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Heat Shock Proteins in Maize[1]

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PAM COOPER AND TUAN-HUA DAVID HO[2]
Department of Botany, University of Illinois, Urbana, Illinois 61801

ABSTRACT

The pattern of protein synthesis in roots of 3-day-old maize seedlings (*Zea mays* L.) is rapidly and dramatically altered when the incubation temperature is raised from 25 to 40°C. One-dimensional sodium dodecyl sulfate gels reveal that although synthesis of the proteins observed at 25°C continues at 40°C, a new set of 'heat shock proteins' (hsp) is induced within 20 minutes of the temperature transition. The hsp have molecular weights of 87, 85, 79, 78, 77, 72, 70, 27, 22, and 18 kilodaltons. The 10 hsp are visible on autoradiograms but not on stained gels, suggesting that the proteins do not accumulate to any great extent.

The induction of the hsp is transitory. With prolonged high temperature treatment, the synthesis of hsp continues for 4 hours in excised roots and for 8 hours in the roots of intact seedlings before declining sharply. Coincident to the decline in synthesis of the 10 hsp is the gradual increase in intensity of three new polypeptides having molecular weights of 62, 49.5, and 19 kilodaltons. These proteins begin to appear about the time that synthesis of the other 10 hsp becomes maximal.

Shifting the temperature back to 25°C also causes a decline in synthesis of hsp, but this decline occurs more rapidly than that seen during prolonged heat shock. A decrease in hsp synthesis becomes apparent 2 hours after the roots are returned to 25°C.

Shifting the temperature from 25 to 45°C results in a pattern of protein synthesis different from that observed after a shift to 40°C. Normal protein synthesis continues, except four proteins, which are produced in small amounts at lower temperatures, show greatly enhanced synthesis at 45°C. These proteins have apparent molecular weights of 83, 81, 68, and 65 kilodaltons. Also, the 10 hsp listed above are not synthesized. It is suggested that at least two distinct high-temperature responses are present in maize, which may reflect the metabolic changes generated at different elevated temperatures.

Protein synthesis in plants responds dramatically to changes in environmental conditions. Water stress leads to the rapid loss of polyribosomes and low levels of protein synthesis in many plants (for review, see 9) including maize seedlings (23). Similar loss of polyribosomes during desiccation occurs in the moss *Tortula ruralis* (4).

Stress also leads to alterations in the pattern of protein synthesis in plants. *Avena* coleoptiles exhibit differential synthesis and degradation of proteins during osmotic stress (5, 6). Nutrient stress in pea root meristem induces the synthesis of a single new polypeptide when overall protein synthesis is declining (30). These observations suggest that a particular stress may induce specific changes in protein synthesis that will enable the plant to cope with that stress. This hypothesis is borne out in the case of maize growing under anaerobic conditions. During anaerobiosis, there is a marked shift in the pattern of protein synthesis. Aerobic protein synthesis ceases, and the synthesis of a few novel proteins begins (26). Among these new anaerobic proteins are the isozymes of alcohol dehydrogenase (25). Presumably, the induction of these proteins allows survival of the plants under conditions of flooding. More recently, it has been reported that high temperatures induce the synthesis of a novel set of hsp[3] in soybean (2, 12) and tobacco (2).

This so-called heat shock response has been most extensively studied in the fruit fly, *Drosophila melanogaster*, the organism in which this phenomenon was first observed (for review, see 1). When *Drosophila* cells are shifted from their normal growth temperature (25°C) to an increased temperature (37°C), there is a cessation of normal protein synthesis with the concomitant synthesis of a small set of hsp. The hsp are not detectable at 25°C and their synthesis at 37°C is rapid; they appear as early as 20 min after heat shock begins (1). The messages coding for the hsp result from new transcription and are preferentially translated during heat shock. That is, normal mRNA are rapidly cleared from the ribosomes to make way for the translation of heat shock mRNA.

While high temperature activates transcription of heat shock genes, it completely represses the transcription of nearly all the other genes. When *Drosophila* cells are returned to 25°C, normal transcription and translation resume, and hsp synthesis gradually declines (17). When heat-shocked cells are returned to 25°C and the resumption of normal transcription is prevented with Actinomycin D, normal protein synthesis will still return (17). Also, *in vitro* translation of mRNA isolated from heat-shocked cells yields both hsp and normal proteins (17). Taken together, these data indicate that normal messages are not degraded during heat shock, but rather are sequestered in some fashion such that they may be reused when the temperature returns to normal. Clearly, the response of *Drosophila* to heat shock is a highly controlled event at both transcriptional and translational levels.

Drosophila, soybean, and tobacco are not the only organisms possessing a heat shock response. Hsp have recently been reported in a variety of organisms, including bacteria (31), birds (10), fish (13), mammals (28), slime molds (18), and yeast (21). The similarity to the heat shock response of *Drosophila* varies from organism to organism. For instance, the heat shock response of soybean strongly resembles that of *Drosophila* (12), whereas the response in yeast is quite different (21).

While some laboratories have localized hsp at the subcellular level in *Drosophila* (29) and higher plants (F. Schoeffl, personal communication), the precise physiological function of the hsp is unknown. They appear to provide for thermotolerance in some manner because the presence of hsp increases the survival rates of *Drosophila* (22) and *Dictyostelium discoideum* (18) when they are subjected to severe temperature stress.

Here we report that corn root tissue also exhibits a heat shock

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[2] To whom reprint requests should be addressed.

[3] Abbreviations: hsp, heat shock protein(s); MS, Murashige and Skoog culture medium.

response. Elevated temperatures induce the synthesis of a set of hsp in both excised roots and roots of intact seedlings. Based on the complexity of hsp induction, we suggest that at least two distinct high-temperature responses are present in maize, which may reflect the metabolic changes generated by two different elevated temperatures.

MATERIALS AND METHODS

Plant Materials and Incubation Conditions. Corn seeds (*Zea mays* L. var A632 × C1042, obtained from Crow's Hybrid Corn Company, Milford, IL) were sterilized for 1 min in 2% NaOCl and rinsed thoroughly with distilled H_2O. The seeds were placed embryo-side down in glass trays upon paper towels kept moist with 0.1 mM $CaCl_2$ solution and germinated in the dark at 29°C. Three-d-old seedlings were used in both intact plant and excised tissue studies.

For experiments involving excised tissue, 1-cm-long root tips were removed from the primary roots by cutting the tips under water with a new razor blade. Each tip was transferred in a droplet of water to a flask containing the major and minor salt solutions of MS, pH 5.5 (24). The flasks were shaken at 120 cycles/min in a constant-temperature bath.

Intact plants still connected to the endosperm were supported on racks and kept in darkened, constant-temperature containers at 100% humidity to prevent dehydration of exposed root surfaces. One rack supported 3 to 4 plants. The last cm of each primary root was suspended in vials containing 1 ml of MS salts.

Although the same results may be obtained using other types of incubation media (*e.g.* 0.1 mM $CaCl_2$ solution or distilled H_2O), all of the experiments described below were performed using MS salts. To prevent excessive bacterial growth during long-term studies, no exogenous carbon source was ever included in the medium. Unless otherwise indicated, experiments were performed using excised root tips.

***In Vivo* Labeling and Extraction of Proteins.** Five excised root tips were incubated in 5 ml MS salts until just prior to pulse labeling with [^{35}S]methionine (>1,000 Ci/mmol; obtained from Amersham). At this point, the medium was removed. The tips were rinsed with fresh medium of the appropriate temperature, and then resuspended in 1 ml fresh medium. Roots were labeled with 25 to 50 μCi/ml of [^{35}S]methionine (see "Results" and figure legends for specific labeling times). After labeling, the medium was removed and the roots rinsed with 1 mM nonradioactive methionine. The roots were weighed and then homogenized in the SDS gel loading sample buffer described by Laemmli (14). The homogenate was boiled for 2 min, then centrifuged for 3 min in an Eppendorf microfuge. The resulting supernatant was analyzed for total uptake of [^{35}S]methionine by counting aliquots dissolved in ACS Scintillation Fluid (Amersham). Incorporation of [^{35}S] methionine into protein was analyzed by TCA precipitation as described by Mans and Novelli (20).

For pulse labeling of roots on intact seedlings, 50 μCi/ml of [^{35}S]methionine was introduced into the vials containing 1 ml of MS salts. Labeling was carried out during the last h of the incubation. After labeling, 1-cm primary root tips were excised from 3 to 4 plants and processed in the manner described above.

All tissue extracts were stored at −20°C.

Gel Electrophoresis of Proteins. *In vivo* synthesized proteins were analyzed by one-dimensional SDS polyacrylamide gel electrophoresis as described by Laemmli (14). The following mol wt standards were run alongside the extracted proteins: phosphorylase B, 92.5 kD; BSA, 66.2 kD; ovalbumin, 45.0 kD; carbonic anhydrase, 31.0 kD; and lysozyme, 14.4 kD.

Either the same number of cpm or the same amount of total extracted protein was loaded into each sample well of 10% polyacrylamide slab gels. Soluble proteins were acetone precipitated to remove SDS and β-mercaptoethanol, and the protein content was determined by the method of Lowry *et al.* (19). Gels were stained in a solution containing 0.025% Coomassie blue R, 25% isopropanol, and 10% acetic acid. They were destained in 7.5% acetic acid.

Autoradiography and Fluorography of *In Vivo*-Synthesized Proteins. After destaining, some gels were prepared for fluorography by treatment with PPO in DMSO prior to drying (3, 15). Autoradiography and fluorography were performed on dried gels using Kodak SB-5 X-ray film. Fluorography was carried out at −80°C.

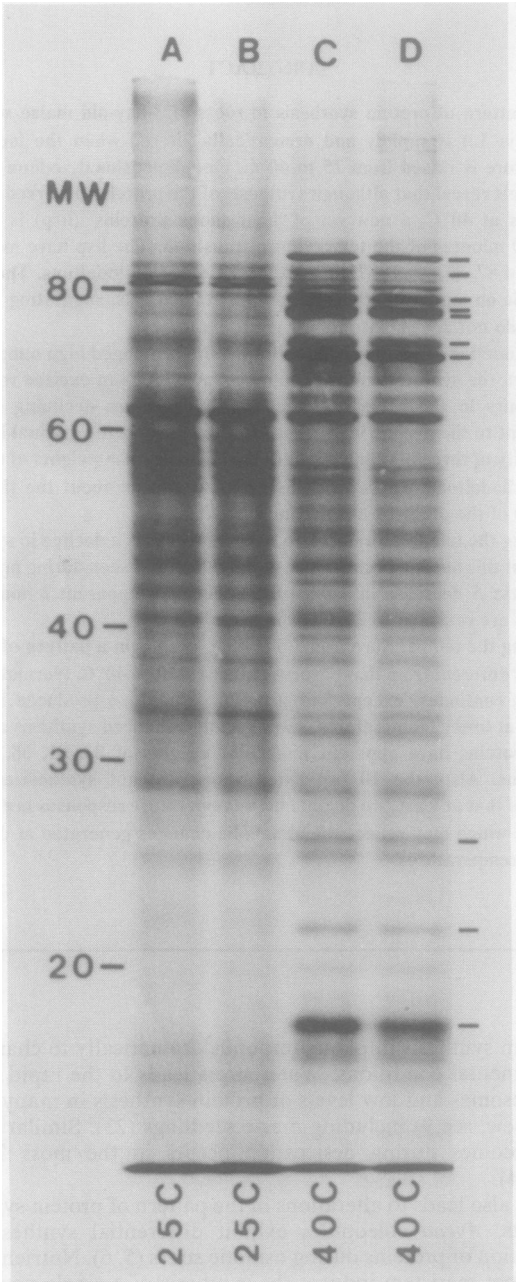

FIG. 1. Pattern of protein synthesis in excised maize roots subjected to 25°C (lanes A and B) or 40°C (lanes C and D) temperatures. One-cm-long root tips were excised from 3-d-old maize seedlings and preincubated ('washed') at 25°C for 4 h prior to further incubation for 2 h at either 25 or 40°C. Labeling with 25 μCi/ml [^{35}S]methionine took place in the final 2 h of incubation. Proteins were separated on a 10% SDS gel and visualized by autoradiography. Mol wt distribution in kD is indicated at the left, and hsp are indicated by bars at the right.

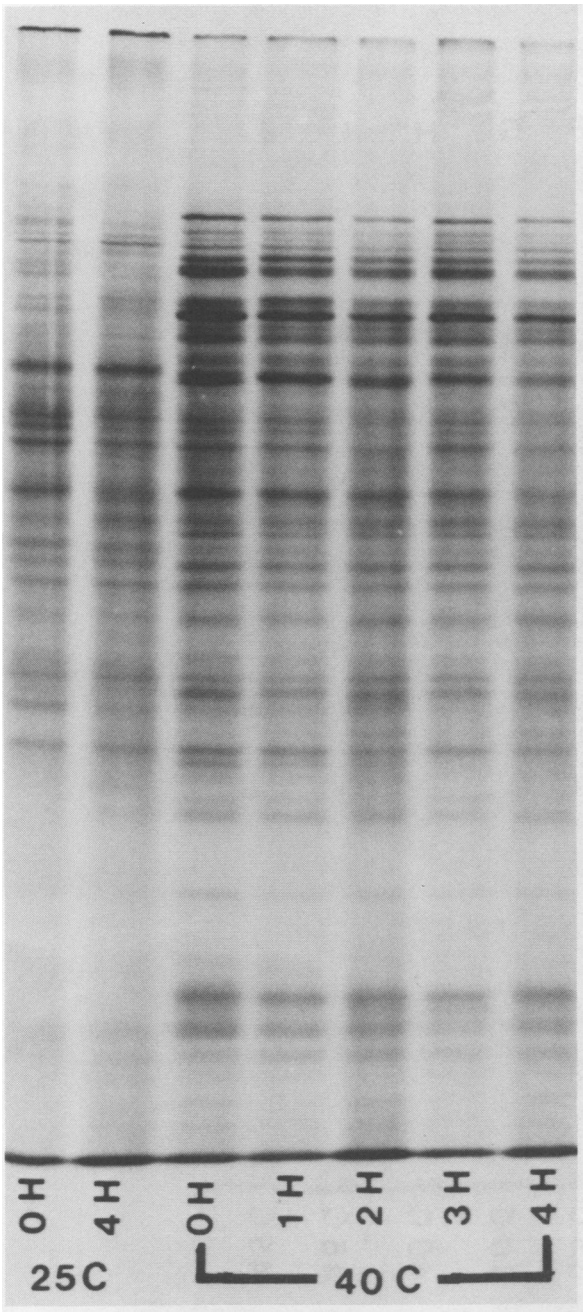

FIG. 2. Effect of preincubation (washing) after excision on normal and hsp synthesis. Roots were excised and incubated for increasing lengths of time at 25°C prior to an additional 1 h of incubation at 25 or 40°C. Labeling with 25 μCi/ml [^{35}S]methionine was carried out in the final hour of incubation. The proteins were separated on a 10% SDS gel and visualized by autoradiography. Temperatures indicated are those used in the final hour of the incubation. Times refer to hours of washing prior to the final hour of temperature treatment.

RESULTS

When excised root tips are transferred to 40°C, there is a significant alteration in the pattern of protein synthesis as compared to control roots incubated at 25°C. As shown by the autoradiogram in Figure 1, lanes C and D, at least 10 new hsp (indicated by bars) are synthesized in response to the elevated temperature, and have apparent mol wt of 87, 85, 79, 78, 77, 72, 70, 27, 22, and 18 kD. These proteins are completely absent in control tissue (Fig. 1, lanes A and B). Most of the proteins synthesized at 25°C continue to be produced at 40°C, so that hsp synthesis occurs in addition to the 'normal' pattern of synthesis when roots are subjected to elevated temperatures.

Effect of Excision on the Heat Shock Response. Gronewald and Hanson (8) have shown that excision injury markedly reduces ion transport capabilities in corn roots, and that preincubating the tissue for 4 hours ('washing') will completely restore these activities to levels seen in undamaged tissues. In our early studies, it was found that freshly cut (unwashed) root tips, in some cases, would not exhibit the heat shock response. Because of this, the following experiments were performed to determine whether excision injury renders the root incapable of responding to high-temperature stress, and whether this ability can be restored by washing the tissues prior to heat shock.

Roots were excised and subjected to 1 h of heat shock after 0, 1, 2, 3, or 4 h of washing at 25°C. The labeling of tissue with [^{35}S] methionine was begun simultaneously with temperature elevation. Control roots were labeled for 1 h at 25°C after 0 or 4 h of washing at 25°C.

As the 25°C control lanes in Figure 2 show, excision itself does not induce the synthesis of stress proteins. Also, cutting injury does not seem to prevent the induction of hsp as seen by the 40°C, 0 h lane. It is not understood why a heat shock response was not exhibited in freshly cut roots in the early experiments.

Because washed tissue consistently gives a heat shock response, all subsequent experiments using excised tissue were performed following a 4 h preincubation at 25°C.

Effect of Temperature on hsp Synthesis. Root tips were subjected to various temperatures for 2 h to determine the effect of temperature on the induction of hsp. As shown in Figure 3, temperature affects the strength and pattern of hsp synthesis. The optimum temperature for the induction of the 10 hsp described above is 40°C, although a lesser response is also observed at 35°C. Little or no induction is seen at temperatures below 35°C. Temperature elevations up to 40°C have no effect on the normal or background pattern of protein synthesis.

A striking change in the pattern of synthesis occurs at 45°C. The background pattern is altered to some extent, particularly in the 40 to 60 kD mol wt range. However, most notable is the disappearance of the 10 hsp synthesized at 40°C and the appearance of four other proteins having apparent mol wt of 83, 81, 68, and 65 kD. These proteins are present at lower temperatures, but at 45°C their synthesis is greatly pronounced. Temperatures above 45°C almost entirely inhibited both normal and hsp synthesis (data not shown).

Time Course of hsp Synthesis: Excised *versus* Intact Tissues. Figure 4 shows the time course of protein synthesis for heat-shocked root tips. The heat shock response is rapid. Figure 4, lane A, shows that hsp can be detected as early as 20 min after the temperature is raised. But the synthesis of hsp is transient. The protein bands on autoradiograms increase in intensity until 3 to 4 h (Fig. 4, lanes D and E), after which synthesis of the 10 hsp declines. This decline is most rapid between 4 and 6 h, and by 10 h the proteins are barely detectable or absent. Accompanying the decline in synthesis of the 10 original hsp is the appearance of three new proteins having mol wt of 62, 49.5, and 19 kD. These proteins begin to appear about the time that synthesis of the 10 hsp becomes maximal, and the intensity of these new proteins increases through 10 h of 40°C treatment. Except for slight increases in hsp 70, 77, and 78, most of the hsp are not detectable on Coomassie blue stained one-dimensional gels (Fig. 5). They also cannot be detected on two-dimensional gels using the highly sensitive silver staining technique (27, data not shown). The fact that hsp are visible on one- and two-dimensional gels only by autoradiography suggests that most of these proteins do not accumulate in great quantities. At no time during prolonged incubation of tissue at 25°C were any hsp detected (data not shown).

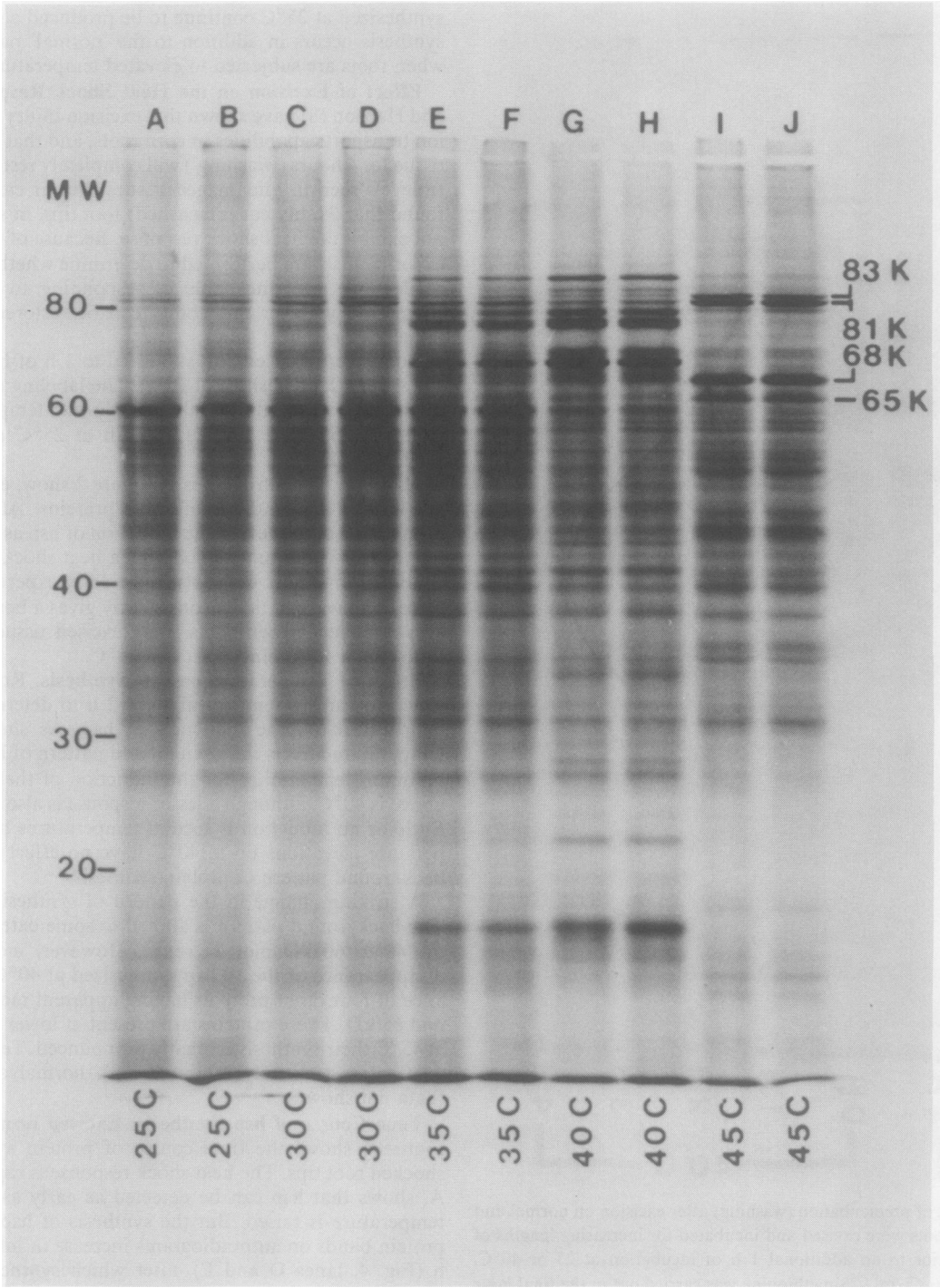

FIG. 3. Optimum temperature for the induction of hsp. Excised roots were incubated at various temperatures for 2 h after 4 h washing at 25°C. Labeling with 25 μCi/ml [^{35}S]methionine was carried out in the final 2 h of the experiment. Proteins were separated on a 10% SDS gel and visualized by autoradiography. Lanes: A and B, 25°C; C and D, 30°C; E and F, 35°C; G and H, 40°C; I and J, 45°C. Mol wt distribution in kD is indicated at the left. Numbers at right indicate mol wt of proteins whose synthesis is enhanced at 45°C.

But is the transient synthesis of hsp an inherent part of the response, or is it artifactual, resulting from damage of the tissue by nutrient stress, prolonged heat stress, or their combined effects? There is a gradual but steady decline of uptake and incorporation of [^{35}S]methionine with time for both control and heat-shocked tissue. However, heat shock does not accelerate this decline (data not shown). These results suggest that the transitory nature of the response is not due to accelerated tissue death from high temperatures, but that nutrient stress might be a factor.

To eliminate the variable of nutrient stress, intact seedlings, to which the endosperm was still attached, were subjected to heat shock for increasing lengths of time. Figure 6 shows the results of such an experiment. Lanes A to D show that no hsp were synthesized in the root tips of intact seedlings incubated continuously at 25°C. Lanes E to H indicate that at 40°C, hsp are produced for up to 8 h before their synthesis declines by 10 h. Thus, it appears that the heat shock response is inherently transient, because synthesis declines after 10 h even in intact seedlings still connected to the endosperm. However, we cannot completely rule out the possibility that the transient response seen in intact

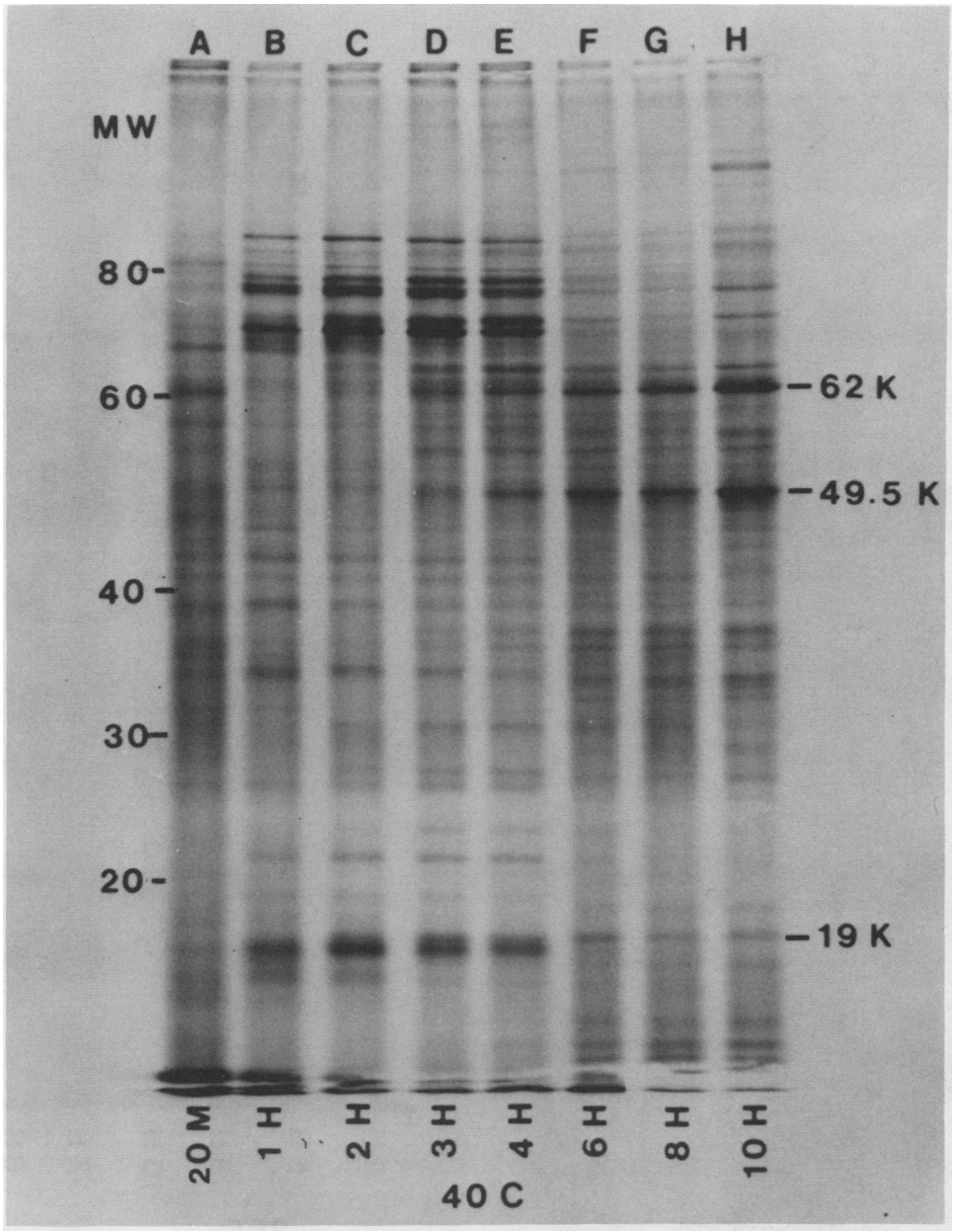

FIG. 4. SDS gel analysis of proteins synthesized by excised maize roots incubated at continuous 40°C. Roots were washed for 4 h at 25°C then transferred to 40°C and incubated for increasing times. Labeling with 50 μCi/ml [^{35}S]methionine was carried out in the final 20 min of the incubation. Proteins were visualized by autoradiography. Lanes: A, 20 min; B, 1 h; C, 2 h; D, 3 h; E, 4 h; F, 6 h; G, 8 h; H, 10 h incubation at 40°C. Mol wt distribution in kD indicated at left. Numbers at right indicate mol wt of proteins synthesized after 6, 8, or 10 h of 40°C treatment.

seedlings may also be a consequence of nutrient deprivation. Prolonged high temperature may interfere with transport of energy reserves out of the endosperm.

As in excised roots, the original 10 hsp do not accumulate in intact roots. However, the appearance of the 62, 49.5, and 19 kD proteins in intact seedlings is variable. Although they are not evident in Figure 6, we have observed them in other experiments performed in an identical fashion.

Recovery from Heat Shock. Figure 7 shows the effect of shifting heat-shocked tissues back to 25°C. Root tips were subjected to 40°C temperature for 90 min and then returned to 25°C for 2, 5, 7, or 9 h. Control tissues were maintained at either constant 25 or 40°C for 90 min. Pulse labeling with [^{35}S]methionine was carried out in the last 30 min of the incubation.

Recovery from heat shock is relatively rapid. As Figure 7, lane C indicates, synthesis of hsp decline after root tips have been transferred back to 25°C for 2 h. After 5 h at 25°C, the hsp are barely detectable (Fig. 7, lane D). After 7 h, the heat-shock pattern is completely absent (Fig. 7, lanes E and F).

DISCUSSION

Elevated temperatures (35–40°C) induce the synthesis of a set of 10 hsp in the roots of maize seedlings. It is unlikely that these proteins are merely the consequence of changes in turnover rates of stability of proteins because in a 2-h period of heat shock, both long term (2 h) and short term (last 20 min of heat shock) labeling yields the same pattern of protein synthesis.

The size distribution of maize hsp is similar but not identical to that of soybean (12). One of the maize hsp has a mol wt of 70 kD. A 70 kD hsp has been reported in numerous eukaryotes (1, 10, 12, 13, 18). Furthermore, antiserum to the 70 kD hsp from chicken

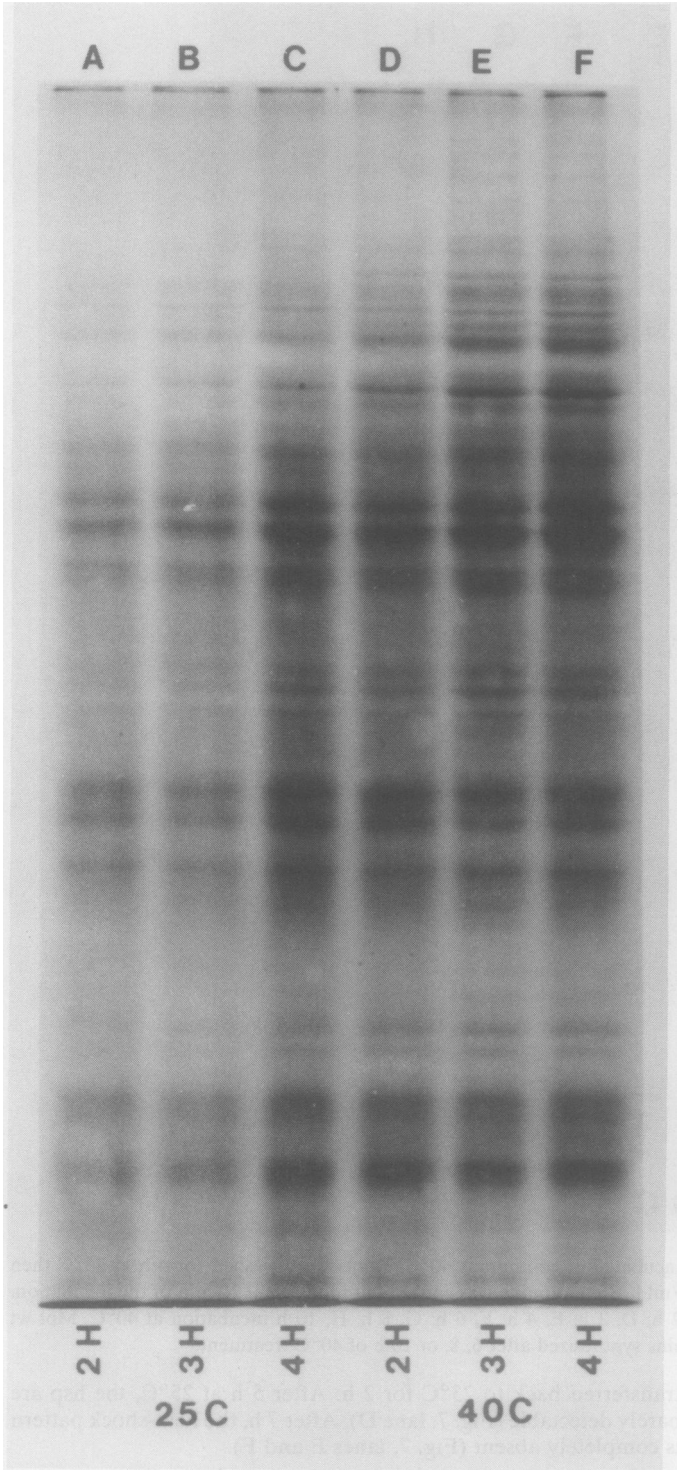

FIG. 5. SDS gel analysis of proteins from excised maize roots incubated at 2, 3, or 4 h at 25 or 40°C after washing for 4 h at 25°C. Each lane contains the same amount of protein. Proteins were visualized by staining with Coomassie blue.

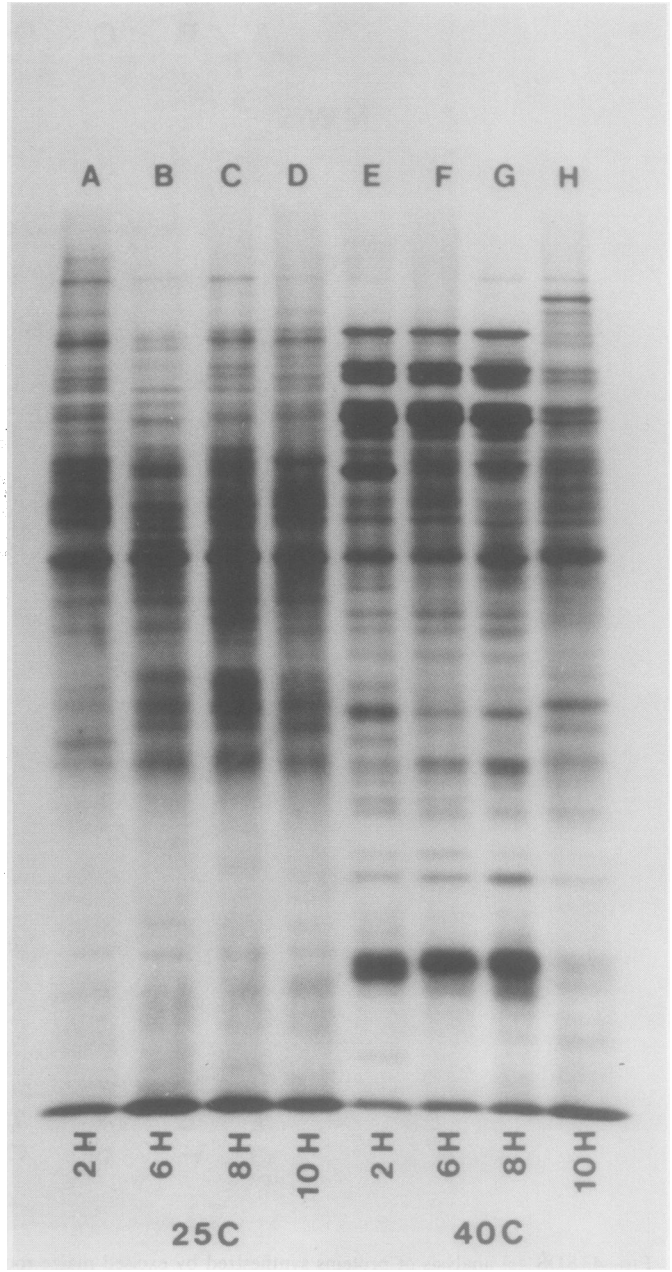

FIG. 6. SDS gel analysis of proteins synthesized by roots from intact maize seedlings incubated at continuous 25 or 40°C for increasing times. Labeling of roots with 50 μCi/ml [^{35}S]methionine was carried out in the last hour of the incubation with the roots still attached to the plants. Proteins were visualized by fluorography. Lanes: A, 25°C, 2 h; B, 25°C, 6 h; C, 25°C, 8 h; D, 25°C, 10 h; E, 40°C, 2 h; F, 40°C, 6 h; G, 40°C, 8 h; H, 40°C, 10 h.

fibroblasts cross-reacts with the 70 kD hsp from numerous other systems, including maize (11). The above observations suggest that this protein has been highly conserved in evolution, and therefore may play a significant role in the maintenance of cellular processes at high temperature.

A unique aspect of the heat shock response in maize is that hsp synthesis occurs in addition to normal protein synthesis. This is very different from the heat shock response of soybean (12) or tobacco (2), in which normal protein synthesis ceases so that only hsp are detectable on a gel. A temperature of 45°C fails to cause the complete cessation of normal protein synthesis in maize. In fact, this temperature induces a very different group of proteins from that observed at 40°C. Lindquist (16) has observed in *Drosophila* that different temperatures will produce varying patterns of hsp synthesis, but the proteins induced are still members of the basic set of hsp produced at the optimum shocking temperature of 37°C. It seems that maize roots are capable of expressing at least two distinct heat shock responses, the type of response being a function of temperature. Because different temperature

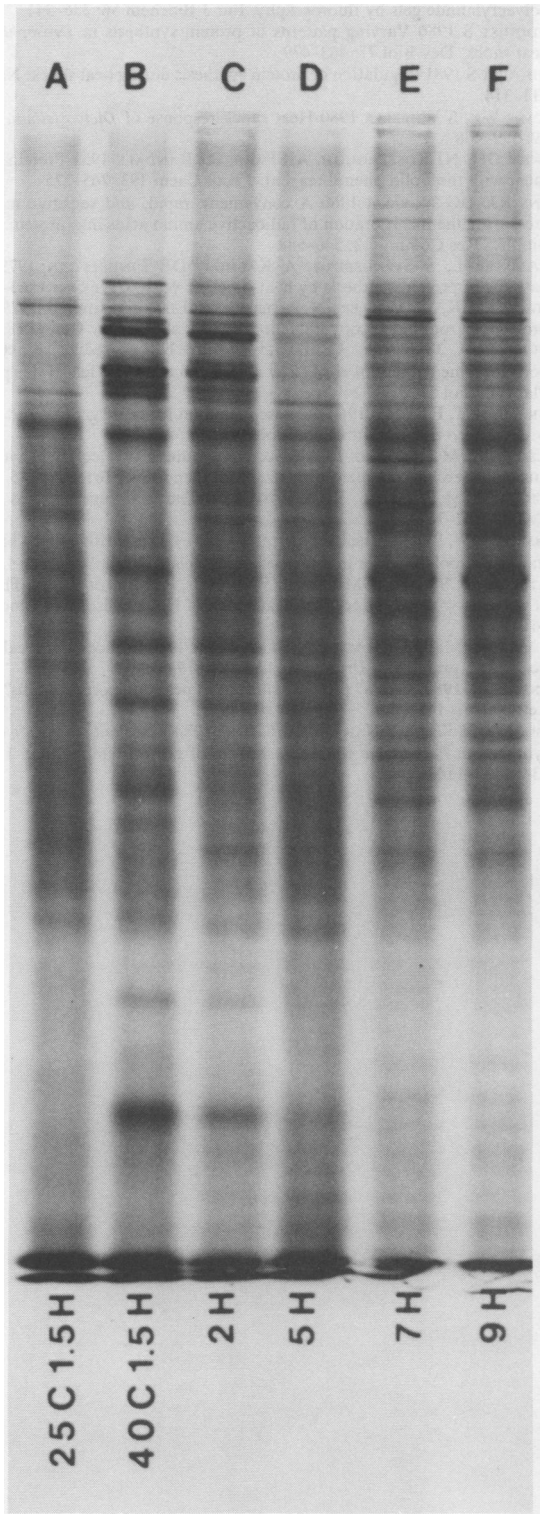

FIG. 7. Recovery from heat shock of excised maize roots. After washing for 4 h at 25°C, roots were heat shocked for 1 h at 40°C, then returned to 25°C for increasing times. Roots were labeled with 50 μCi/ml [^{35}S]methionine during the last 30 min of the incubation. Proteins were visualized by autoradiography. Lanes: A, continuous incubation at 25°C for 1.5 h; B, continuous incubation at 40°C for 1.5 h; C to F, 1 h at 40°C, then transferred to 25°C for: C, 2 h; D, 5 h; E, 7 h; F, 9 h.

ranges may have different effects on cellular processes, it would not be unreasonable for the plant to possess more than one set of hsp to enable it to compensate for any temperature it might experience during the course of a day.

Another interesting aspect of the heat shock phenomenon in maize is the transience of the response at continuous 40°C temperature. This transient response occurs in both excised and intact roots. The duration of the response is longer in intact seedlings than in excised roots. Although other causes for the transience have not been ruled out, it may be that the nutrient status of the roots affects their ability to maintain hsp synthesis. However, transient synthesis has been noted in organisms under conditions in which the carbon source is never limiting (2, 21). Thus, it is possible that transience is an inherent part of the heat shock response in maize, too. In yeast subjected to continuous high temperatures, the pattern of protein synthesis returns to normal after a period of hsp synthesis (21). In tobacco, all protein synthesis, including hsp synthesis, ceases after a prolonged period of heat shock (2), indicating that protein synthesis in the cells eventually succumbs to high temperatures. In maize roots, protein synthesis neither stops nor returns to normal after the interval of hsp synthesis. Instead, a different pattern or protein synthesis arises which includes the strong induction of three new polypeptides. Whether these polypeptides represent turnover products of the hsp or are completely new gene products remains to be investigated.

High temperature is the external signal for the induction of hsp, and we have shown that hsp synthesis will eventually cease if that signal is removed. How the signal is perceived by the cell and whether its effects on protein synthesis are direct or indirect are questions that remain largely unanswered. There is some evidence that in *Drosophila* the effects of high temperature are indirect. A number of chemical inhibitors of respiration will activate certain heat shock genes (1), implying that respiratory distress is the immediate signal for initiation of hsp synthesis. From the present study we can only conclude that the heat shock responses and tissue damage appear to be unrelated. Excision injury does not seem to prevent the synthesis of hsp at elevated temperatures. Also, because excision injury alone will not induce the proteins which are synthesized at high temperatures, the heat shock response must not be part of a more general response to wounding.

A third interesting feature of the heat shock response in maize is that hsp do not accumulate as indicated by our inability to detect them on stained gels. They may therefore be turned over rather rapidly. This observation is probably related to the transient nature of the heat shock response in maize. However, in at least two other systems, *i.e. Drosophila* (1) and soybean (12), hsp are accumulated after prolonged treatment at elevated temperature. The significance of the differences between these two types of heat shock responses is not understood at this point.

The translational inhibitor cycloheximide and the transcriptional inhibitor cordycepin both prevent hsp synthesis in excised roots; cordycepin will specifically block hsp synthesis without affecting normal synthesis (P. Cooper, unpublished data). These results suggest that RNA synthesis is required for the induction of the hsp in maize roots. Experiments examining the effects of heat shock on the synthesis of specific RNA in maize are in progress. Data from such experiments will directly establish whether the synthesis of specific RNA is an essential part of the heat shock response.

We intend to investigate further the heat shock response of maize in hopes of determining if hsp play any regulatory role and what the nature of the role is at high temperature. Subcellular localization and tissue specificity of the proteins may give some clue to the mode of action of the hsp. Also, by investigating varieties of maize known to be particularly heat sensitive or heat tolerant for the presence of hsp, we may be able to determine in what way, if any, hsp provide for thermotolerance in the maize plant.

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