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Acquisition of Thermotolerance in Soybean Seedlings[1]

SYNTHESIS AND ACCUMULATION OF HEAT SHOCK PROTEINS AND THEIR CELLULAR LOCALIZATION

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ABSTRACT

When soybean *Glycine max* var Wayne seedlings are shifted from a normal growth temperature of 28°C up to 40°C (heat shock or HS), there is a dramatic change in protein synthesis. A new set of proteins known as heat shock proteins (HSPs) is produced and normal protein synthesis is greatly reduced. A brief 10-minute exposure to 45°C followed by incubation at 28°C also results in the synthesis of HSPs. Prolonged incubation (e.g. 1–2 hours) at 45°C results in greatly impaired protein synthesis and seedling death. However, a pretreatment at 40°C or a brief (10-minute) pulse treatment at 45°C followed by a 28°C incubation provide protection (thermal tolerance) to a subsequent exposure at 45°C. Maximum thermoprotection is achieved by a 2-hour 40°C pretreatment or after 2 hours at 28°C with a prior 10-minute 45°C exposure. Arsenite treatment (50 micromolar for 3 hours) also induces the synthesis of HSP-like proteins, and also provides thermoprotection to a 45°C HS; thus, there is a strong positive correlation between the accumulation of HSPs and the acquisition of thermal tolerance under a range of conditions.

During 40°C HS, some HSPs become localized and stably associated with purified organelle fractions (*e.g.* nuclei, mitochondria, and ribosomes) while others do not. A chase at 28°C results in the gradual loss over a 4-hour period of the HSPs from the organelle fractions, but the HSPs remain selectively localized during a 40°C chase period. If the seedlings are subjected to a second HS after a 28°C chase, the HSPs rapidly (complete within 15 minute) relocalize in the organelle fractions. The relative amount of the HSPs which relocalize during a second HS increases with higher temperatures from 40°C to 45°C. Proteins induced by arsenite treatment are not selectively localized with organelle fractions at 28°C but become organelle-associated during a subsequent HS at 40°C.

The induction of HSPs[3] has been shown to be a universal response to thermal stress in a wide range of organisms (5–7, 9, 15, 21, 24, 30, 34). Earlier work showed that when the growth temperature of soybean seedlings was shifted from 28°C to 40°C the pattern of protein synthesis changed rapidly; *i.e.* normal protein synthesis decreased and a new set of proteins (HSPs) was induced (15). In *Drosophila* the changes in protein synthesis during HS were shown to result from changes in the production and utilization of mRNA (5, 25). While there is no definitive information on the exact role or function of HSPs, the evidence from work with *E. coli* (34), yeast (24), and *Dictyostelium* (21) mutants, along with the specific cellular localization of HSPs (3, 4, 16, 22, 26, 32, 33) generally supports the view that HSPs provide protection or thermotolerance for the organism to otherwise lethal temperatures.

In this report, we present three lines of evidence supporting the role of HSPs in the acquisition of thermotolerance in plants. The criteria for thermoprotection are based on both the growth of soybean seedlings after a 2-h treatment at the lethal temperature of 45°C and the level of amino acid incorporation at this temperature. First, evidence is presented that the two conditions which stimulate the production of HSPs, *i.e.* a brief exposure to 45°C followed by incubation at 28°C or a somewhat longer exposure to 40°C, provide thermoprotection. Second, some HSPs become selectively localized in cellular organelles during HS and relocalize during a second heat shock after delocalization by a chase at 28°C. A third line of evidence is based on the induction by arsenite of HSP-like proteins. In soybean (results of this study) and other systems (5, 14), this respiratory inhibitor stimulates the production of electrophoretically similar proteins that provide thermoprotection and which become localized only during HS in the soybean seedlings. These observations support the view that the accumulation and selective localization of HSPs impart thermoprotection to soybean seedlings to otherwise lethal temperatures.

MATERIALS AND METHODS

Plant Material. Soybean seeds (*Glycine max* var Wayne) were germinated in rolls of moist Chem-pak at 28°C in a dark growth chamber. For growth analysis, 30 seeds were germinated overnight as described above (length of axis was about 1 cm) and then incubated in 1% sucrose, 1 mM K-phosphate (pH 6.0) in shaking water baths at various temperature regimes. After treatment, the seedlings were replanted in moist Chem-pak rolls and grown at 28°C. Seedling length was measured every 24 h for 72 h.

***In Vivo* Labeling and Extraction of Labeled Proteins.** For amino acid incorporation measurements, five 2-d-old seedlings (3–4 cm in length) without cotyledons were incubated in 5 ml of incubation buffer (1% sucrose, 1 mM K-phosphate [pH 6.0], chloramphenicol [50 μg/ml], and 100 μCi of [^{3}H]leucine). For cellular localization of HSPs, 50 to 100 seedlings (5–10 g, without cotyledons) were incubated in 18 to 20 ml of media with 400 to 600 μCi [^{3}H]leucine. Labeled seedlings were rinsed thoroughly with nonradioactive leucine before protein extraction or isolation of cellular organelles. For protein extraction, seedlings were homogenized in 10 ml of 50 mM Tris-HCl (pH 8.5), 2% SDS, 2% mercaptoethanol, and 1 mM PMSF at room temperature. The homogenate was centrifuged for 30 min at 10,000 rpm in a JA-20 rotor (Beckman) and filtered through a layer of Miracloth.

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[3] Abbreviations: HSP, heat shock protein; HS, heat shock; PMSF, phenylmethylsulfonyl fluoride.

Table I. *Influence of Preincubation at 40°C on Growth of Soybean Seedlings following a 2-Hour Heat Shock at 45°C*

Thirty germinating seedlings with the embryonic axis protruding about 1 cm from the seed coat were subjected to the temperature treatments shown below. After the treatment periods, they were replanted in moist Chem-pak rolls and placed in a dark 28°C incubator. The length of seedlings was measured at each of the times shown.

Heat Shock Condition	Length			% of Seedlings (72 h) at Following Range (cm)		
	24 h	48 h	72 h	<5	5–10	>10
		cm				
1. Normal 28°C	6.0 ± 0.9	13.9 ± 1.9	21.2 ± 1.9	0	0	100
2. 45°C (2 h)	2.3 ± 0.3	2.5 ± 0.3	3.0 ± 0.3	100	0	0
3. 40°C (15 min) → 45°C (2 h)	2.5 ± 0.3	3.9 ± 1.1	6.8 ± 0.3	43	40	17
4. 40°C (30 min) → 45°C (2 h)	2.6 ± 0.4	3.7 ± 1.3	6.0 ± 0.3	55	24	21
5. 40°C (1 h) → 45°C (2 h)	3.3 ± 0.5	7.0 ± 1.0	12.9 ± 1.9	0	3	97
6. 40°C (2 h) → 45°C (2 h)	3.3 ± 0.5	6.7 ± 1.5	12.0 ± 3.0	7	7	86
7. 40°C (2 h)	5.2 ± 0.6	11.3 ± 1.8	19.4 ± 2.3	0	0	100
8. 40°C (15 min) → 28°C (4 h) → 45°C (2 h)	2.6 ± 0.3	4.5 ± 1.1	8.8 ± 2.9	11	48	41
9. 40°C (30 min) → 28°C (4 h) → 45°C (2 h)	2.7 ± 0.3	5.0 ± 1.3	9.5 ± 3.0	16	20	64
10. 40°C (2 h) → 28°C (4 h) → 45°C (2 h)	3.2 ± 0.5	6.4 ± 3.1	11.9 ± 3.1	4	20	76

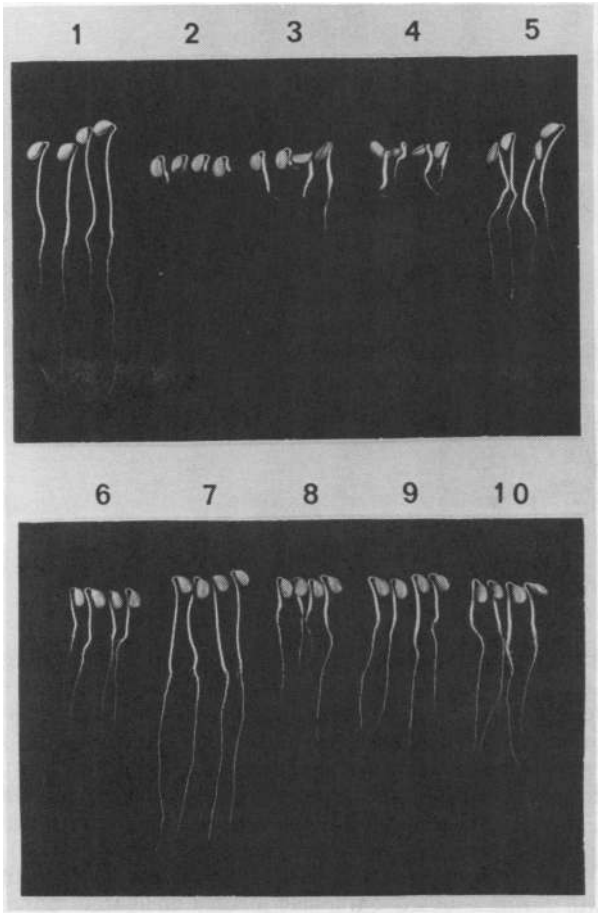

FIG. 1. Development of thermotolerance to 45°C in soybean seedlings by a 40°C pretreatment. Seedlings 72 h after treatment as described in Table I. (1), 28°C, 2 h; (2), 45°C, 2 h; (3), 40°C, 15 min → 45°C, 2 h; (4), 40°C, 30 min → 45°C, 2 h; (5), 40°C, 1 h → 45°C, 2 h; (6), 40°C, 2 h → 45°C, 2 h; (7), 40°C, 2 h; (8), 40°C, 10 min → 28°C, 4 h → 45°C, 2 h; (9), 40°C, 30 min → 28°C, 4 h → 45°C, 2 h; and (10), 40°C, 2 h → 28°C, 4h → 45°C, 2 h.

A sample of each filtrate was blotted on 3-mm filter paper as described by Mans and Noveli (23) for determination of amino acid incorporation. For SDS-gel electrophoresis, the filtrate was precipitated with 5 volumes of acetone and stored at −20°C overnight. The precipitates were pelleted, dried, and dissolved in sample buffer for electrophoresis.

Ribosome Isolation. Seedlings were homogenized in medium containing 0.5 M sucrose, 200 mM Tris-HCl (pH 8.8), 30 mM $MgCl_2$, 100 mM KCl, and 1 mM DTT with a Polytron equipped with a PT-20 probe. The homogenate was filtered through a layer of Miracloth and centrifuged at 20,000*g* for 15 min. Triton X-100 (final concentration 1%) was added to the postmitochondrial supernatant, and ribosomes were pelleted as described previously (15).

Isolation of Mitochondria. Seedlings (10 g) were homogenized in 20 ml of medium containing 0.4 M mannitol, 25 mM MOPS (pH 7.8), 5 mM KCl, 8 mM cysteine, 1 mM EGTA, and 0.1% (w/v) BSA with mortar and pestle. Isolation and purification of mitochondria followed the method of Forde *et al.* (10). Purified mitochondria were recovered from the interface between 1.2 M sucrose and 1.4 M sucrose layers.

Isolation of Nuclei. Seedlings (10 g) were homogenized in a buffer containing 25 mM Mes-NaOH buffer (pH 6.0), 20 mM KCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1.0 M sucrose, and 1 mM PMSF to obtain crude nuclei pellets (8). The crude nuclei suspension was layered over four layers of Percoll discontinuous gradients (10%, 20%, 35%, and 50%), and the gradients were centrifuged for 30 min at 10,000 rpm in JA-20 rotor (Beckman). The nuclei fraction was recovered from the interlayer of 35% and 50% Percoll by centrifugation at 10,000*g* for 10 min after dilution with an equal volume of homogenization medium. The nuclei pellets were washed twice with the same medium.

Gel Electrophoresis and Fluorography of *In Vivo* Synthesized Proteins. Proteins prepared as described above were dissolved in a sample buffer of 2.3% SDS containing 5% mercaptoethanol. One-dimensional SDS-gel electrophoresis was carried out according to Laemmli (18) and equivalent amounts of protein samples were loaded unless otherwise specified. For two-dimensional gels, the method of O'Farrell was followed (28). Fluorography of the gels was accomplished using ENHANCE (New England Nuclear) and preflashed Kodak film (XAR-5).

Table II. *Protection of Seedling Growth by a Brief 45°C Treatment Followed by Incubation at 28°C and a Subsequent 2-Hour Heat Shock at 45°C*

Thirty germinating seedling with the embryonic axis protruding about 1 cm from seed coat were subjected to the temperature regimes shown below. After each treatment, seedlings were planted in moist Chem-pak rolls, grown in a dark incubation at 28°C, and the lengths measured at the indicated times.

Heat Shock Condition	Length			% of Seedlings (72 h) at Following Range (cm)		
	24 h	48 h	72 h	<5	5–10	>10
		cm				
1. Normal 28°C	5.2 ± 0.7	10.9 ± 1.5	19.1 ± 2.5	0	3	97
2. 45°C (10 min)	4.9 ± 0.4	9.7 ± 1.4	16.1 ± 2.0	0	0	100
3. 45°C (2 h)	2.2 ± 0.3	2.2 ± 0.3	3.2 ± 1.2	94	6	0
4. 45°C (10 min) → 28°C (30 min) → 45°C (2 h)	2.2 ± 0.3	2.9 ± 0.7	4.3 ± 2.1	70	30	0
5. 45°C (10 min) → 28°C (1 h) → 45°C (2 h)	2.9 ± 0.5	4.1 ± 1.3	6.8 ± 3.0	47	40	13
6. 45°C (10 min) → 28°C (2 h) → 45°C (2 h)	3.2 ± 0.3	6.1 ± 0.7	11.2 ± 1.5	0	17	83
7. 45°C (10 min) → 28°C (3 h) → 45°C (2 h)	3.3 ± 0.4	5.9 ± 0.7	11.0 ± 1.4	0	13	87

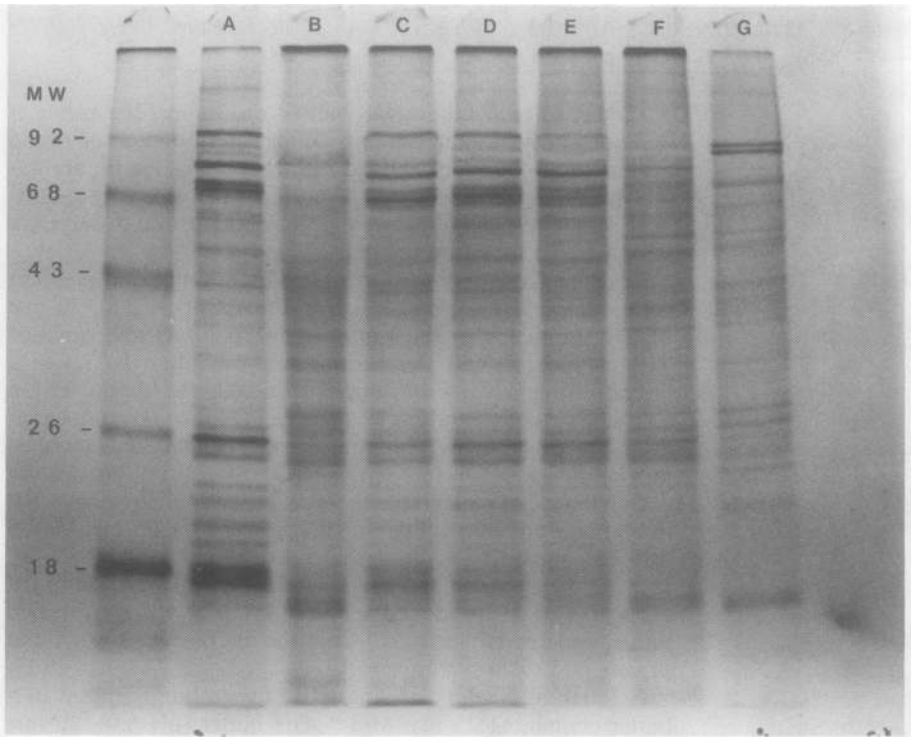

FIG. 2. Induction of HSP synthesis at 28°C by a short pretreatment at 45°C. Fluorogram of SDS-polyacrylamide slab gel of proteins isolated from soybean seedlings labeled during 28°C incubation following a 10-min incubation at 45°C. Lane A, 40°C HS; lane B, label during the 1st h; lane C, label during the 2nd h; lane D, label during the 3rd h; lane E, label during the 4th h; lane F, label during the 5th h, at 28°; lane G, 28°C control. Mol wt standards are in order of decreasing size: 92, 68, 43, 26, and 12 kD shown in the left of lane A.

RESULTS

During the study of amino acid incorporation into proteins at elevated temperatures, a precipitous drop in amino acid incorporation above 40°C was observed. However, prior treatment of soybean seedlings at 40°C for 2 h permitted higher levels of protein synthesis at the otherwise nonpermissive HS temperatures (*i.e.* above 42.5°C) (17). Growth of seedlings was also shown to be protected during a subsequent incubation at 45°C for 2 h (normally a lethal treatment) by a preincubation at 40°C. Based on the pattern of protein synthesis over a temperature range of 30°C to 42.5°C and the level of amino acid incorporation, HS at 40°C is considered the optimum temperature for induction of HSP synthesis in soybean seedlings. In this study, we have investigated how the synthesis and accumulation of HSPs at 40°C provides protection to seedlings. The germinating seedlings (embryogenic axis about 1 cm long) were incubated at 40°C for varying lengths of time prior to a 2-h 45°C treatment. The subsequent growth in length at 28°C is shown in Table I, and typical seedlings 72 h after each treatment are shown in Figure 1. Seedlings incubated for 1 h at 40°C were almost as well protected as those treated for 2 h at 40°C. Preincubation at 40°C for more than 2 h did not provide additional protection (data not shown). A preincubation of less than 1 h at 40°C is not sufficient to give thermoprotection, but as little as 15 min at 40°C followed by 2 to 4 h at 28° C causes the seedlings to develop significant levels of thermoprotection (Table I; Fig. 1, no. 8).

A brief, 10-min HS pulse at 45°C will also protect seedling growth during a subsequent longer incubation at 45°C, provided there is an adequate intervening incubation at 28°C (Table II). The pattern of protein synthesis during this intervening incubation at 28°C is shown in Figure 2. It is evident that a large proportion of proteins synthesized during the 2nd and 3rd h at 28°C are HSPs. Data in Table II show that under conditions where HSP synthesis is not fully developed (*i.e.* less than 1 h after a brief HS), seedlings are not maximally protected. Only after HSP synthesis is well established (*i.e.* 2 h after a brief HS

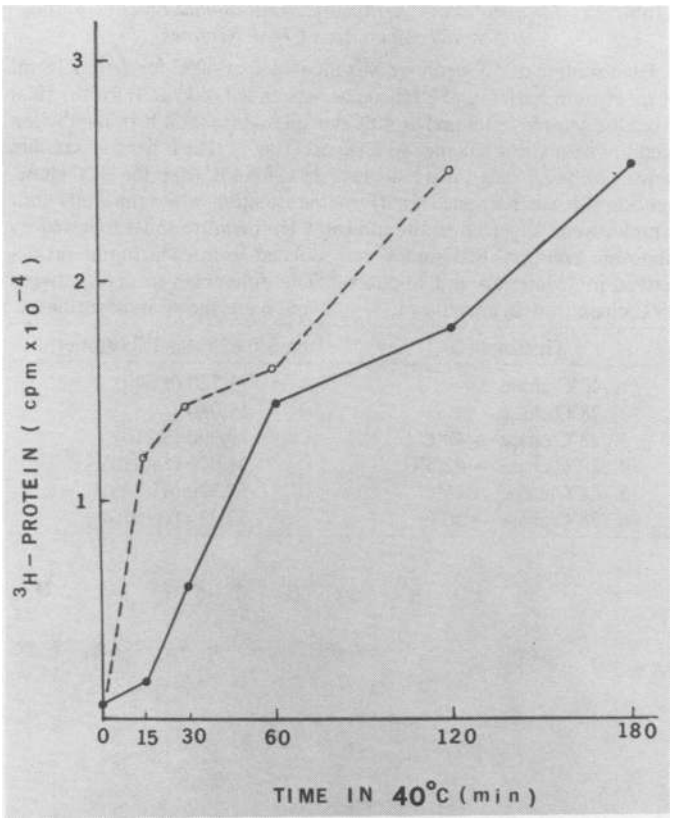

FIG. 3. [^{3}H]Leucine incorporation in soybean seedlings at 45°C in relation to preincubation at 40°C for various time. Six seedlings were incubated in 6 ml of incubation medium at 40°C. After the indicated time at 40°C, samples were either incubated for 4 h at 28°C followed by incubation at 45° (O– – –O) or transferred straight to 45°C for 2 h (●——●); 100 μCi of [^{3}H]leucine were added during the terminal 2-h incubation. After 2 h labeling, the seedlings were rinsed, and ^{3}H incorporation into protein was determined by measuring hot TCA-insoluble radioactivity.

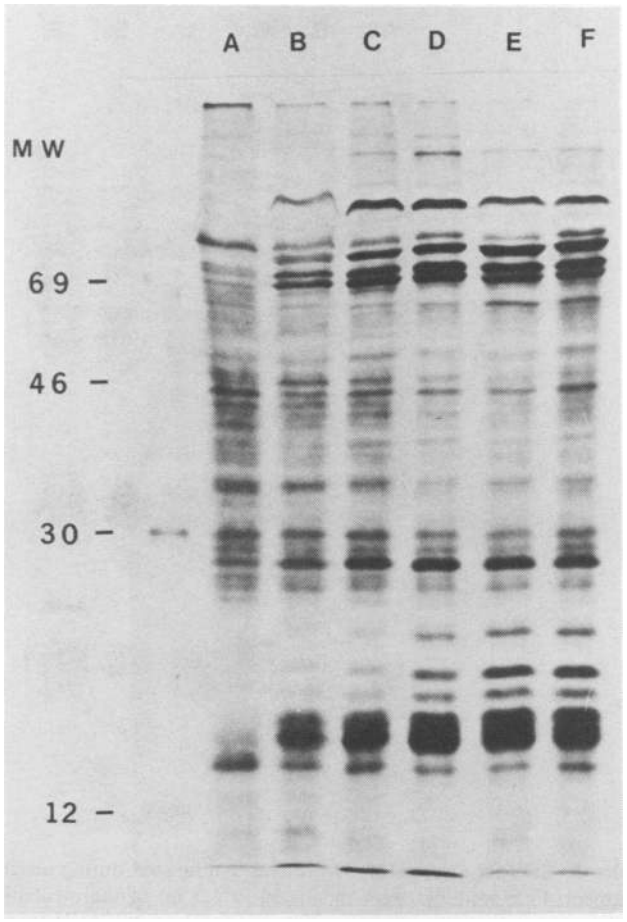

FIG. 4. Pattern of HSP synthesis at 45°C. Fluorogram of SDS-polyacrylamide slab gel of proteins extracted from soybean seedlings labeled at 45°C following various preincubations at 40°C as shown in Figure 4. Lane A, 0 time; lane B, 15 min; lane C, 30 min; lane D, 1 h; lane E, 2 h; lane F, 3 h; mol wt standards, 69, 46, 30, and 12 kD are shown in the left lane of lane A.

at 45°) do seedlings show thermal protection.

Another criterion for thermoprotection relates to [^{3}H]leucine incorporation at 45°C. Seedlings incubated directly at 45°C incorporate very little [^{3}H]leucine into protein (16). However, substantial protein synthesis occurs during incubation at 45°C, following a preincubation of 30 min or more at 40°C (Fig. 3). A 30-min incubation at 40°C followed by 2 to 4 h at 28°C prior to incubation at 45°C permits significant incorporation of [^{3}H]-leucine into protein. These levels of protein synthesis at 45°C correlate well with the levels of seedling growth noted in Table I and with the attendant level of HSP accumulation under these various HS regimes. The level of amino acid incorporation noted in these analyses does not necessarily relate directly to the rate of protein synthesis; however, the levels of amino acid incoporation observed in these experiments do reflect closely the level of HS-mRNA which accumulate during these treatment regimes (unpublished data and 16). Accordingly, the levels of incorporation which indicate thermoprotection of protein synthesis likely relate closely to rates of protein synthesis. Figure 4 shows that the proteins synthesized at 45°C under thermoprotected conditions are mostly HSPs.

The next line of evidence which indicates a close relationship between the acquisition of thermotolerance and the accumulation of HSPs is based on the effects of arsenite treatment. Arsenite at a concentration of 50 μM (Fig. 5C) is optimal for inducing proteins of mol wt of 92, 84, 68–70, 27, and 15–18 kD. This pattern of protein synthesis is similar to that induced by HS. The proteins induced by arsenite are not present in the same proportion as in HS; there appears to be less low molecular HSP synthesis (15–18 kD) relative to the 68–70, 84, and 92 kD bands. It also seems that synthesis of normal 28°C proteins is less affected by arsenite treatment than by HS and that additional proteins are synthesized in response to arsenite. However, since arsenite induces HS-like proteins in soybean seedlings, we investigated the possibility that arsenite treatment would provide thermoprotection. The results in Table III show that a 3-h treatment with arsenite (50 μM) at 28°C provides a certain degree of thermoprotection as measured by both growth and protein synthesis. However, these treatments do not give as much protection as the optimal conditions shown in Tables I and II. This may be due to the toxic effect of arsenite or to the relatively slower accumulation of HSPs in the presence of arsenite. A longer period of HSP accumulation in response to arsenite might well lead to a higher level of thermal protection.

Localization of HSPs. In order to investigate how HSPs that are synthesized during a pretreatment at 40°C provide thermoprotection at 45°C, soybean seedlings were labeled with [^{3}H]-leucine at 40°C to induce synthesis and accumulation of HSPs. Seedlings were then chased at 28°C, in buffer containing nonradioactive leucine (1 mM), for 4 h prior to shifting to 45°C. The labeled HSPs made at 40°C are stable during the 4-h chase; in fact, HSPs are also stable for 12 h or more (15). Table IV shows the amount of labeled proteins associated with the ribosomal

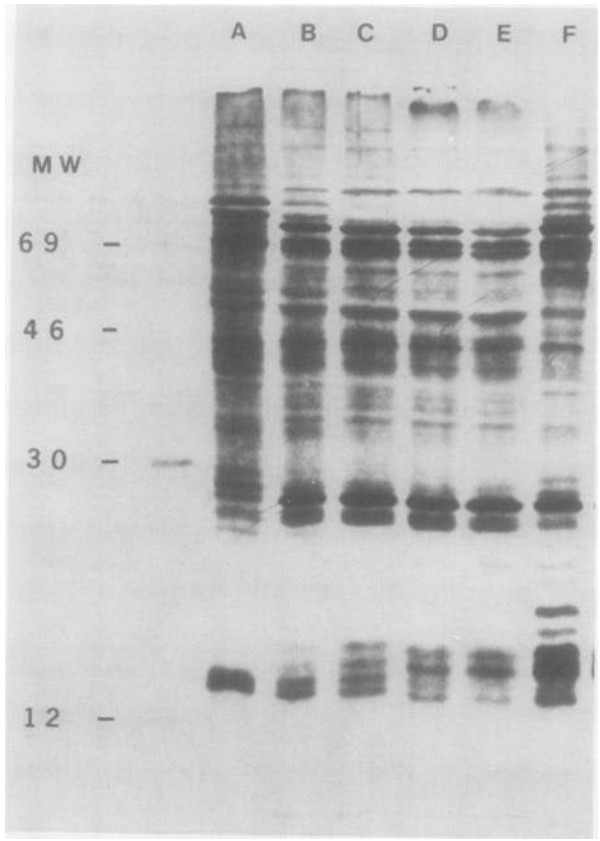

FIG. 5. SDS-gel analysis of ^{3}H-proteins synthesized during arsenite treatment. Five seedlings were incubated in 5.5 ml of the incubation medium containing arsenite up to 100 μM for 3 h at 28°C; [^{3}H]leucine (100 μCi) was added for 2 h after the 1-h pretreatment. Total proteins were extracted and separated on SDS-acrylamide gel. Equal cpm were loaded in each lane. Lane A, no arsenite; lane B, 25 μM arsenite; lane C, 50 μM arsenite; lane D, 75 μM arsenite; lane E, 100 μM arsenite; lane F, HS at 40°C.

Table III. *Development of Thermoprotection in Response to Arsenite Treatment*

Thirty germinating seedlings with the embryonic axis protruding about 1 cm from the seed coat were pretreated with arsenite (50 μM) for 3 h at 28°C. Seedlings were then rinsed with water and subjected to 45°C for 2 h and planted as described in Table I. To measure [^{3}H]leucine incorporation, 2-d-old seedlings were incubated at 28°C in 6 ml of incubation medium containing 50 μM arsenite for 3 h, and [^{3}H]leucine (120 μCi) was added during a subsequent 2-h 45°C incubation. After these treatments, the seedlings were rinsed with 1 mM leucine and homogenized as described in "Materials and Methods." Aliquots of the homogenate (50 μl) were used to measure hot TCA-precipitable radioactivity.

Heat Shock Condition	Length (72 h)	[^{3}H]Leucine Incorporation
	cm	*cpm*
28°C Control	20.9 ± 3.1	
Arsenite 50 μM (3 h)	15.9 ± 1.8	
45°C (2 h)	3.9 ± 1.2	940
Arsenite (3 h) → 45°C (2 h)	9.8 ± 1.6	12,050

fractions 1 h after a second HS at 40°C, 42.5°C, 45°C, or 50°C. The association of labeled proteins with ribosomes is temperature dependent, increasing up to 45°C and declining at 50°C. The ^{3}H-proteins associated with the ribosomal fraction are primarily the 15–18 kD HSPs; some 69–70 kD HSPs also associate with the

Table IV. *Localization of ^{3}H-Proteins in Ribosomal Fraction during Different Temperatures Chase Regimes*

Each sample of 50 seedlings was incubated at 40°C for 3 h in 18 ml of incubation buffer, and [^{3}H]leucine was added (400 μCi) for the final 2 h. One sample remained at 40°C for an additional 4 h in incubation medium with 1 mM leucine (40°C) chase (row 1). The other five samples were also chased with 1 mM leucine at 28°C for 4 h. After the 28°C chase, one sample was harvested for ribosome isolation while the other four samples were subjected to the indicated temperature shifts followed by ribosome isolation. Ribosomes were isolated from each sample as described in "Materials and Methods." The differences in cpm between 28°C chase (row 2) and rows 1, 3, 4, 5, and 6 are shown in parentheses.

Treatment	cpm/0.625 A_{260} Ribosomes
1. 40°C chase	25,720 (9,640)
2. 28°C chase	16,080
3. 28°C chase → 40°C	19,290 (3,210)
4. 28°C chase → 42.5°C	31,070 (14,490)
5. 28°C chase → 45°C	57,320 (41,240)
6. 28°C chase → 50°C	32,330 (16,250)

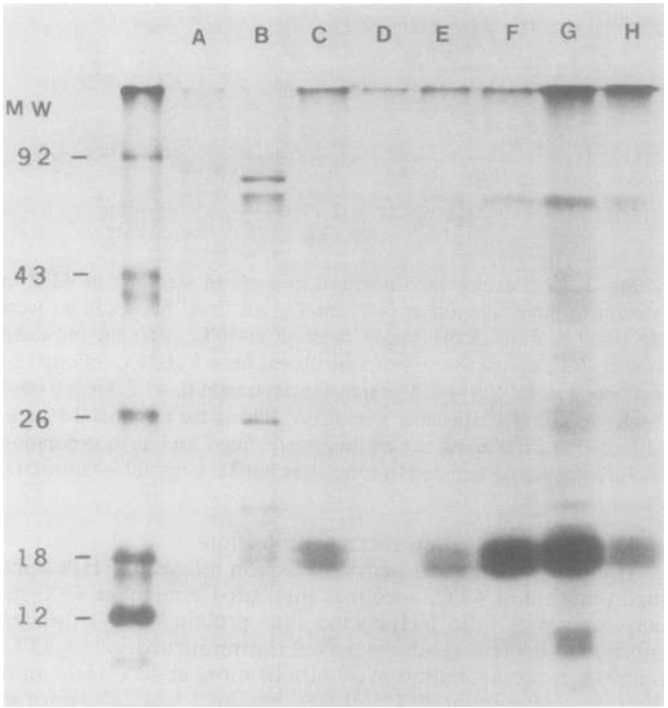

FIG. 6. Fluorogram of SDS-polyacrylamide gel pattern of ^{3}H-proteins associated with the ribosome fraction under HS conditions. Fifty seedlings per sample were incubated at 40°C for 3 h and labeled the last 2 h with [^{3}H]leucine. A chase with 1 mM leucine was done at 40°C or at 28°C for 4 h; the seedlings were then incubated at several HS temperatures (1 h) as shown in Table IV. Lane A, 28°C total proteins; lane B, 40°C total proteins; lane C, 40°C chase; lane D, 28°C chase; lane E, 40°C after 28°C chase; lane F, 42.5°C after 28°C chase; lane G, 45°C after 28°C chase; lane H, 50°C after 28°C chase. Lanes C, D, E, F, G, and H reflect treatments of rows 1, 2, 3, 4, 5, and 6 in Table IV, respectively. Mol wt standards, 92, 43, 26, 18, and 12 are shown in the lane to the left of lane A.

ribosome fraction (Fig. 6). The other HSPs with mol wt of 92, 84, and 27 kD are not localized in ribosomal fractions but are found primarily in the postribosomal supernatant (data not shown). Although there is TCA-precipitable ^{3}H-protein associated with the ribosomal fraction from the 28°C chase, no labeled HSPs are seen on SDS-gel analysis (Fig. 6D). A much greater level (up to 10 ×) of ^{3}H-HSPs become associated with the

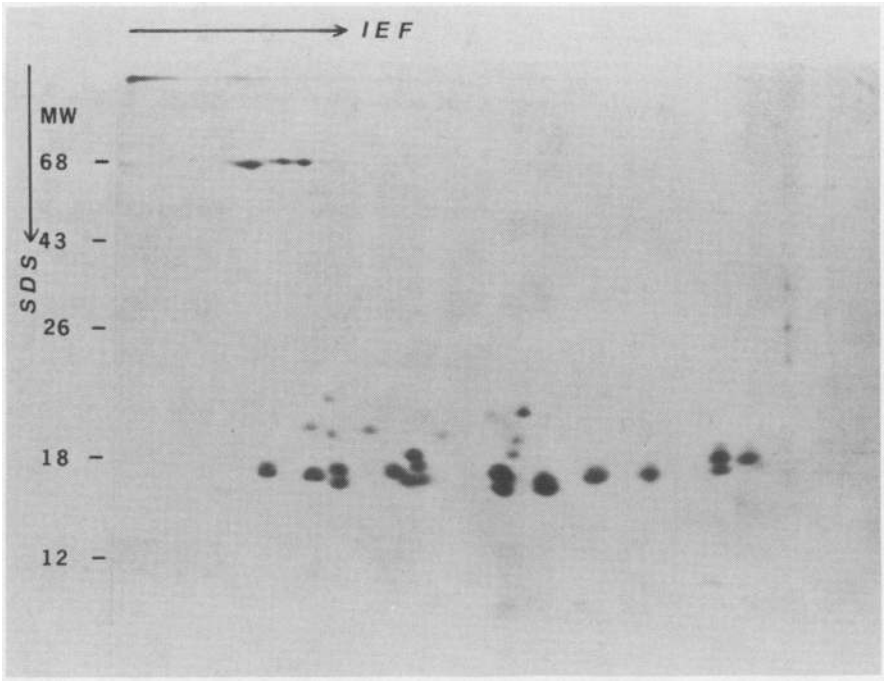

FIG. 7. Two-dimensional O'Farrell polyacrylamide gel and analysis of ^{3}H-proteins associated with ribosomes. Protein sample described in Figure 6, lane G was separated by two-dimensional gel analysis as described in "Materials and Methods."

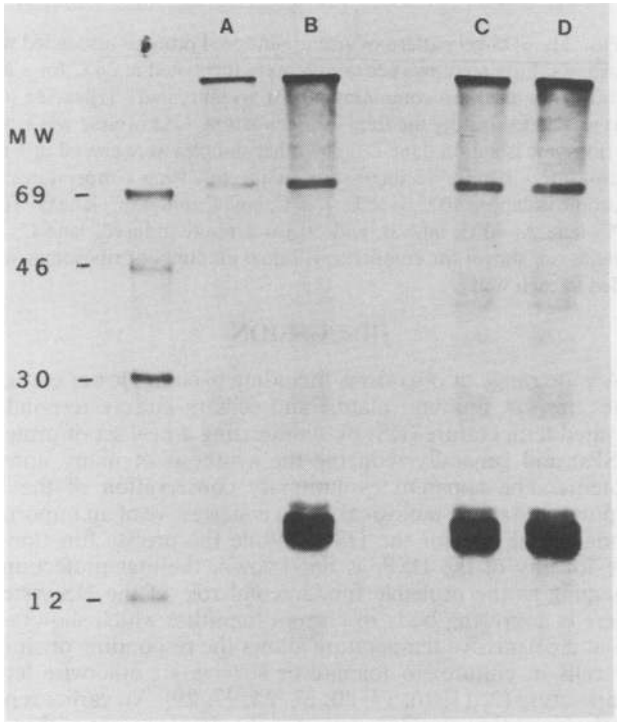

FIG. 8. Time course studies of localization of HSPs in the ribosome fraction. Fifty seedlings per sample were treated and labeled as described in Figure 6. After a 4-h chase at 28°C, the samples were subjected to a 45°C incubation for 15 min, 30 min, or 1 h. Ribosomes were isolated from each sample, and proteins were extracted for SDS-acrylamide gel analysis. Lane A, 28°C chase; lane B, 15 min at 45°C; lane C, 30 min at 45°C; and lane D, 1 h at 45°C.

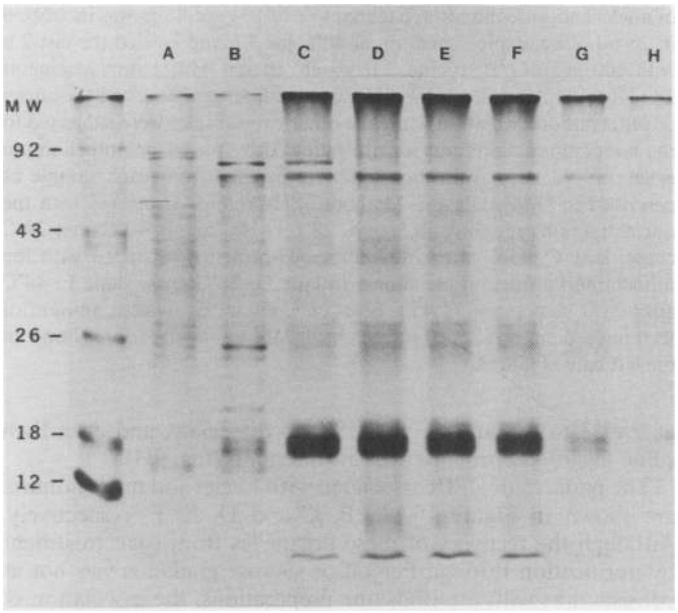

FIG. 9. Time course studies of delocalization (chase) of HSPs from the ribosome fraction. Soybean seedlings were labeled at 40°C as indicated in Figure 6. After 2 h labeling, they were chased in 1 mM leucine for an additional 30 min and then shifted to 45°C for 30 min before being transferred to 28°C. Equivalent amounts of ribosomes was added to each well. Fluorogram of SDS-polyacrylamide gel fractionated proteins from the ribosome fraction; proteins were extracted from seedlings incubated at 28°C for 0 time, lane C; 30 min, lane D; 1 h, lane E; 2 h, lane F; 3 h, lane G; and 4 h, lane H. Lane A, 28°C total proteins; lane B, 40°C total proteins. Mol wt standards are shown in lane to left of lane A.

ribosome fraction following a 45°C shift than from a comparable 40°C shift (Table IV, row 5 *versus* row 3). The HSPs associated with the ribosome fraction separate into a complex pattern on two-dimensional O'Farrell gels (Fig. 7). The 15–18 kD bands of one-dimensional SDS gel are resolved into more than 20 proteins, and the 69–70 kD protein band resolves into three spots.

The association of the HSPs with the ribosome fraction is a very quick process; within 15 min most of the proteins that will localize have already done so (Fig. 8, B *versus* D). HSPs do not chase out of the ribosomal fraction and into the soluble supernatant fraction as quickly, however; Figure 9 shows that it takes

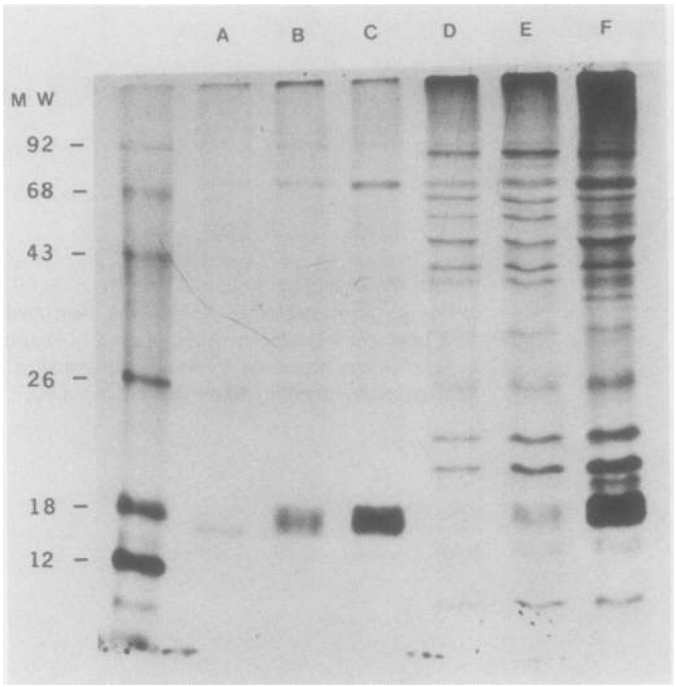

FIG. 10. SDS-gel analysis of ^{3}H-proteins associated with the fractions of nuclei and mitochondria. Each sample of 10 g seedlings was incubated in 25 ml of incubation medium at 40°C for 3 h and labeled the last 2 h with 600 μCi of [^{3}H]leucine. They were chased with 1 mm leucine at 28°C for 4 h. After the 28°C chase, one sample was harvested for nuclei or mitochondria isolation while the other two samples were subjected to the temperature shifts for 30 min followed by nuclei or mitochondria isolation. Nuclei or mitochondria were isolated from each sample as described in "Materials and Methods." ^{3}H-proteins associated with the nuclei fraction are shown in lane A, 28°C chase; lane B, 40°C after 28°C chase; lane C, 45°C after 28°C chase. ^{3}H-proteins associated with the mitochondrial fraction are shown in lane D, 28°C chase; lane E, 40°C after 28°C chase; lane F, 45°C after 28°C chase. Equivalent amount of seedling protein was added to each well. Mol wt standards are shown in the left lane of lane A.

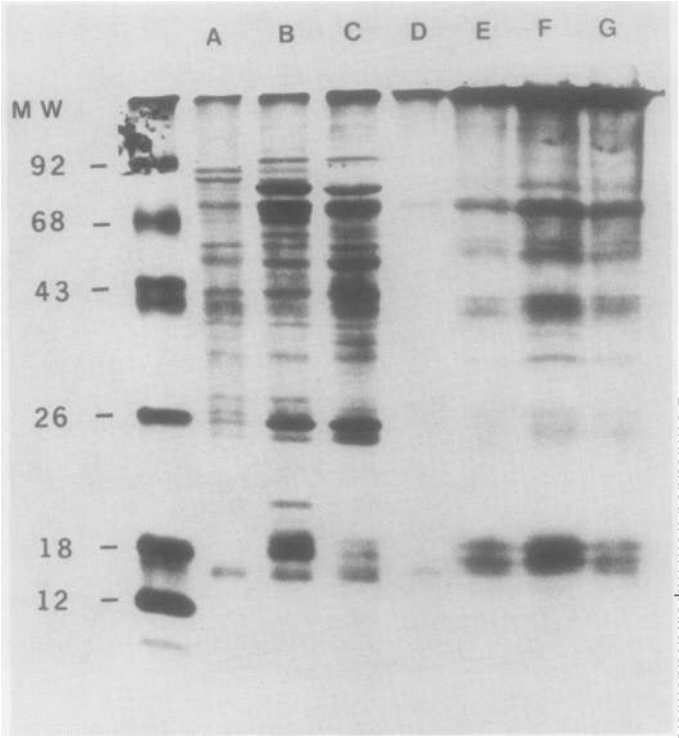

FIG. 11. SDS-gel pattern of arsenite-induced proteins associated with ribosomes. Fifty seedlings per sample were incubated at 28°C for 3 h in 18 ml of the medium containing 50 μM arsenite, and [^{3}H]leucine (400 μCi) was added during the final 2-h incubation. One sample was rinsed for ribosome isolation (lane D). The other samples were chased in 1 mM leucine for 1 h prior to incubation at the following temperatures for ribosome isolation: 40°C, lane E; 42.5°C, lane F; and 45°C, lane G. Total 28°C, lane A; 40°C, lane B; and 50 μM arsenite-induced, lane C; ^{3}H-proteins are shown for comparison. Equal amounts of ribosomes were added to each well.

at least 3 to 4 h at 28°C for HSPs to dissociate, and even then some 69–70 kD proteins appear to remain (Fig. 9H).

The patterns of HSPs associated with nuclei and mitochondria are shown in Figure 10, A, B, C and D, E, F, respectively. Although the recovery of these organelles from each treatment by purification through Percoll or sucrose gradients was not as consistent as with the ribosome preparations, the association of 69–70 and 15–18 kD proteins with these organelles was similar to that found in the ribosome fraction. Additional HSPs with sizes of about 24 and 22 kD were found associated preferentially with the mitochondrial fraction in a temperature-dependent manner. The HSPs which associated preferentially with the mitochondrial fraction are not mitochondrial in origin (unpublished data), and they do not appear to chase significantly at either 28°C or 40°C.

The arsenite-induced proteins that appear to provide thermoprotection behave like HSPs relative to localization patterns. A set of arsenite-induced proteins are shown in Figure 11 to localize into the ribosomal fraction when the tissue is subjected to HS at 40°C, 42.5°C, or 45°C for 1 h (Fig. 1, E, F, and G, respectively); these proteins are not localized during arsenite induction at 28°C (Fig. 11D)., The localized arsenite-induced proteins are similar in size and electrophoretic properties to the HSPs found in the ribosomal fraction.

DISCUSSION

A wide range of organisms including bacteria, lower eurakaryotes, insects, animals, plants, and cells in culture respond to elevated temperature (HS) by synthesizing a new set of proteins (HSPs) and generally reducing the synthesis of many normal proteins. The apparent evolutionary conservation of the HS response across the biological world is suggestive of an important fundamental role for the HSPs. While the precise function or role for any of the HSPs is not known, thermal protection is emerging as the probable fundamental role of the HS system. There is a growing body of current literature which shows that HS at a permissive temperature allows the responding organism (or cells in culture) to tolerate or survive an otherwise lethal temperature (2, 11, 16, 17, 20, 21, 24, 27, 29). An earlier report by Yarwood (35) also demonstrated the development of thermal tolerance or 'adaptation' to high temperature (*e.g.* 55°C) or 'challenge heat' by pretreatment with a nonlethal 'predisposition heat' (*e.g.* 50°C for 20 s) followed by several hours at the normal growing temperature. The evidence presented here and preliminary data previously reported (16, 17) demonstrate that germinating soybean seedlings develop thermal tolerance to an otherwise nonpermissive (lethal) temperature (*e.g.* 2 h at 45°C) by prior HS at a permissive temperature (*e.g.* 40°C for 2 h). Several different treatment regimes of permissive HS result in the development of thermotolerance in the soybean seedling. These treatments include: (a) a 1 to 2-h continuous HS at 40°C followed by 45°C incubation; (b) a 30-min HS at 40°C followed by 2 to 3 h at 28°C prior to the shift to 45°C; (c) a 10-min HS at 45°C

followed by about 2 h at 28°C prior to the shift to 45°C; and (d) treatment of seedlings with 50 μM arsenite at 28°C for 3 h or more prior to the shift to 45°C. The important feature which these treatments have in common is the induction of synthesis and accumulation of HSPs (prior to incubation at the potentially lethal temperature). It seem highly likely that it is the HSPs in the soybean seedling which accumulate during these treatments that provide thermal protection to the subsequent 45°C incubation. However, one report dealing with HS of pollen and the development of thermal tolerance might question this interpretation (1). Since mutations in *E. coli* (34), yeast (24), and *Dictyostelium* (21) which affect HSP synthesis also prevent the development of thermotolerance in the organism, a strong argument can be made for a crucial role(s) of HSPs in the development of protection to otherwise lethal temperatures. This view would indicate that HSPs which accumulate during the permissive HS would be involved in generating a state or condition which allows HS mRNAs and HSPs to be synthesized at otherwise nonpermissive temperatures; the data presented here and earlier (17, 31) show in fact that both HS mRNA and HSP synthesis do occur at 45°C if the seedlings had earlier been exposed to one of the conditions noted above. We envision a role(s) for the HSPs in protecting vital functions and structures (*e.g.* transcription, translation, energy production machinery) during high temperature HS to allow the HS response and to permit normal functions to rapidly return when favorable temperatures are reestablished. It is known that recovery of normal mRNA and protein synthesis occurs rapidly when the temperature is shifted back to normal (*e.g.* 28°C) (15, 30). The resumption of normal protein synthesis utilizes mRNAs conserved during HS (5) as well as that newly synthesized during recovery (30).

The acquisition of thermotolerance appears to depend not only upon the synthesis of HSPs but also on their selective cellular localization. In the soybean seedlings, several HSPs become selectively localized in or associated with nuclei, mitochondria, and ribosomes in a state that causes them to isolate in gradient-purified fractions of these organelles. Specifically, the complex group of 15–18 kD HSPs selectively localize in these fractions during HS of soybean seedlings. Lesser amounts of the 68–70 and 92 kD HSPs seem to localize in this way. Some additional 22–24 kD HSPs seem to selectively localize in the mitochondrial fraction. The 27 kD and 84 kD HSPs seem not to associate with any organelle fractions and to remain as 'soluble' supernatant proteins during HS.

The selective localization of HSPs is temperature dependent. The HSPs (except the 22–24 kD HSPs of the mitochondrial fraction) chase from the organelle fractions during a 4-h incubation at 28°C; they remain organelle-associated during a chase at HS temperature. Arsenite-induced HSPs do not localize at 28°C, but they do become organelle-associated during a subsequent HS. Also, a second HS following a 4-h 28°C chase results in rapid (within 15 min) reassociation of HSPs with the organelle fractions. The level of HSP reassociation during this second HS is also temperature dependent.

The nature of the association of the HSPs with the organelle fractions of soybean is not known. One approach to gaining some insight into this phenomenon would be immunofluorescent localization of some of the HSPs with specific HSP antibodies. Such studies with *Drosophila* have shown that the 70 kD HSP (by far the most abundant HSP in that system) becomes an interband chromosomal protein during HS (3, 19, 26, 32). Other HSPs of *Drosophila* (4) and *Dictyostelium* (22) also seem to become nuclear localized during HS. In chick fibroblasts, the 70 kD protein seems to be primarily a cytoskeleton protein during HS (30). The precipitation of the 70 and 72 kD HSPs of animal cells with a monoclonal antibody to a cell surface glycoprotein has led to the suggestion that HSPs may mediate an association between the cell membrane and cytoskeleton (12). Since there are many more species of HSP in plants (6, 9, 15) than in animal systems, selective association with organelle fractions other than nuclei may be largely restricted to plants (16). While there is not a substantial amount of data available to date, and not complete agreement even among those studies on the specifics of localization of HSPs (5, 30), the data do seem adequate to conclude that selective localization of HSPs during HS is important to their function(s) in the development of the homeostatic state or acquisition of thermotolerance.

The unique structure of the four small HSPs of *Drosophila* may relate to their role in selective localization and development of thermotolerance. The nucleotide sequence of the genes for the four small *Drosophila* HSPs (13) shows a striking homology from position 85 to position 195 (three of the genes have the same nucleotide at 77% of these positions and all four genes have the same nucleotide at 37% of the positions). Further, there is striking homology of this region of these HSPs and the α-crystallin protein of mammals; for a region containing 76 amino acids, the same amino acid in α-crystallin is used at 39 positions in three of the HSPs and at least one of the HSPs has the same amino acid at 53 positions. The derived amino acid sequence of these HSPs shows striking and somewhat alternating regions of hydrophobicity. The known aggregation of α-crystallin protein into 800 kD complexes coupled with the striking homology with these *Drosophila* HSPs is suggestive that these properties may serve as a basis for selective localization of these HSPs and provision of structural interactions which relate directly to thermal protection.

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