 930-base insert, was shown by in vitro translation of hybrid-selected RNA to encode a part of the 83,000-M_r heat shock protein of *N. crassa* (Fig. 2). The insert from this plasmid hybridized under nonstringent conditions (data not shown) with the hsp83 gene (pUTX17) of *S. cerevisiae* (12) but did not

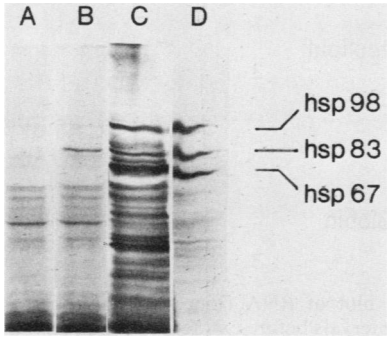

FIG. 2. Translation product of RNA that was hybrid selected by plasmid pNcHsp-83. The translation products from a reticulocyte lysate to which plasmid-selected RNA was added (B) and the products without added RNA (A) are shown. Proteins were radiolabeled with 50 μCi of [^{35}S]methionine. Also shown are the total translation products of poly(A)$^+$ RNA (0.5 μg) (C) and the in vivo synthesis pattern of heat-shocked cells (D); the three high-molecular-weight heat shock proteins are indicated. The proteins were separated in an SDS–10% polyacrylamide slab gel and autoradiographed for 19.5 h.

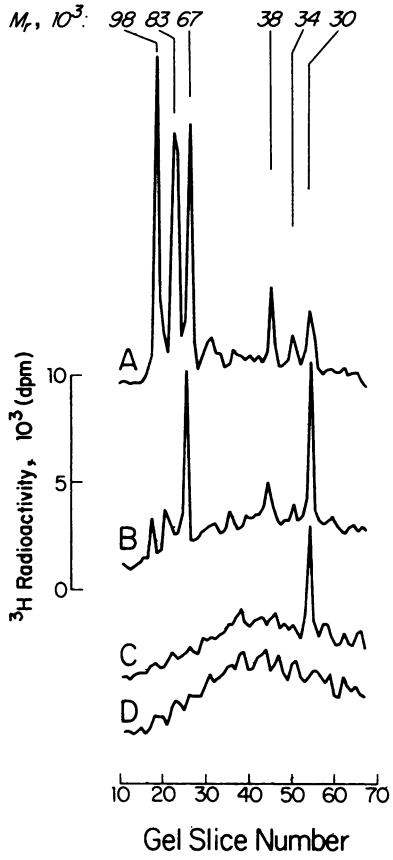

FIG. 3. Translation product of RNA that was hybrid selected by plasmid pNcHsp-30. The translation products from a reticulocyte lysate to which plasmid-selected RNA was added (C) and the products when no RNA was added (D) are shown. Proteins were radiolabeled with 3.3 μCi of [^{3}H]leucine. Also shown are the total translation products of poly(A)$^+$ RNA (B) and the in vivo protein synthesis pattern of heat-shocked mycelial cells (A). The prominent heat shock proteins of mycelial cells are indicated. The proteins were separated by SDS electrophoresis in cylindrical 10% polyacrylamide gels, and the tracking dye bromophenol blue migrated 90 mm.

cross-hybridize with the hsp70 gene (B8) of *D. melanogaster* (17). Seven hybrid plasmids were complementary to an mRNA of 1,400 bases that was expressed only during heat shock (Fig. 1). The largest of these plasmids (pNcHsp-30), which contains a 1,230-base insert, was determined by hybrid selection and in vitro translation to encode the 30,000-M_r heat shock protein of *N. crassa* (Fig. 3). The heat shock proteins encoded by these cloned cDNAs, pNcHsp-83 and pNcHsp-30, are synthesized during heat shock by both conidiospores and mycelial cells.

Measurement of specific RNA content of cells. RNA was extracted from dormant conidiospores of *N. crassa* and from spores incubated at 45°C for 5, 15, 30, and 45 min. RNA was also isolated from mycelial cultures (grown 7 h at 30°C) before transfer and at 5, 15, 30, 60, and 90 min after transfer to a 45°C water bath. Rabbit globin mRNA was added (5 μg) to each cellular extract. Equal amounts of total RNA from each treatment were applied in replicate to nitrocellulose filters which were subsequently hybridized in parallel to four radiolabeled probes: pNcHsp-83 and pNcHsp-30, which encode heat shock sequences; AV48, which contains full-length cDNA for the proteolipid subunit of the mitochondrial ATPase-ATP synthase of *N. crassa* (40), a nuclear gene

TABLE 1. Quantity of specific sequences in extracted RNA

RNA source and no. of min at 45°C	Total RNA (mg)	Hybridized dpm for specified probe and amt (μg) of assayed RNA[a]					
		pNcHsp-83		pNcHsp-30		AV48	
		10	2.5	10	2.5	10	2.5
Dormant spore	12.57	1,281	461	1,019	419	133	38
5	12.36	14,241	3,455	20,342	5,171	291	106
15	12.69	31,494	8,482	49,021	12,909	198	66
30	11.58	32,181	9,017	43,367	11,624	260	57
45	10.20	16,891	4,419	16,935	4,200	587	174
Mycelium (7 h)	7.59	4,065	2,002	650	452	7,467	2,017
5	6.38	11,551	3,269	5,113	1,502	5,094	1,465
15	5.96	29,584	7,040	19,485	4,899	3,750	1,068
30	6.15	71,589	19,097	57,114	15,168	2,556	580
60	8.74	66,741	17,142	73,683	19,733	1,359	366
90	8.08	57,446	14,753	72,528	16,831	1,475	403

[a] Two concentrations (10 and 2.5 μg) of RNA from each cell preparation were applied to nitrocellulose filters and probed with nick-translated plasmids that contained cDNA sequences either for one of the heat shock proteins (pNcHsp-83 or pNcHsp-30) or for the ATPase proteolipid subunit (AV48). Background radioactivity has been subtracted for the values shown.

product; and pRB1.95, containing the 5′ portion and flanking sequences of the β1 gene for rabbit β-globin (19). The nitrocellulose filters were autoradiographed, and the areas containing bound RNA were cut out for counting by scintillation spectrometry. Background counts were subtracted, and hybridized radioactivity was found to increase linearly with the increase in RNA bound to the filter, a fourfold increase for each (Table 1) and a twofold increase for proteolipid spore RNA (Fig. 4). These experiments were performed two or more times, and the data presented are typical results.

To estimate the amount of specific mRNA sequences originally present in the cells, we determined the original RNA content of each preparation, expecting that the RNA content of *N. crassa* cells would change during heat shock. The total RNA content was determined by dividing the final yield of RNA for the preparation by its own extraction efficiency, and its extraction efficiency was determined by hybridization of a β-globin sequence to the globin mRNA added to the cell extract, compared with hybridization to a known amount of globin mRNA (Fig. 5). This estimated total RNA content of each preparation was used to adjust the hybridized radioactivity for each experimental probe. For example, if the original total RNA in the cell extract was 10 mg, the hybridized counts for the 10 μg of applied RNA were multiplied by 1; the radioactive counts from a cell preparation originally containing 12 mg of RNA were multiplied by 1.2. The estimated total RNA content ranged from 6.0 to 8.7 mg for mycelial cells (approximately 1 g [fresh weight] at harvest) and from 10.2 to 12.7 mg for spores (1 g at inoculation) exposed to 45°C (Table 1).

These normalized counts from RNA hybridization to a specific DNA probe do not indicate the actual number or amount of specific RNA molecules present in the cells; instead, they reflect the relative amounts of specific sequences present in cellular RNA from different sources, provided these RNAs were hybridized in parallel to the same radiolabeled probe.

Kinetics of RNA accumulation during heat shock. Quantification of the hybridized radioactivity indicated that mRNAs for hsp83 and hsp30 are induced together and to a similar extent in spores and mycelial cells after exposure to 45°C, but the kinetics of this induction are different in the two cell types. In mycelial cells (Fig. 6 and 7) the increase of both mRNAs began as soon as 5 min after transfer to 45°C

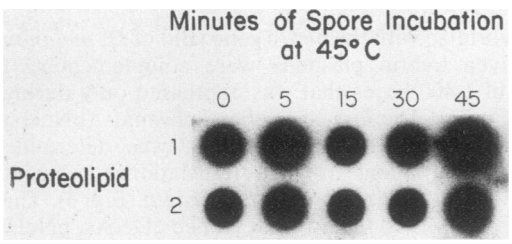

FIG. 4. Dot blot of RNA from spores incubated at 45°C for different time intervals before extraction. Cellular RNA (20 μg [row 1] and 10 μg [row 2]) was applied to a nitrocellulose filter and hybridized to the proteolipid cDNA insert that was radiolabeled to a high specific activity (4.0×10^7 dpm/40 ng). The filter was exposed to X-ray film for 24 h, and the hybridized radioactivity was quantified by scintillation spectrometry. The disintegrations per minute for RNA from dormant spores and from spores extracted after 5, 15, 30, and 45 min of incubation were (for 10 μg of RNA) 782, 1,806, 559, 790, and 2,910, respectively. The corresponding figures for 20 μg of RNA were 1,556, 3,448, 1,099, 1,788, and 6,061 dpm. When the two measurements for each time point were averaged and adjusted for total cellular RNA content, the resultant figures were 1,961 dpm for 20 μg of dormant spore RNA and 4,363, 1,407, 1,950, and 6,060 for RNA from spores incubated at 45°C for 5, 15, 30, and 45 min, respectively.

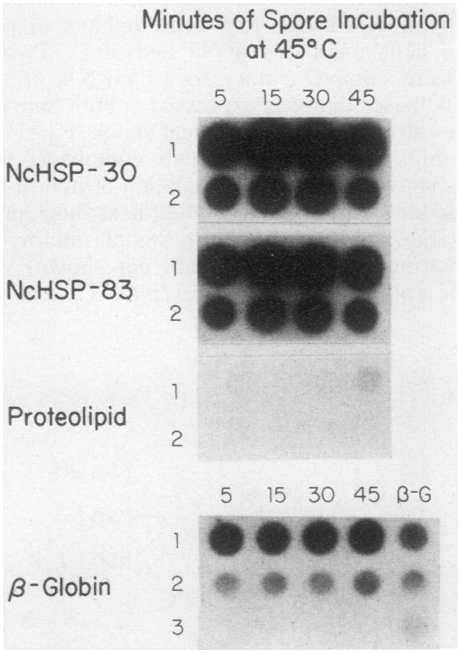

FIG. 5. Dot blot of RNA from spores incubated at 45°C for different time intervals before extraction. Cellular RNA (10 μg [row 1] and 2.5 μg [row 2]) was applied to the nitrocellulose filters. In the β-G lane of the β-globin filter, 1 ng (row 1) and 0.25 ng (row 2) of pure globin mRNA were applied, along with 2.5 μg of *E. coli* tRNA as carrier; 2.5 μg of tRNA alone was applied in row 3. The plasmids used as probes (500 ng) were nick translated to a radioactivity of 2.0×10^7 dpm (pNcHsp-83), 3.2×10^7 dpm (pNcHsp-30), 2.0×10^7 dpm (proteolipid AV48), and 3.2×10^7 dpm (globin pRB1.95). The blots were hybridized in 2 to 2.5 ml each and autoradiographed with two intensifying screens for 3.75 h (pNcHsp-83, pNcHsp-30, and pRB1.95) or 15 h (AV48).

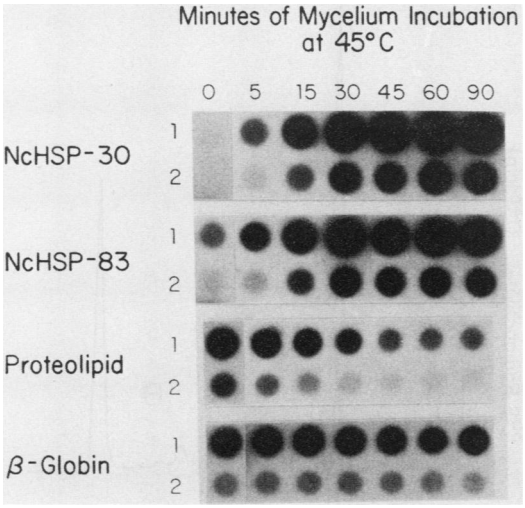

FIG. 6. Dot blots of RNA from mycelial cells exposed to 45°C for different time intervals before extraction. Cellular RNA (10 μg [row 1] and 2.5 μg [row 2]) was applied to the nitrocellulose filters. The hybridization probes (500 ng of each plasmid) were nick translated to a radioactivity of 3.5×10^7 dpm (pNcHsp-83), 2.1×10^7 dpm (pNcHsp-30), 2.0×10^7 dpm (proteolipid probe AV48), and 4.0×10^7 dpm (globin probe pRB1.95). Each blot was hybridized in 2 ml and was autoradiographed with two intensifying screens for 3.75 h (pNcHsp-83 and pNcHsp-30) or 15 h (AV48 and pRB1.95).

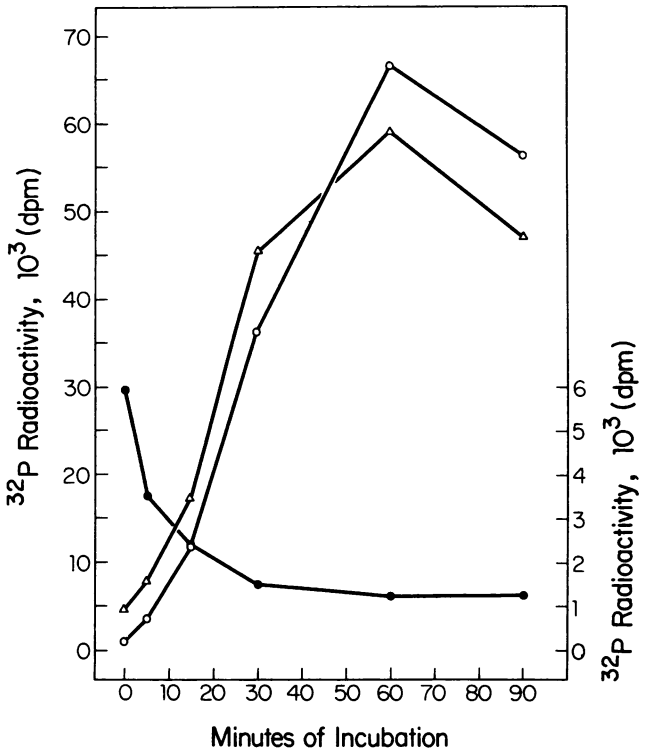

FIG. 7. Kinetics of RNA accumulation in mycelial cells exposed to 45°C. Curves represent hsp83 (△), hsp30 (○), and the proteolipid subunit of the mitochondrial ATPase (●). The scale on the right ordinate applies to the proteolipid. The radioactive counts from the dot blots of Fig. 6 were adjusted for total cellular RNA content and plotted.

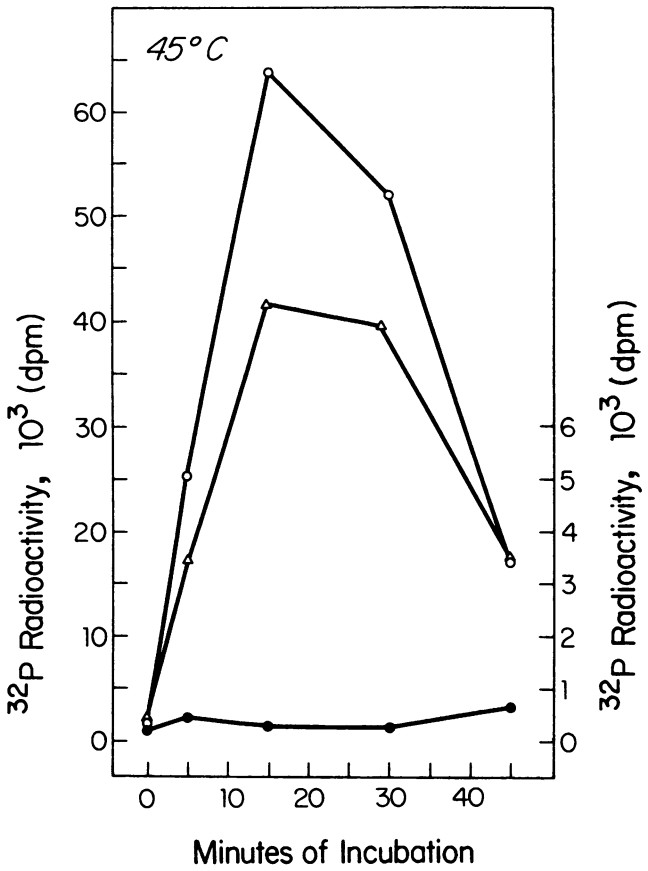

FIG. 8. Kinetics of RNA accumulation in ungerminated conidiospores exposed to 45°C during activation. Curves represent hsp83 (△), hsp30 (○), and the proteolipid subunit of the mitochondrial ATPase (●). The scale on the right ordinate applies to the proteolipid. The radioactive counts from the dot blots of Fig. 5 were adjusted for total cellular RNA content and plotted.

and the increase reached its maximum around 60 min of exposure to heat shock; by 90 min the level of these two heat shock-induced mRNAs began to decrease. Spores incubated at 45°C (Fig. 5 and 8) displayed a much more rapid response than did mycelial cultures. The expression of both heat shock mRNAs was strongly induced by 5 min and reached its maximum as soon as 15 min of exposure, and by 45 min the RNA levels had declined considerably.

There was a dramatic decrease in RNA for the proteolipid subunit of the mitochondrial ATPase after transfer of mycelial cells to 45°C. This decrease in a normal cellular mRNA was evident by 5 min of exposure, and the RNA reached its lowest point by about 60 min of cellular exposure to 45°C (Fig. 7), kinetics that are conversely similar to the induced expression of heat shock mRNAs. However, there was no observed increase by 90 min in the level of proteolipid mRNA to accompany the decrease in heat shock mRNAs at this time. Even by 120 min of heat treatment (data not shown), when heat shock RNA concentration had declined severely, there was no increase in the concentration of proteolipid sequences in the mycelial RNA above the concentration at 60 min of heat shock. In contrast, ungerminated spores, whose heat shock transcripts decreased substantially by 45 min, accumulated new proteolipid transcripts during recovery from heat shock. The

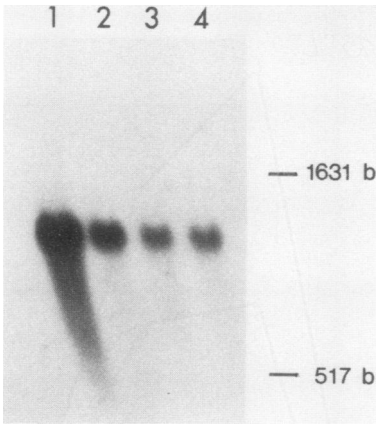

FIG. 9. Northern blot of ATPase proteolipid RNA. The RNA (20 μg) isolated from mycelial (7-h) cells before transfer (lane 1) to 45°C and 30 min (lane 2), 60 min (lane 3), and 90 min (lane 4) after heat shock was subjected to electrophoresis and blotted to nitrocellulose. The blot was hybridized in 12 ml to 250 ng of plasmid AV48 (proteolipid) that was nick translated to a radioactivity of 9.45×10^7 dpm. Film exposure was for 40 h with two intensifying screens. Molecular weight markers were *Hin*fI restriction fragments of pBR322. b, Bases.

proteolipid mRNA of these spores increased fourfold between 15 and 45 min of heat shock (Fig. 4 and 8).

Log phase mycelial cells contain a substantial amount of ATPase proteolipid RNA, and although it decreases dramatically during heat shock, it never disappears; at least 20% of the proteolipid mRNA originally present in the cells appeared to be stably retained or synthesized during heat shock (Fig. 7). We determined that the proteolipid mRNA was intact in mycelial cells during heat shock and was not partially degraded or present as an unprocessed precursor. The same cellular RNAs that were used in the dot blots (0, 30, 60, and 90 min at 45°C) were separated electrophoretically and hybridized, after blotting, with the radiolabeled ATPase proteolipid probe. Autoradiography of the Northern blot (Fig. 9) indicated that the proteolipid sequences in the RNA from heat-shocked mycelial cells are accounted for by intact, mature proteolipid mRNA.

Synthesis of ATPase proteolipid peptide during heat shock. These results indicate that there is no increase in a normal cellular RNA at a time during heat shock (90 min) when mycelial cells are found to resume synthesis of normal proteins (31). To determine specifically for the ATPase proteolipid peptide its recovery kinetics of synthesis, we immunoprecipitated this peptide subunit both from unfractionated cell extracts of mycelia and from mitochondrial fractions. We found that between 30 and 60 min of heat shock the synthesis of this subunit was reduced to 2% or less of its pre-heat shock level (Fig. 10). However, synthesis of the proteolipid subunit resumed by 60 to 90 min of heat shock, increasing with each subsequent 30-min radiolabeling interval up to 150 to 180 min of heat shock, when the rate of synthesis (at least briefly) reached a plateau (Fig. 10A). The level of incorporation of radiolabeled amino acids into total cellular proteins was similar during each of these labeling intervals (data not shown).

The extent of proteolipid accumulation by mitochondria appears to depend on the rate of peptide synthesis, rather than on differences in membrane translocation efficiency. The proteolipid subunit of the mitochondrial ATPase is synthesized in the cytoplasm as a peptide precursor with a

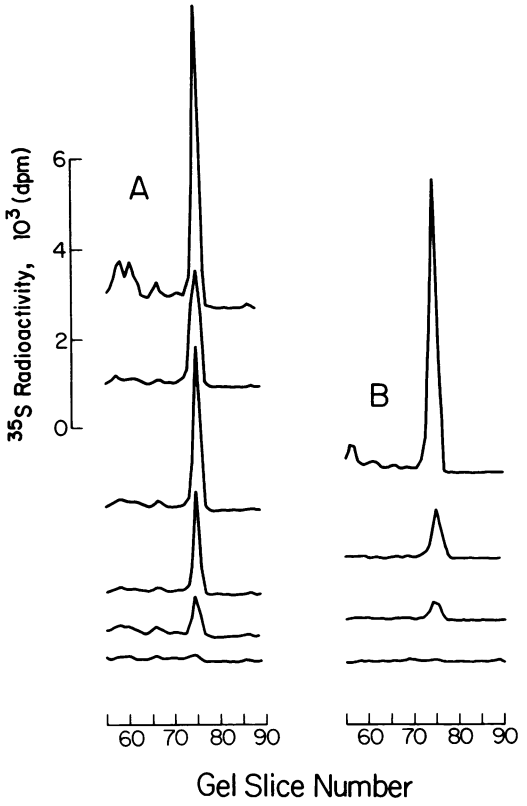

FIG. 10. Synthesis of the proteolipid subunit of mitochondrial ATPase during heat shock. Proteins immunoprecipitated by proteolipid antibody were separated electrophoretically in SDS-12.5% polyacrylamide cylindrical gels. The tracking dye cytochrome *c* migrated 65 mm. (A) Immunoprecipitation from cellular extracts of mycelial cells that were radiolabeled with 2.5 μCi of [^{35}S]methionine per ml for 30 min before harvest, beginning at 6.5 h of growth at 30°C (top tracing) and (from bottom to top) at 30, 60, 90, 120, and 150 min after transfer to 45°C. (B) Immunoprecipitation from a mitochondrial fraction of mycelial cells that were radiolabeled with 1 μCi of [^{35}S]methionine per ml for 30 min before harvest, beginning at 6 h of growth at 30°C (top tracing) and (from bottom to top) at 30, 90, and 150 min after transfer to 45°C.

long amino-terminal extension, which is not removed until after mitochondrial import. The 15,000-dalton precursor (40) should have a different electrophoretic mobility (44) than that of the 8,000-dalton mature subunit. The proteolipid peptides immunoprecipitated both from cell extracts and from mitochondria had the electrophoretic mobility of the mature subunit, which indicates that import has already occurred in both experiments.

DISCUSSION

The integrated response of *N. crassa* to heat shock resembles in specific ways the contrasting reactions of *D. melanogaster* and *S. cerevisiae*. Like the latter, *N. crassa* resumes normal protein synthesis at elevated temperature, but like *D. melanogaster*, it cannot grow or develop at that temperature. To examine the molecular mechanisms of *N. crassa* for initially expressing only heat shock genes and later resuming normal protein synthesis at elevated temperature, we compared the kinetics of RNA increase in two developmental stages of *N. crassa*, in conidiospores that were metabolically inactive before heat shock and in expo-

nentially growing mycelial cells. At both stages, *N. crassa* responded to heat shock by accumulating heat shock transcripts, but the two developmental stages apparently differed in the basis for resuming normal protein synthesis.

The spores displayed a large and rapid increase (at least 20-fold) in the levels of hsp83 and hsp30 RNA that peaked by 15 min. The heat shock response of mycelial (7-h) cells, which required 60 min for maximum accumulation of heat shock RNAs, was considerably slower than that of spores. In addition, the mRNA for a representative normal protein, the proteolipid subunit of the mitochondrial ATPase, declined to its lowest point at 60 min in mycelial cells, and its concentration did not increase in these cells even by 120 min of heat shock. Ungerminated spores, on the other hand, showed a substantial increase (fourfold) in proteolipid mRNA by 45 min of heat shock, compared with its low level at 15 min.

Despite these differences at the RNA level, spores and mycelial cells appear to resume normal protein synthesis at the same time during heat shock. We earlier reported that spores initiate synthesis of normal proteins, typified by actin, by 60 to 90 min of incubation at high temperature (31). In the current study we found that mycelia begin to synthesize another normal protein, the ATPase proteolipid subunit, at the same time, or between 60 and 90 min of heat shock. Since there is no increase in the proteolipid mRNA content of mycelia at this time, changes in mRNA levels alone cannot account for the repressed synthesis of this peptide early in heat shock or for its renewed synthesis during recovery. Instead, it seems likely that a change in translational discrimination occurs when mycelial cells are shifted to 45°C, and there is a reversion in preference after 60 min of exposure to heat shock. Spores, on the other hand, at elevated temperature show an increase in a normal mRNA (for the proteolipid subunit) that appears coordinated with their initiation of normal protein synthesis, which suggests that translational control is less important for the heat shock response of spores.

This apparent difference in regulation between spores and mycelia of *N. crassa* may be related to the disparate amounts of normal mRNAs they contain. Dormant spores contain much less proteolipid mRNA, for example, than do growing mycelial cells. Even with a substantial decline (80% for proteolipid mRNA), heat-shocked mycelial cells retain a high enough level of these messages to reinitiate synthesis of normal proteins with little or no new transcription. Spores, on the other hand, may require new transcription for their increased synthesis of normal proteins. In addition to this difference in regulation between the two developmental stages, there are two heat shock proteins (with M_rs of 38,000 and 34,000) that are synthesized by *N. crassa* mycelia (31) but not by spores.

We think it is likely that the synthesis of many normal proteins by *N. crassa* mycelia is regulated at the translational level during heat shock. Our screening of the heat shock cDNA library from mycelial cells indicates that 24% of the sequences were enriched in cells incubated at 45°C, whereas 14% were enriched in cells grown at 30°C, and these latter messages were only moderately reduced at 45°C (data not shown). This suggests that the synthesis of many normal proteins is depressed in mycelia during the first 60 min of heat shock, despite a significant cellular content of their mRNAs.

For *N. crassa* the predominant mechanism for regulating normal protein synthesis during heat shock apparently depends on the developmental stage. In using selective translation to regulate protein synthesis, *N. crassa* mycelia resemble the cells of *D. melanogaster*, which retain normal mRNAs during heat shock, although they are not translated (39). The preserved mRNAs are translated into normal proteins after temperature shiftdown when recovery occurs in this organism (22). In contrast, the asexual spores of *N. crassa*, like yeast cells (22, 25), may require new transcription for initiating normal protein synthesis during heat shock.

The means by which translational components of *D. melanogaster* cells discriminate between normal mRNAs and heat shock mRNAs during heat shock remain unknown. Scott and Pardue (37) showed that the lysates from heat-shocked cells display the same translational preference for heat shock mRNAs as the intact cells do and that this selectivity can be overcome by the addition of a ribosome fraction from non-heat-shocked cells. Other workers have examined the contribution of the long 5′ leader sequence of heat shock mRNAs which is characteristically deficient in secondary structure, and they have found that this sequence (or some part of it) is both necessary (27) and sufficient (18) for making mRNAs readily translatable during heat shock. How this distinguishing structural feature of heat shock mRNAs is related to heat shock-induced changes in cellular components of translation is not yet known.

A transient heat shock response, such as that displayed by *N. crassa*, offers an important advantage for studying the molecular bases for translational selectivity. A reversion in translational preference occurs during prolonged heat shock, independently of a change in temperature. This makes it possible to analyze molecular modifications that might affect translational discrimination at high temperature without the complicating effects of a temperature shift (34).

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LITERATURE CITED

1. **Ashburner, M., and J. J. Bonner.** 1979. The induction of gene activity in *Drosophila* by heat shock. Cell **17**:241–254.
2. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Bonnen, A., and R. Brambl.** 1983. Germination physiology of *Neurospora crassa* conidia. Exp. Mycol. **7**:197–207.
5. **Brambl, R.** 1975. Characteristics of developing mitochondrial genetic and respiratory functions in germinating fungal spores. Biochim. Biophys. Acta **396**:175–186.
6. **Capetanaki, Y. G., J. Ngai, C. N. Flytzanis, and E. Lazarides.** 1983. Tissue-specific expression of two mRNA species transcribed from a single vimentin gene. Cell **35**:411–420.
7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**: 5294–5299.
8. **Cox, R. A.** 1968. The use of guanidinium chloride in the isolation of nucleic acids. Methods Enzymol. **12**:120–129.
9. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation of

calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23–28.
10. **Deeley, R. G., J. I. Gordon, A. T. H. Burns, K. P. Mullinix, M. Binastein, and R. F. Goldberger.** 1977. Primary activation of the vitellogenin gene in the rooster. J. Biol. Chem. **252**:8310–8319.
11. **Deng, G., and R. Wu.** 1981. An improved procedure for utilizing terminal transferase to add homopolymers to the 3′ termini of DNA. Nucleic Acids Res. **9**:4173–4188.
12. **Farrelly, F. W., and D. B. Finkelstein.** 1984. Complete sequence of the heat shock-inducible *HSP90* gene of *Saccharomyces cerevisiae*. J. Biol. Chem. **259**:5745–5751.
13. **Groudine, M., and C. Casimir.** 1984. Post-transcriptional regulation of the chicken thymidine kinase gene. Nucleic Acids Res. **12**:1427–1446.
14. **Gubler, U., and B. J. Hoffman.** 1983. A simple and very efficient method for generating cDNA libraries. Gene **25**:263–269.
15. **Hickey, E., and L. A. Weber.** 1982. Preferential translation of heat-shock mRNAs in HeLa cells, p. 199–206. *In* M. J. Schlesinger, M. Ashburner, and A. Tissières (ed.), Heat shock from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**: 193–197.
17. **Ingolia, T. D., E. A. Craig, and B. J. McCarthy.** 1980. Sequence of three copies of the gene for the major *Drosophila* heat shock induced protein and their flanking regions. Cell **21**:669–679.
18. **Klemenz, R., D. Hultmark, and W. J. Gehring.** 1985. Selective translation of heat shock mRNA in *Drosophila melanogaster* depends on sequence information in the leader. EMBO J. **4**:2053–2060.
19. **Lacy, E., R. C. Hardison, D. Quon, and T. Maniatis.** 1979. The linkage arrangement of four rabbit β-like globin genes. Cell **18**:1273–1283.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Landfear, S. M., P. Lefebvre, S. Chung, and H. F. Lodish.** 1982. Transcriptional control of gene expression during development of *Dictyostelium discoideum*. Mol. Cell. Biol. **2**:1417–1426.
22. **Lindquist, S.** 1981. Regulation of protein synthesis during heat shock. Nature (London) **293**:311–314.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. USA **72**:1184–1188.
25. **McAlister, L., and D. B. Finkelstein.** 1980. Alterations in translatable ribonucleic acid after heat shock of *Saccharomyces cerevisiae*. J. Bacteriol. **143**:603–612.
26. **McAlister, L., S. Strausberg, A. Kulaga, and D. B. Finkelstein.** 1979. Altered patterns of protein synthesis induced by heat shock of yeast. Curr. Genet. **1**:63–74.
27. **McGarry, T. J., and S. Lindquist.** 1985. The preferential translation of *Drosophila* hsp70 mRNA requires sequences in the untranslated leader. Cell **42**:903–911.
28. **Miller, M. J., N.-H. Xuong, and E. P. Geiduschek.** 1979. A response of protein synthesis to temperature shift in the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **76**: 5222–5225.
29. **Mitchell, H. K., and L. S. Lipps.** 1978. Heat shock and phenocopy induction in *Drosophila*. Cell **15**:907–918.
30. **Neidhardt, F. C., R. A. VanBogelen, and V. Vaughn.** 1984. The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. **18**:295–329.
31. **Plesofsky-Vig, N., and R. Brambl.** 1985. The heat shock response of *Neurospora crassa*: protein synthesis and induced thermotolerance. J. Bacteriol. **162**:1083–1091.
32. **Plesofsky-Vig, N., and R. Brambl.** 1985. The heat shock response of fungi. Exp. Mycol. **9**:187–194.
33. **Rave, N., R. Crkvenjakov, and H. Boedtker.** 1979. Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. Nucleic Acids Res. **6**:3559–3567.
34. **Scharf, K.-D., and L. Nover.** 1982. Heat-shock-induced alterations of ribosomal protein phosphorylation in plant cell cultures. Cell **30**:427–437.
35. **Schlesinger, M. J., M. Ashburner, and A. Tissières (ed.).** 1982. Heat shock from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Schloss, J. A., C. D. Silflow, and J. L. Rosenbaum.** 1984. mRNA abundance changes during flagellar regeneration in *Chlamydomonas reinhardtii*. Mol. Cell. Biol. **4**:424–434.
37. **Scott, M. P., and M. L. Pardue.** 1981. Translational control in lysates of *Drosophila melanogaster* cells. Proc. Natl. Acad. Sci. USA **78**:3353–3357.
38. **Southern, E.** 1979. Gel electrophoresis of restriction fragments. Methods Enzymol. **68**:152–176.
39. **Storti, R. V., M. P. Scott, A. Rich, and M. L. Pardue.** 1980. Translational control of protein synthesis in response to heat shock in *D. melanogaster* cells. Cell **22**:825–834.
40. **Viebrock, A., A. Perz, and W. Sebald.** 1982. The imported preprotein of the proteolipid subunit of the mitochondrial ATP synthase from *Neurospora crassa*: molecular cloning and sequencing of the mRNA. EMBO J. **1**:565–571.
41. **Vogel, H. J.** 1956. A convenient growth medium for *Neurospora*. Microb. Genet. Bull. **13**:42.
42. **Wenzler, H., and R. Brambl.** 1981. Mitochondrial biogenesis during fungal spore germination: catalytic activity, composition, and subunit biosynthesis of oligomycin-sensitive ATPase in *Botryodiplodia*. J. Biol. Chem. **256**:7166–7172.
43. **Zaitlin, M., and V. Hariharasubramanian.** 1970. An improvement in a procedure for counting tritium and carbon-14 in polyacrylamide gels. Anal. Biochem. **35**:296.
44. **Zimmermann, R., B. Hennig, and W. Neupert.** 1981. Different transport pathways of individual precursor proteins in mitochondria. Eur. J. Biochem. **116**:455–460.