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Heat shock proteins of higher plants

(protein patterns/soybean seedlings)

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ABSTRACT **The pattern of protein synthesis changes rapidly and dramatically when the growth temperature of soybean seedling tissue is increased from 28°C (normal) to about 40°C (heat shock). The synthesis of normal proteins is greatly decreased and a new set of proteins, "heat shock proteins," is induced. The heat shock proteins of soybean consist of 10 new bands on one-dimensional NaDodSO$_4$ gels; a more complex pattern is observed on two-dimensional gels. When the tissue is returned to 28°C after 4 hr at 40°C, there is progressive decline in the synthesis of heat shock proteins and reappearance of a normal pattern of synthesis by 3 or 4 hr. *In vitro* translation of poly(A)$^+$RNAs isolated from tissues grown at 28 and 40°C shows that the heat shock proteins are translated from a new set of mRNAs induced at 40°C; furthermore, the abundant class mRNAs for many of the normal proteins persist even though they are translated weakly (or not at all) *in vivo* at 40 or 42.5°C. The heat shock response in soybean appears similar to the much-studied heat shock phenomenon in *Drosophila*.**

Protein synthesis responds rapidly and dramatically to stress in a wide range of organisms. In soybean seedlings exposed to anaerobic conditions or incubation in dinitrophenol there is a fast read-out of polyribosomes which results in a rapid transition from polyribosomes to mostly monoribosomes and a new low rate of protein synthesis (1). Water stress in maize seedlings similarly leads to a rapid loss of polyribosomes and low levels of protein synthesis (e.g., refs. 2 and 3). In the case of anaerobiosis, much of the pre-stress mRNA persists for several hours during the anaerobic treatment in both soybean (1) and maize (4). In the case of maize roots, at least, the anaerobic treatment also results in the synthesis of a small number of new mRNAs and proteins and, as noted above, a greatly decreased (or no) level of translation of the pre-stress mRNAs (4, 5).

These results together with those relating to the much-studied heat shock phenomenon of *Drosophila* (see ref. 6) suggest that these changes in the patterns of mRNA and protein synthesis result from the induction by the stress agent of some regulatory event(s). In *Drosophila* (6) and a number of other systems studied to date (e.g., refs. 7–10) a change from the normal growth temperature to an increased temperature (e.g., 25°C to 37°C in the case of *Drosophila*) results in the shut-off (or reduction) of normal protein synthesis in concert with the induction of a set of "heat shock proteins." The heat shock response seems to be representative of a more general stress response because a wide range of stress agents induce the heat shock proteins in *Drosophila* (6) and a set of similar proteins in other systems (e.g., refs. 7 and 9).

Except for the alcohol dehydrogenases induced by anaerobiosis in maize (4), the identity of the stress proteins remains obscure. There is some progress, however, in localizing at the subcellular level some of the heat shock proteins in *Drosophila* (e.g., refs. 11 and 12).

We have initiated studies to analyze the influence of a number of common stresses encountered by plants on the transcription and translation patterns of the stressed plants. Here we report on the effect of temperature transitions (heat shock) on the pattern of protein synthesis in soybean. A temperature shift from 28–30°C (normal) to 40°C (heat shock) leads to a rapid and dramatic reduction in the level of polyribosomes and a shift in the pattern of protein synthesis. The pattern of induced proteins is reminiscent of the heat shock proteins of *Drosophila* and the anaerobic proteins of maize.

METHODS

Plant Material. Soybean seeds (*Glycine max*, variety Wayne) were germinated either in moist vermiculite or in rolls of moist Chem-pak at 28–30°C in the dark. Two-day-old seedlings (1.5–2.5 cm in length) grown in rolls or 1-cm slices of hypocotyl of 4-day germinated (vermiculite) seedlings (excised 1.5–3.5 cm below the cotyledons) were used in these experiments. The various tissues were incubated in 1% sucrose/1 mM K phosphate, pH 6.0, containing chloramphenicol (50 μg/ml); 2,4-dichlorophenoxyacetic acid (10 μg/ml) was included in the medium for studies with hypocotyl slices.

Polyribosome Isolation. Hypocotyl slices were incubated as described above for the times indicated in Fig. 1. Typically, 10 g of tissue was homogenized in 12 ml of 0.5 M sucrose/30 mM $MgCl_2$/100 mM KCl/200 mM Tris·HCl, pH 8.8/1 mM dithiothreitol (buffer A); diethylpyrocarbonate was added, to 0.1%, immediately before homogenization of the tissue. Homogenization was at full speed for 10–15 sec at 0–4°C in a Polytron equipped with a PT-20 probe. The homogenate was centrifuged at 12,000 rpm for 15 min (Sorvall SS 34 rotor) and filtered through Miracloth. Triton X-100 was added to a final concentration of 1%. Ribosomes were pelleted by layering 16 ml of the cleared homogenate over a 7-ml pad of 1.5 M sucrose containing one-quarter strength buffer A and centrifuging for 2.5 hr at 56,000 rpm in a Beckman Ti 60 rotor. The pellets were suspended in one-quarter strength buffer A and about 6 A_{260} units of ribosomes was layered over an 11-ml linear sucrose gradient (12–36% in the same buffer); this mixture was centrifuged for 1.5 hr at 36,000 rpm in a Beckman SW 41 rotor.

Extraction of *in Vivo*-Labeled Proteins. About 1 g of seedling tissue was incubated with shaking in 5 ml of buffer (as noted above) containing 200–500 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]leucine. The tissue was rinsed, chilled, and homogenized at 0–4°C in 10 ml of 0.5 M ammonium sulfate/0.1% mercaptoethanol/50 mM Tris·HCl, pH 7.9/1 mM EDTA. This was followed by preparation of the proteins as described by Zurfluh and Guilfoyle (13). Alternatively, the tissue was homogenized

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at room temperature in 50 mM Tris·HCl, pH 8.5/2% NaDodSO$_4$/1% mercaptoethanol/1 mM phenylmethylsulfonyl fluoride. In the latter case the homogenate was centrifuged for 30 min at 15,000 rpm and filtered through a layer of Miracloth; the proteins were precipitated with 4 vol of acetone and stored at −20°C. The observed patterns of heat shock protein synthesis were essentially identical with the two methods of protein extraction.

Purification of Poly(A)$^+$RNA. Total RNA was extracted from excised hypocotyl tissue after incubation for 3 hr at either 28°C or 40°C and poly(A)$^+$RNA was purified as described by Silflow and Key (14) except that a 3.0 M NaCl precipitation step was substituted for pelleting the RNA through CsCl prior to oligo(dT) fractionation.

***In Vitro* Translation of Poly(A)$^+$RNA.** Poly(A)$^+$RNAs from hypocotyl slices incubated at 28°C or 40°C were translated in a wheat germ S30 preparation (15) and in the reticulocyte lysate system (16). Typically, 0.5 μg of poly(A)$^+$RNA was translated in 26.3 μl of standard reaction containing 22 μCi of [^{3}H]leucine; in addition, the reaction mixture contained placental RNase inhibitor (17) (kindly provided by T. J. Guilfoyle) and 0.1 mM phenylmethylsulfonyl fluoride. Incorporation ranged from 70,000 to 90,000 cpm/μl of assay mixture for unheated poly(A)$^+$RNA or 100,000–140,000 cpm/μl for heat-denatured (70°C for 1–2 min) poly(A)$^+$RNA; endogenous incorporation was 1500–3000 cpm.

Gel Electrophoresis and Fluorography of *in Vivo*- and *in Vitro*-Synthesized Proteins. Proteins prepared as described above were routinely dissolved in 2.3% NaDodSO$_4$ containing 5% mercaptoethanol and boiled for 1 min. For one-dimensional NaDodSO$_4$ gel electrophoresis according to Laemmli (18), the dissolved proteins were loaded directly at 20,000–50,000 cpm per well. For two-dimensional gels run according to O'Farrell (19), urea and Ampholines (a 1:4 mixture of pH 3.5–10 and pH 5–7 resulting in a pH gradient of 4.5–8.5 after running) were added to final concentrations of 8 M and 0.2%, respectively, to the protein samples; samples loaded contained 500,000–1,000,000 cpm. Fluorography of the gels was accomplished by using EN^{3}HANCE (New England Nuclear) and preflashed Kodak film (XR-5).

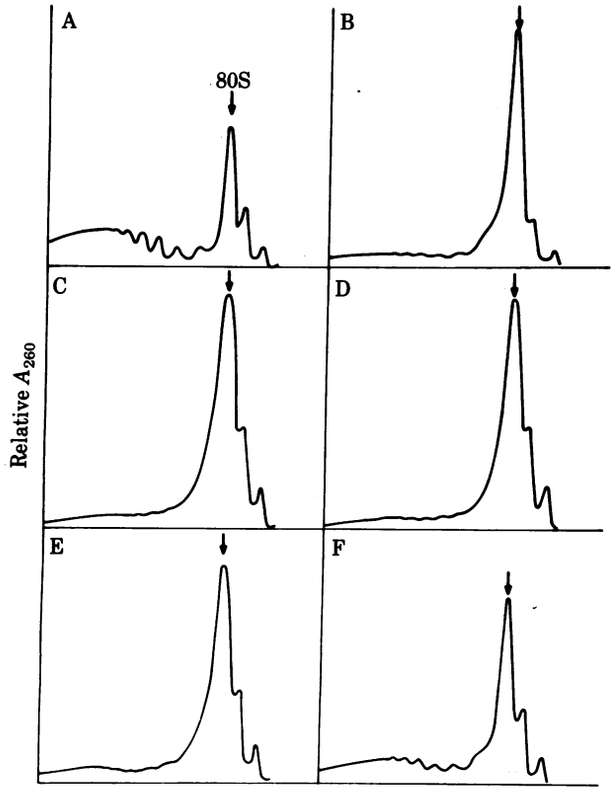

FIG. 1. Polyribosome transitions after transfer of excised soybean hypocotyl from 30°C to 40°C. Polyribosomes were isolated from hypocotyl tissue grown at 30°C without incubation of excised tissue (*A*) or after 2 hr of incubation of excised tissue (*F*) or from tissue incubated at 40°C for 15 min (*B*), 30 min (*C*), 1 hr (*D*), or 2 hr (*E*). Fractionation was achieved on 12–36% linear sucrose gradients. The 80S monoribosome peak is indicated; subunits sedimented to the right and polyribosomes sedimented to the left.

RESULTS

State of Polyribosomes. When excised soybean hypocotyl was incubated at 40°C, there was a rapid shift of polyribosomes into monoribosomes and subunits (Fig. 1). This transition was maximal within 15–30 min and was followed by the accumulation of a much reduced level of polyribosomes by 2 hr. These transitions occurred against the backdrop of a gradual decrease in the relative level of polyribosomes in the 30°C incubated excised hypocotyl during incubation. Also, the total level of ribosomes recovered from heat shocked tissue after a 2-hr incubation was decreased by 20–30% (data not shown), indicating a possible degradation of ribosomes at the higher temperature. Also, 40°C was the near optimal temperature for the polyribosome-to-monoribosome transition during the initial 1 hr of treatment.

***In Vivo* Patterns of Protein Synthesis.** Fig. 2 shows the patterns of protein synthesis in tissues incubated at temperatures from 30 to 42.5°C. Several new bands (indicated by arrows) of proteins were induced whereas other bands were relatively increased or decreased dramatically, the relative change dependent upon the incubation temperature. Assessment of the relative rates of protein synthesis under the different temperature regimens is complicated by effects of the higher temperatures on amino acid uptake. However, the results from several experiments indicate that the relative rate of total protein synthesis is decreased only 20–40% at temperatures up to 40°C (data not shown); above 40°C, the relative incorporation decreased precipitously but again the interpretation is affected by greatly reduced uptake of [^{3}H]leucine. The pattern of proteins synthesized at 42.5°C compared to that at lower temperatures suggests that the large decline in [^{3}H]leucine incorporation between 40 and 42.5°C resulted from an almost complete inhibition of synthesis of those proteins synthesized normally at the lower temperatures with continued synthesis of the heat shock proteins. There was a gradual increase in relative synthesis of the heat shock proteins as the temperature increased from 30°C up to 40°C; above 37.5°C there was a marked loss in capacity to synthesize the normal proteins, and little or no synthesis of them occurred at 42.5°C. This new pattern of protein synthesis resulting from heat shock was common to the root and hypocotyl of intact seedlings and to excised hypocotyl slices (data not shown).

This new pattern of protein synthesis was induced during the first hour of incubation at 40°C and persisted for at least 4 hr (Fig. 3). The relative proportion of labeling in the heat shock proteins increased between hr 1 and hr 2 at 40°C and was maintained through hr 4. A similar spectrum of proteins was synthesized during the next 4-hr period at 40°C (data not shown).

Recovery from the heat shock state with resumption of the synthesis of a normal pattern of proteins was relatively rapid after return of the tissue to 30°C after 4 hr at 40°C (Fig. 4). Within 1 hr, and more obvious by 2 hr, there was a shift from synthesis of predominantly heat shock proteins to synthesis of

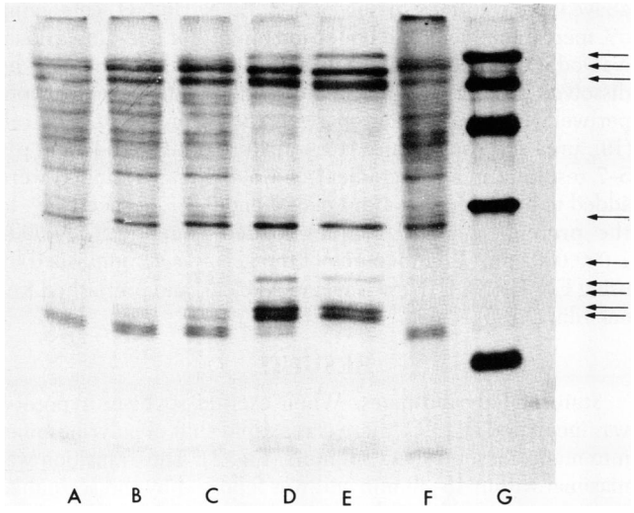

FIG. 2. Optimal temperature for induction of the heat shock proteins. Excised hypocotyl tissues were incubated at various temperatures for 3 hr; 300 μCi of [^{3}H]leucine was added for the final 2 hr of incubation. The proteins were fractionated on NaDodSO$_4$/polyacrylamide gels and visualized by fluorography. Lanes: A, 30°C; B, 35°C; C, 37.5°C; D, 40°C; E, 42.5°C; F, 30°C; G, molecular weight standards in order of decreasing size: 92,500, 69,000, 46,000, 30,000, and 12,300. Arrows denote major heat shock bands.

the normal 30°C proteins; this transition continued through hr 3 of recovery, resulting in the synthesis of an essentially normal pattern of proteins during hr 4 at 30°C.

The pattern of heat shock proteins was simple on the one-dimensional NaDodSO$_4$ gels shown above. It was more complex when the proteins were fractionated on two-dimensional O'Farrell gels (Fig. 5); each of the major heat shock bands resolved into two or more protein spots on these gels. For example, the two major low molecular weight heat shock bands (15,000–18,000) of the NaDodSO$_4$ gels resolved into at least 8–10 components on the two-dimensional gels. Thus it was somewhat difficult to determine the exact number of the proteins induced by the heat shock treatment.

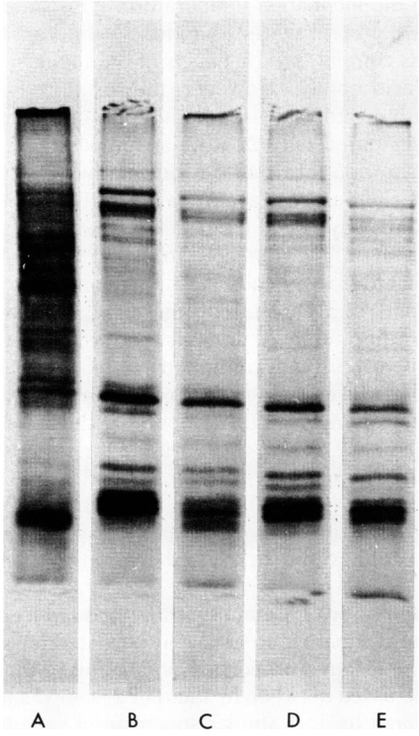

FIG. 3. Induction of heat shock proteins. Fluorogram of NaDodSO$_4$/polyacrylamide gel fractionated proteins isolated from excised hypocotyl incubated for varying times after transfer to 40°C and labeled in 500 μCi of [^{3}H]leucine. Lanes: A, 30°C, labeled 4 hr; B, 40°C, labeled 4 hr; C, 40°C, labeled during first hour; D, 40°C, labeled during second hour; E, 40°C, labeled during fourth hour.

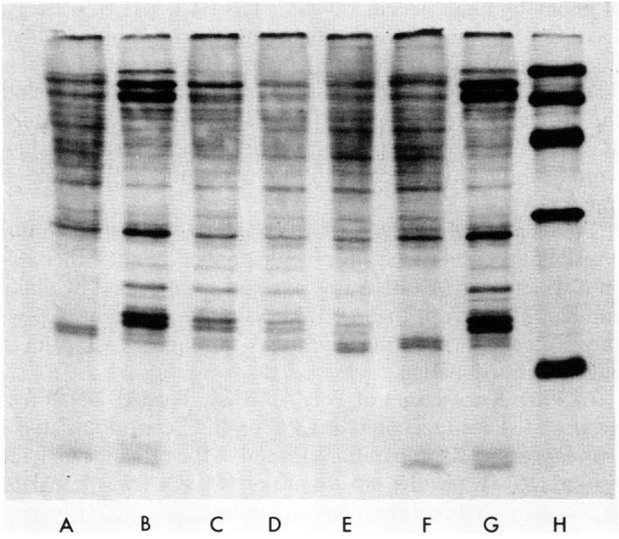

FIG. 4. Recovery from heat shock. NaDodSO$_4$/polyacrylamide gel fractionation of proteins labeled for 1 hr with 500 μCi of [^{3}H]leucine at various times after transfer of tissue from 40°C to 30°C. Lanes: A, 4-hr label at 30°C; B, 4-hr label at 40°C; C, label during first hour at 30°C after 4 hr at 40°C; D, label during second hour at 30°C after 4 hr at 40°C; E, label during third hour after 4 hr at 40°C; F, label during fourth hour after 4 hr at 40°C; G, 40°C, labeled 4 hr as in B; H, molecular weight standards as in Fig. 2.

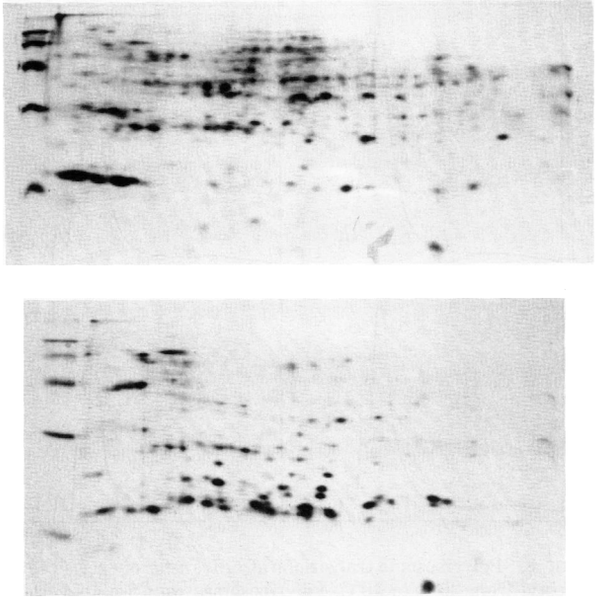

FIG. 5. Fluorogram of two-dimensional gels of control (*Upper*) and heat shock (*Lower*) proteins. Soybean seedlings were incubated for 4 hr at the indicated temperature, with 500 μCi of [^{3}H]leucine being added after 1 hr of incubation.

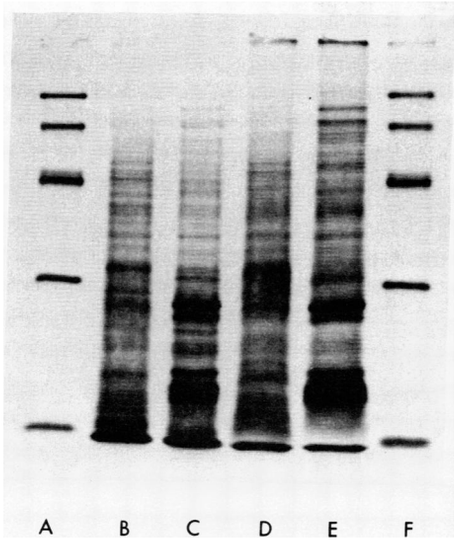

FIG. 6. *In vitro* translation products of poly(A)$^+$RNA isolated from control (lanes B and D) and heat shock (lanes C and E) excised hypocotyl tissue shown as fluorogram of NaDodSO$_4$/polyacrylamide gels of ^{3}H-labeled proteins synthesized in a wheat germ (lanes B and C) or reticulocyte lysate (lanes D and E) system. Lanes A and F, molecular weight standards as in Fig. 2.

***In Vitro* Translation of Poly(A)$^+$RNAs.** Poly(A)$^+$RNA was purified from hypocotyl slices that had been incubated for 3 hr at 30°C or at 40°C. These RNAs were translated in a standard wheat germ or reticulocyte lysate system and fractionated on one-dimensional NaDodSO$_4$ gels (Fig. 6). In general the translation products were similar for the 30°C and 40°C RNAs except that an additional eight or so bands were translated from 40°C RNA and these appear to correspond to the major *in vivo* labeled heat shock proteins. The relative intensity was much greater for the low molecular weight than for the higher molecular weight heat shock bands in comparison to the relative intensity of the *in vivo*-labeled heat shock proteins. This difference likely reflects the lower efficiency of translation of large mRNAs relative to smaller ones in the *in vitro* protein synthesizing systems. This does not detract, however, from the fact that the appearance of the new set of proteins synthesized *in vivo* at 40°C results from the production of a new set of mRNAs for the heat shock proteins.

Additionally, it appears that many of the normal 30°C abundant mRNAs persist at the higher temperature even though they were poorly translated *in vivo* at 40 or 42.5°C. As with the *in vivo*-labeled proteins (Fig. 5), the two major low molecular weight (15,000–18,000) bands of *in vitro*-synthesized heat shock proteins resolved into 8–10 protein spots in the two-dimensional gel system (data not shown). This suggests that the several protein components of *in vivo*-labeled proteins as seen on two-dimensional gels do not result from postsynthetic modification of a common protein resulting in altered pIs of that protein but rather are translated from different mRNAs.

DISCUSSION

Different tissues of the soybean seedling respond to temperature transitions (heat shock) in a manner somewhat analogous to animal systems including the much studied heat shock response in *Drosophila* (6). A similar pattern of heat shock protein synthesis has been reported recently for tobacco and soybean cells growing in solution culture (20) as that reported here for soybean seedling tissue. After a shift of soybean seedlings from the normal growing temperature of about 30°C up to 40°C there is a rapid (within 15 min) conversion of polyribosomes into monoribosomes followed by reappearance and maintenance of a low level of polyribosomes similar to that reported for heat-shocked *Drosophila* (e.g., ref. 21). There is a much decreased translation of the 30°C mRNAs at 40–42.5°C associated with the initiation of translation of a set of poly(A)$^+$mRNAs which give rise to a new set of proteins, commonly referred to as heat shock (or stress) proteins. On one-dimensional NaDodSO$_4$ gels the pattern of heat shock proteins appears similar to that of the *Drosophila* heat shock proteins, especially the bands in the 65,000–85,000 molecular weight range; the major low molecular weight heat shock protein bands of soybean are relatively smaller, however. Also the number of the major heat shock proteins of soybean appears to be greater.

Both the induction of and recovery from the heat shock response are fast. The transitions occur early after the shift from 30°C to 40°C (or the reverse), with appearance of the new pattern of proteins occurring within the first hour of the shift; a near "steady-state" pattern is achieved during the first 2 hr after this shift up. The normal 30°C pattern of protein synthesis is not achieved until about 3 hr after the shift down.

The *in vitro* translation of poly(A)$^+$RNA from normal and heat shocked tissue coupled with the *in vivo* patterns of protein synthesis at the two temperatures indicate that (*i*) new mRNAs are synthesized in response to heat shock for induction of the heat shock proteins and (*ii*) some translational event renders the 30°C mRNAs poorly translatable at 40–42.5°C even though many of the abundant (at least) poly(A)$^+$RNAs persist at the higher temperature for some time. These observations are consistent with and similar to those on the induction of the heat shock proteins in *Drosophila* (6, 22, 23) and the anaerobic proteins in maize (4).

The data reported here are consistent with the rapid induction of synthesis of a new set of mRNAs by heat shock and a rapid turn-off of their synthesis and turn-over of the already synthesized heat shock mRNAs upon return to the normal temperature. Preliminary experiments using cloned DNAs complementary to total poly(A)$^+$RNA from heat shock tissue (40°C for 3 hr) indicate that these conclusions are correct (unpublished data).

If, as suggested for the heat shock (stress) response in *Drosophila* (6), the response is homeostatic and designed to "correct" or protect from abnormalities in cellular processes, one would expect this to be a highly conserved genetic system. Certainly, crop plants such as soybeans growing under normal environmental conditions of our summers experience diurnal temperature variations, and often higher temperatures, similar to those used in this study. Thus, a rapidly responding heat shock system such as that indicated by these studies may play an important role in plant survival under extremely high temperatures. A similar suggestion was made for the anaerobic proteins of maize and survival under flooding conditions (4).

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1. Lin, C. Y. & Key, J. L. (1967) *J. Mol. Biol.* **26**, 237–247.
2. Hsiao, T. C. (1970) *Plant. Physiol.* **46**, 281–285.
3. Morilla, C. A., Boyer, J. S. & Hageman, R. H. (1973) *Plant. Physiol.* **51**, 817–824.
4. Sachs, M. M., Freeling, M. & Okimoto, R. (1980) *Cell* **20**, 761–767.
5. Sachs, M. M. & Freeling, M. (1978) *Mol. Gen. Genet.* **161**, 111–115.
6. Ashburner, M. & Bonner, J. J. (1979) *Cell* **17**, 241–254.
7. Keeley, P. M. & Schlesinger, M. J. (1978) *Cell* **15**, 1277–1286.

8. Keeley, P. M., Aliperti, G. & Schlesinger, M. J. (1980) *J. Biol. Chem.* **255**, 3230–3233.
9. Guttman, S. D., Glover, C. V. C., Allis, C. D. & Gorovsky, M. A. (1980) *Cell* **22**, 299–307.
10. McAlister, L. & Finkelstein, D. B. (1980) *J. Bacteriol.* **143**, 603–612.
11. Velazquez, J. M., DiDomenico, B. J. & Lindquist, S. (1980) *Cell* **20**, 679–689.
12. Arrigo, A.-P., Fakon, S. & Tissieres, A. (1980) *Dev. Biol.* **78**, 86–103.
13. Zurfluh, L. L. & Guilfoyle, T. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 357–361.
14. Silflow, C. & Key, J. L. (1979) *Biochemistry* **18**, 1013–1018.
15. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2330–2334.
16. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256.
17. Blackburn, P. (1979) *J. Biol. Chem.* **254**, 12484–12487.
18. Laemmli, U. K. (1970) *Nature (London)* **277**, 680–685.
19. O'Farrell, P. (1975) *J. Biol. Chem.* **250**, 4007–4021.
20. Barnett, T., Altschuler, M., McDaniel, C. N. & Mascarenhas, J. P. (1980) *Dev. Gen.* **1**, 331–340.
21. McKenzie, S. S., Henikoff, S. & Meselson, M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1117–1121.
22. Tissieres, A., Mitchell, H. K. & Tracy, U. M. (1974) *J. Mol. Biol.* **84**, 389–398.
23. Mirault, M.-E., Clearmont-Goldschmidt, M., Moran, L., Arrigo, A. P. & Tissieres, A. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 819–827.