Novel Regulation of Heat Shock Genes during Carrot Somatic Embryo Development

J. Lynn Zimmerman,1 Nestor Apuya, Kamel Darwish,2 and Cynthia O’Carroll
Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Maryland 21228

We have determined that somatic embryos of carrot exhibit a number of interesting and unusual properties when exposed to heat shock at different times in their development. Specifically, we have seen that mid-globular embryos can be arrested irreversibly in their development when heat-shocked, whereas all other stages of embryogenesis, both before and after this stage, are fully capable of normal development after the stress. In investigating the molecular basis of this developmental sensitivity to heat shock, using a cloned heat shock gene encoding a small heat shock protein, we have determined that globular embryos both synthesize and accumulate significantly less heat shock mRNA when compared with embryos of any other stage or to callus suspension cells. In fact, there appears to be no transcriptional induction of heat shock gene expression in response to heat shock during this time period; the gene is expressed at the same relatively low level both before and after heat shock. However, in spite of the low level of heat shock mRNA available, globular embryos synthesize the full complement of heat shock proteins in response to heat treatment. The globular embryos appear to accomplish this by translating the existing heat shock mRNAs at an elevated rate, which compensates for the low level of available mRNA. Once the embryos have progressed beyond the globular stage of development, regulation at the transcriptional level resumes, and the embryos again exhibit normal development after heat shock.

INTRODUCTION

All organisms respond to heat exposure by inducing the transcription and subsequent translation of a specific set of genes, the heat shock (HS) genes (see recent reviews by Lindquist, 1986; Bond and Schlesinger, 1987). In general, the molecular regulation of the response is imposed at both the transcriptional and translational levels; upon heat shock, HS mRNAs are selectively transcribed and accumulate, and are also selectively translated even though non-heat shock (NHS) mRNAs remain in the cells for many hours (Lindquist, 1981, 1986). The selective transcription of HS genes involves the interaction between a trans-acting “heat shock transcription factor” (Parker and Topol, 1984; Wu, 1984, 1985) and some conserved cis-acting elements, including the “heat shock consensus element” (Pelham, 1985; Wu, 1984, 1985) and some conserved cis-acting elements, including the “heat shock consensus element” (Pelham, 1986), which often occur as overlapping elements, within 100 bp 5’ to the transcription initiation site (Gurley et al., 1986; Pelham, 1987). The selective translation of HS mRNAs appears to result from the specific interaction between this altered translational machinery (Storti et al., 1980; Scott and Pardue, 1981) and sequences that reside in the transcribed but nontranslated leader region of the mRNA (McGarry and Lindquist, 1985).

In addition to this typical transcriptional-induction/translational-selection scheme of HS gene regulation, which normally occurs during a eukaryotic heat shock response, there are some examples of the uncoupling of transcriptional and translational control. For example, in Xenopus oocytes, hsp 70 mRNA is present in substantial amounts both before and after heat shock, but the hsp 70 protein is only synthesized after heat shock (Bienz, 1982; Bienz and Gurdon, 1982). There are also a number of examples of time periods during which subsets of the heat shock genes are expressed without heat shock (often associated with oogenesis and morphogenesis; Bienz and Gurdon, 1982; Sirotkin and Davidson, 1982; Witting et al., 1983; Zimmerman, Petri, and Meselson, 1983), as well as periods during which heat shock genes are uninducible (often during early embryogenesis) in species as diverse as Drosophila (Graziosi et al., 1983), sea urchin (Rocchetti et al., 1982), Xenopus (Heikillik et al., 1985), mouse (Witting et al., 1983), and yeast (Petko and Lindquist, 1986). In each of these cases where HS genes are uninducible, the embryos or organisms are extremely thermosensitive; relatively mild heat shock is generally lethal.

We have analyzed heat shock gene regulation during the development of somatic embryos from carrot cells in culture, and have observed yet another scenario for heat shock gene regulation and expression that is develop-
mentally dependent and that appears to have a profound impact on the process of somatic embryogenesis. Specifically, early embryos pass through a period of development during which heat shock gene regulation shifts from the transcriptional to the translational level, and then, with further development, shifts back to the transcriptional level. Superimposed upon this shift in the level of gene regulation is a precise and dramatic arrest of embryo development after exposure to heat shock. This study focuses on these two phenomena and the relationship between them.

RESULTS

Heat Shock Interrupts Somatic Embryo Development

Carrot cells growing in suspension culture can be induced to develop into somatic embryos by simply removing the auxin 2,4-D from the growth medium (Steward, 1958). As shown in Figure 1, development proceeds through a series of morphologically distinct stages termed the globular, heart, torpedo, and cotyledon or plantlet stages, and typically spans a period of several days. In contrast to this normal pattern of somatic embryo development, we have found that the exposure of carefully timed globular stage embryos to heat shock (37°C, 2 hr to 3 hr) can permanently arrest their development. As also shown in Figure 1, when mid-globular embryos (day 8 of development for this particular cell line) are exposed to heat shock, the embryos increase in size about twofold, but do not proceed to the heart stage or beyond. No other stage of somatic embryogenesis shows equivalent developmental sensitivity to heat shock.

The sensitivity of globular embryos to heat shock is quite precise; these embryos show a specific window of heat shock sensitivity that is generally only 2 hr to 4 hr long during the mid- to late-globular stage (Zimmerman et al., 1989). This window may occur on a different day for each different cell line, but is always constant for a given line, and is keyed to the developmental timing of that line. For example, our initial observation of the developmental interference was made using a line (line 8) which reached the mid-globular stage by day 5, and was maximally sensitive for 2 hr on day 5 (Zimmerman et al., 1989), whereas the line described and characterized here (line P2) develops more slowly, reaching the mid-globular stage on day 8, and showing peak sensitivity over approximately a 4-hr period on day 8. We have also observed the same phenomenon in two other independently derived carrot cell culture lines.

Many lines of evidence lead us to conclude that the heat-shocked globular embryos are arrested in development rather than killed outright by heat shock. First, heat-shocked embryos incorporate \(^{3}H\)-thymidine at appreciable rates in the 48 hr following a heat shock, as judged from TCA-precipitable counts of embryo extracts and the examination of autoradiograms of embryo sections (C. O’Carroll, unpublished data). Second, two recognized tests for plant cell viability, the reduction of triphenyltetrazolium chloride (TTC; Bennett and Loomis, 1949; Stepokus, 1971), and the retention and metabolism of fluorescein diacetate dye (Widholm, 1972) are positive with heat-shocked globular embryos. The ability of cells to reduce TTC has long been regarded to be a relatively sensitive and quantifiable measurement of viability. As shown in

Figure 1. Heat-Shocked Globular Embryos Are Arrested in Development.

Individual globular embryos were selected by hand and individually cultured in microtiter wells such that their progression through somatic embryo development could be followed. The panel on the left (Heat Shocked) follows a globular embryo that was exposed to 37°C for 3 hr on day 8 (mid-globular stage for this line) and then returned to 22°C for the remainder of the experiment. The panel on the right (Non Heat Shocked) follows an equivalent mid-globular embryo from the same embryogenesis as it develops into a heart stage (day 13), a torpedo stage (day 15), and, finally, a large plantlet stage (day 21), a portion of which is shown at the same magnification for comparison. The differentiated vascular tissue is evident in both the day 15 and day 21 NHS embryos. Magnification = ×100.
Figure 2, heat-shocked and non-heat-shocked embryos show equivalent levels of TTC reduction, both immediately after HS treatment and for at least 48 hr following heat shock; boiled embryos show very low levels of TTC reduction ability. Moreover, as shown in Figure 3, both heat-shocked and non-heat-shocked globular embryos (Figures 3A and 3B, respectively) are able to take up, metabolize, and retain the fluorescent dye fluorescein diacetate. If the dye is cleaved intracellularly to release free fluorescein, the cells fluoresce a bright green. Both non-heat-shocked and heat-shocked globular embryos fluoresce equivalently. If the embryo is dead (as is the boiled globular embryo of Figures 3B and 3D), it does not fluoresce. (Note the absence of fluorescence of the boiled embryo in Figure 3B.) However, dead cells and embryos can be stained by the exclusion dye phenosafranine (Widholm, 1972), which results in the embryos appearing a deep red to brown when illuminated by white light. The boiled globular embryo of Figure 3D readily takes up this dye, and appears much darker than either the non-heat-shocked (Figure 3C) or heat-shocked (Figure 3D) globular embryos that exclude this dye. Thus, based on these observations, we conclude that heat shock does not kill globular embryos, but, rather, arrests them at the globular stage of development.

Finally, we have determined that the heat shock arrest of globular embryo development can be avoided by pre-shocking the embryos (at 37°C for 3 hr) prior to the sensitive window; that is, the embryos can be “thermoprotected.” The protection can last for approximately 48 hr.

Globular Embryos Show no Transcriptional Induction of Heat Shock Genes

In investigating the molecular basis of the heat shock sensitivity of globular embryos, we analyzed the mRNAs that accumulate in heat-shocked somatic embryos relative to those induced by heat shock of callus suspension cells. The results are shown in Figure 4. Total RNAs from heat-shocked callus suspension cells and from progressively more advanced somatic embryos (embryogenesis induction = day 0) were displayed on a methylmercury agarose gel, blotted to GeneScreen, and hybridized successively with a carrot heat shock gene probe (HS-17.5, which encodes a low molecular weight heat shock protein; Zimmerman et al., 1989) and with a probe for 18S rRNA (pXlrl4a; Sollner-Webb and Reeder, 1979) to verify equivalent loading. This analysis clearly shows that all embryo stages, and especially globular embryos (days 8 to 10) accumulate significantly less (about 50%) heat shock mRNA when compared with the level of HS mRNA in callus suspension cells. Torpedo stage (day 14) and plantlet stage (day 19) embryos also accumulate less heat shock mRNA than callus cells (about 50%), but more than globular embryos. Parallel analysis of non-heat shock mRNA from the same somatic embryo time points showed no detectable signal, even when 3 times the RNA was run per lane (data not shown). Thus, it appears that, in somatic embryos, heat shock either modestly induces transcription of HS mRNAs or leads to the stabilization of pre-existing mRNA; direct analysis of transcription favors the latter interpretation, as described below.

The hybridization band in all lanes of the blot shown in Figure 4 is fairly broad, and likely reflects the presence of transcripts from different members of the gene family related to the HS-17.5 probe; this clone hybrid-selects mRNAs encoding at least five different heat shock proteins (hsp5) (C.H. Hwang and J.L. Zimmerman, unpublished data). Moreover, the positions of hybridization suggest that perhaps different subsets of HS mRNAs accumulate at different stages; for example, the band in day 1 seems to indicate a slightly larger mRNA than that in days 5 and 8, and by days 14 and 19 there may be two or more bands. This complexity can only be resolved using genespecific oligonucleotide probes.

To determine whether the decreased heat shock mRNA accumulation seen in globular embryos is reflective of low heat shock gene transcription during this developmental stage, nuclear run-off experiments (Marzluff and Huang, 1984) were performed. Transcripts that were being actively synthesized at the time of nuclei isolation are extended in vitro in the presence of a radioactive ribonucleotide (32P-CTP), and are hybridized to cloned DNA sequences immobilized on a filter. The results of analyzing run-off transcripts from heat-shocked and non-heat-shocked callus suspension cells and somatic embryos are shown in Figure 5A and are quantitated in Figure 5B. Hybridizations to the heat shock clone HS-17.5 revealed that callus suspension cells dramatically induce transcription of the HS gene in response to heat shock, as expected. In contrast, globular embryos show no detectable transcriptional induction of the HS-17.5 gene (or of any cross-hybridizing gene family

![Figure 2. Heat Shock Does not Kill Globular Embryos, as Determined by TTC Reduction.](image-url)

Cell viability was assessed by the ability of embryos to reduce TTC (Bennett and Loomis, 1949) both before (NHS) and after (HS) heat shock at 37°C and after boiling. TTC reduction is measured by final absorbance at 485 nm. Measurements were performed immediately after heat shock (0 hr) and at 48 hr after the heat shock.
The viability of heat-shocked globular embryos was also evaluated by exposure of the embryos to fluorescein diacetate and phenosafranine dyes, according to Widholm (1972). If cells are alive, they will metabolize fluorescein diacetate to generate free fluorescein, which fluoresces a bright green when exposed to light of the appropriate wavelength (excitation = 485 nm, barrier filter = 520 nm). If cells are dead, they will not fluoresce. Dead cells, on the other hand, will stain with the exclusion dye phenosafranine, and will appear a deep red to brown when illuminated by white light.

(A) Non-heat-shocked globular embryo stained with both phenosafranine and fluorescein diacetate, viewed with 485 nm light. 
(B) A heat-shocked globular embryo (left) and a heat-killed (boiled) globular embryo (right), stained with both phenosafranine and fluorescein diacetate, viewed with 485 nm light. Note that only the heat-shocked globular embryo fluoresces (i.e., only the heat-shocked embryo is alive by this criterion).
(C) The same non-heat-shocked globular embryo as in (A), viewed with bright-field illumination.
(D) The same embryos as in (B), viewed with bright-field illumination. Note that the boiled (dead) embryo on the right stains darkly with the phenosafranine, giving it a deep red to brown appearance, whereas the heat-shocked embryo is much lighter, resembling the non-heat-shocked embryo of (C). Magnification = \times400.

members). The transcriptional induction begins to resume at the heart/torpedo stage of embryogenesis, and is clearly active at the plantlet stage, although heat shock induction never reaches the same level seen in callus suspension cells. Hybridization of run-off transcripts to the rRNA clone, pXlr14a, consistently revealed that heat shock of callus suspension cells clearly suppresses rRNA transcription, whereas heat shock of embryos has a less dramatic effect on rRNA transcription (i.e., the levels are almost constant before and after heat shock).

Globular Embryos Show Typical Heat Shock Protein Induction

To evaluate the consequences of the lack of transcriptional induction of HS genes on the overall heat shock response of globular embryos, we analyzed the hspS of heat-shocked callus suspension cells and somatic embryos. As shown in Figure 6, a comparison of the two-dimensional PAGE separation of \textsuperscript{35}S-methionine-labeled polypeptides, isolated from heat-shocked cells or developing embryos,
somatic embryos, and globular embryos in particular, synthesize and accumulate significantly less HS mRNA in response to heat shock, these embryos synthesize the same spectrum of hsps as those produced by callus suspension cells or embryos of any other stage of development.

This result could mean that either the protein encoded by our gene probe is not detectable in standard two-dimensional PAGE analysis (which we do not believe, based on previous hybrid-selection/in vitro translation experiments; C.H. Hwang and J.L. Zimmerman, unpublished data), or that there is sufficient HS-17.5 mRNA in globular embryos (albeit significantly less than in other stages) to direct the translation of equivalent levels of the appropriate

shows no reproducible difference in either the number, position, or relative intensity of hsp spots. All samples were labeled by incubating essentially equivalent mass quantities of cells or embryos with equivalent quantities of \textsuperscript{35}S-methionine (100 \textmu Ci) for an equivalent time (the last 2 hr of a 3-hr heat shock at 37\degree C). We have performed identical analyses on samples from many other developmental time points and from two other embryogenic cell culture lines and, in each case, have seen no developmental difference in the hsp profiles. Thus, although all

Figure 4. Somatic Embryos Accumulate Much Less Heat Shock mRNA after Heat Shock.

Ten micrograms of total RNA from each sample were electrophoresed through a methylmercury/agarose gel. The induction of somatic embryogenesis corresponds to day 0. The globular stage spans days 8 to 10, the heart/torpedo stage corresponds to day 14, and day 19 cultures contain early plantlet stage embryos. Equivalent loading of each lane was verified by reprobing the blot with an 18S rRNA probe.

Figure 5. Somatic Embryos Transcribe Significantly Less Heat Shock mRNA Compared with Callus Suspension Cells after Heat Shock.

Run-off transcription experiments were performed using nuclei isolated from each of the heat-shocked (HS) and non-heat-shocked (NHS) tissues. Equivalent counts (5 \times 10^6 cpm) of purified run-off transcripts were used to hybridize (for 48 hr) to slot blots containing 5 \mu g each of HS-17.5, 18S rRNA, and pGEM-3Z DNAs. (A) Autoradiograms of hybridizations to HS-17.5 and 18S rRNA probes. (B) Quantitation of the hybridizations shown in (A).

Densitometric analysis was performed on autoradiograms exposed for 3 hr (18S rRNA) and for 20 hr (HS-17.5). The induction of the HS-17.5 and inhibition of 18S rRNA transcription were calculated as the ratio of the peak areas for HS and NHS for each stage after the subtraction of background observed by hybridization with pGEM-3Z (data not shown).
hsp(s). If this is true, and there is approximately 25-fold less HS mRNA available for translation, then we reasoned that each message must be translated at a significantly higher rate (or efficiency) in heat-shocked globular embryos. To test whether heat-shocked embryos are more active in protein synthesis than heat-shocked callus suspension cells, we quantitated the specific activity (i.e., counts per minute $^{35}$S-methionine incorporated per microgram of total protein) of polypeptides synthesized in the last 2 hr of a 3-hr heat shock of callus cells and somatic embryos. As shown in Figure 7, the specific activity of proteins synthesized in HS embryo samples is consistently higher than that achieved in HS callus samples. The most extreme increase is seen at day 8 (mid-globular), when heat shock results in more than an order of magnitude greater incorporation of $^{35}$S-methionine (relative to the non-heat-shocked equivalent). Furthermore, the ratio of NHS:HS protein specific activity changes dramatically in the embryo samples compared with callus samples; for callus, the ratio of NHS:HS specific activity is approximately 5:1, whereas in embryo samples, that ratio ranges from 1:4 (day 14) to 1:14 (day 8). These unusual results suggest to us that not only are HS mRNAs being selectively translated (as occurs during a "normal" heat shock), but also that HS mRNAs are being translated more efficiently in somatic embryos, and in particular in globular embryos, than they are in callus suspension cells; that is, that there is some mechanism for "translational compensation" in effect that is activated during development.

**DISCUSSION**

**Lack of Transcriptional Induction of Heat Shock Genes in Globular Embryos**

Many eukaryotes have been shown to have complex developmental regulation of heat shock genes, encompassing periods of heat shock gene transcription in the absence of heat shock (often associated with oogenesis and morphogenesis), as well as periods during which heat shock genes are transcriptionally uninducible (often during early embryogenesis). The transcription of the HS-17.5 gene in globular embryos interestingly falls into both categories; the gene is transcribed at a low level prior to heat shock, and is transcriptionally uninducible even when these embryos are heat-shocked. In other eukaryotes where HS gene transcription is observed without heat shock (e.g., Drosophila, Sirotkin and Davidson, 1982; Zimmerman et al., 1983; Xenopus, Bienz and Gurdon, 1982), the levels of HS mRNA are quite high (e.g., about 10$^7$ molecules per Drosophila embryo; Zimmerman et al., 1983) and represent only a few members of the total heat shock gene set. In carrot suspension cells and embryos, the level of HS-17.5 mRNA is much lower in the absence of heat shock, and was only detected in nuclear run-off experiments (but not in RNA gel blot analysis); hence, the presence of HS mRNA in carrot embryos is probably not functionally equivalent.
to what was reported in Drosophila or Xenopus oocytes. However, protein data of Pitto et al. (1983) are consistent with the possibility that other HS mRNAs may be present in non-heat-shocked carrot cells and embryos at even higher levels than the HS-17.5 transcripts. This possibility can only be tested by using many more cloned heat shock genes as probes for mRNA synthesis and accumulation.

The counterpoint to the developmental induction of the HS genes is the developmental incompetence to transcribe the gene set in response to heat shock. Carrot somatic embryos show such an incompetent period extending from at least day 1 of development through the globular stage; transcriptional induction of the HS-17.5 gene can again be observed, beginning at approximately the heart/torpedo stage of embryogenesis, and increases in magnitude in plantlet stage embryos. One unusual aspect of the heat shock incompetent period in carrot somatic embryos is that it occurs against a background of active mRNA synthesis rather than occurring because of generalized transcriptional repression (i.e., because of little to no RNA polymerase II activity); such a generalized repression is often the reason for similar heat shock incompetent periods in animal embryos that utilized stored or maternal mRNAs for early protein synthesis. In this respect, the heat shock incompetent period of carrot more closely resembles that which occurs in yeast once the yeast cells are committed to sporulate (Petko and Lindquist, 1986), a process that proceeds with active mRNA synthesis for sporulation-specific genes (Percival-Smith and Segal, 1984). Thus, in both these cases, the inability to induce HS mRNA synthesis is not due to generalized transcriptional repression, but, rather, must be more selective, if not specific, for the heat shock gene set. There may be some transient, developmentally regulated modification (or lack of modification) of the heat shock transcription factor in these systems. We are now testing this hypothesis.

Translational Compensation for Heat Shock Protein Production

In spite of the relatively low level of HS mRNA synthesis and accumulation seen in somatic embryos, we can detect no qualitative changes in the 35S-methionine-labeled heat shock proteins produced by either callus suspension cells or embryos of any stage. In the experiments shown, and in many other equivalent analyses performed with this and another embryogenic cell line, no protein spots are missing or reduced in amount in heat-shocked somatic embryos. Moreover, the amount of 35S-methionine incorporated by heat-shocked somatic embryos is consistently greater than that incorporated by heat-shocked callus suspension cells during an equivalent labeling period. Quantitation of overall protein specific activity demonstrates this trend. Assuming that the methionine pool size is the same in callus suspension cells and somatic embryos, the higher specific activity of proteins from heat-shocked embryos (globular stage in particular) can best be explained by a combination of selective translation of heat shock mRNAs (as normally occurs during heat shock) and an unusually high rate of translation of those mRNAs (to compensate for the low level of HS mRNA in somatic embryos). We do not believe that the enhancement of hsp stability is a contributing factor to the increase in 35S-methionine incorporation in somatic embryos because our previous measurement of hsp stability from callus suspension cells showed these proteins to be very stable for at least 12 hr following the labeling, even if the cells were returned to normal growing temperatures (Hwang and Zimmerman, 1989). Thus, we favor the hypothesis that these somatic embryos possess some mechanism for the enhancement of hsp synthesis that allows the specific translation of HS mRNAs at an even higher than normal HS rate. We do not yet know whether this results from enhancement of translation initiation or elongation, or whether both steps may be functioning more efficiently in these cells.

Heat Shock Arrests the Development of Globular Embryos

We have seen that the exposure of mid- to late-globular embryos to a brief heat shock during a 2-hr to 4-hr window of sensitivity dramatically and irreversibly blocks further development of these embryos. We have demonstrated, by a number of criteria, that these embryos are not killed outright by the heat shock, but, rather, arrest at the globular stage. Many arrested embryos increase about twofold in size, and this appears to be, in part, due to cell division because these embryos incorporate 3H-thymidine to approximately the same level as non-heat-shocked globular embryos over a 48-hr period.

The most central question regarding the developmental arrest of globular embryos by heat shock is, "What is the relationship between the arrest and the inability of these embryos to induce heat shock gene transcription?" We believe this relationship is not as simple as it might first appear (i.e., little HS mRNA leads to HS sensitivity). The two primary reasons for suspecting a more complex relationship are the following. First, the time frame of maximal HS sensitivity (2 hr to 4 hr on day 8) is much more restricted than the period of transcriptional incompetence (all of the globular stage). Second, our data show that, in spite of having relatively little HS mRNA, the embryos produce the entire spectrum of heat shock proteins (through some kind of "translational compensation" mechanism); the cells should, therefore, be protected from heat shock. In fact, we believe that the embryos are protected from heat shock (hence, they remain viable), and that the developmental arrest, while triggered by heat shock, is not due to some deleterious effect of the heat or to a defective heat shock response in general. Rather, we speculate that the developmental arrest we have observed is an example of a
classic "phenocopy" phenomenon, as described most extensively in Drosophila.

Phenocopies are nonheritable developmental abnormalities that closely resemble known mutants and that can be induced experimentally by treatment with a variety of chemicals or by brief heat shock exposure (see Eberlein, 1986, for review) during particular periods of development. It is generally believed that the heat shock-induced developmental abnormality results from the interruption of essential gene expression due to the strong transcriptional/translational bias for HS mRNAs during heat stress. There are a number of examples of heat-induced phenocopies in Drosophila development. Phenocopies of the mutant bi- thorax (an adult phenotype) are induced by heat shock during the first 6 hr of embryo development (Santamaria, 1979), implying that, in this case, the expression of some essential gene greatly precedes its phenotypic expression. Other Drosophila phenocopies such as multiple wing hair reflect closer spacing of heat shock sensitivity and phenotype; here, heat shock at different specific times through the pupal stage yields multiple hairs on different parts of the adult fly (Mitchell and Lipp, 1978; Petersen and Mitchell, 1987). Phenocopies are not restricted to Drosophila; similar developmental interference by heat shock has been reported in Xenopus and in mammalian embryogenesis (Elsdale, Pearson, and Whitehead, 1976; Witting et al., 1983).

Several characteristics are common among phenocopies in all systems. First, they result from heat shock exposure during a brief and specific period of development (often during periods of rapid growth and morphogenesis). Second, they occur during periods of heat shock competence (i.e., if cells cannot induce a heat shock response, heat shock is lethal rather than mutagenic). And, third, phenocopies can be prevented by prior exposure to a mild heat shock; that is, the cells can be thermoprotected. The HS arrest of globular embryo development described here exhibits all of these characteristics. First, the enlarged globular embryo phenotype we often see is essentially indistinguishable from a number of the temperature-sensitive variants of carrot cells blocked in somatic embryogenesis, as described by Schnall, Cooke, and Cress (1988). Second, our protein analysis and viability studies indicate that the cells of the embryo are able to survive the heat stress. And, finally, we have seen that we can avoid the developmental arrest by preshocking the cells up to 48 hr prior to the sensitive window.

We believe that the reason that the developmental interference is so severe during the sensitive window on day 8 is because the translational control of HS gene expression is so strong during this same time, thus stringently blocking the production of some essential stage-specific gene product that is only produced or effective over a very brief period. Without this essential determinant, development cannot proceed. Whether a similar heat shock-sensitive period also occurs during normal zygotic embryogenesis is yet to be determined, but we believe that the reproducibility and precision of this phenomenon in in vitro developing embryos strongly suggests that it is a fundamental characteristic of globular embryo development. Moreover, we believe that this developmental perturbation we have observed in our system is helping to define a critical period in higher plant embryogenesis and to focus our attention on the molecular events accompanying the globular to heart transition.

METHODS

Plant Material

Callus suspension cell cultures were initiated from single hypocotyls derived from sterilized seeds of Daucus carota (cv Danvers Half-Long). Cells were grown in Murashige and Skoog's medium (MS; 1962), supplemented with 5 μM 2,4-D and 3% sucrose, and were transferred biweekly.

The development of somatic embryos was initiated essentially as described by Schnall et al. (1988). Embryogenic cell clusters were selected by passing a culture through a series of sieving screens and collecting the 90-μm to 150-μm fraction. The cells were washed several times and resuspended in MS medium lacking auxin at a low cell density (1 x 10^5 cells/mL). Development proceeded at somewhat different rates for individual lines, but was always constant for any given line.

Measurements of Cell Viability

Cell viability was assessed using several techniques. First, the ability of cells and embryos to reduce TTC was performed according to Krishnan, Nguyen, and Burke (1989), as adapted from Bennett and Loomis (1949). Briefly, 0.25 g of embryos, wet weight, either heat-shocked or non-heat-shocked, were vacuum-infiltrated for 30 min with 0.8% (w/v) TTC in 50 mM sodium phosphate buffer (pH 7.4), and incubated in the dark for 18 hr. Samples were washed four times with distilled H_2O, resuspended in 95% ethanol, and boiled to dryness. The resulting powder was resuspended in 3 mL of 95% ethanol, the solution clarified, and the absorbance read at 485 nm. As a reference, a standard curve was generated by mixing known percentages of heat-killed (boiled) embryos with non-heat-shocked embryos and determining the final A_485 of each mixture.

Second, cell viability was assessed based on the ability of heat-shocked and non-heat-shocked embryos to both exclude the dye phenosafranine and metabolize the compound fluorescein diacetate, thus producing fluorescence when viewed under appropriate illumination. Embryos were exposed either separately or in combination to 0.05% phenosafranine and/or 0.0005% fluorescein diacetate (diluted from a fresh stock suspended in acetone) in MS medium. Embryos were incubated for 5 min in the dark, and then viewed with white light for phenosafranine, or with blue light (excitation filter = 485 nm, barrier filter = 520 nm) for fluorescein.

As described by Widholm (1972), live cells do not stain with phenosafranine, but dead cells appear a deep red to brown. In contrast, live cells metabolize fluorescein diacetate, cleaving off
the acetate to release free fluorescein, which is retained by live cells and causes them to fluoresce brightly with proper illumination.

**RNA Isolation and Analysis**

RNA was isolated from cells or embryos using the method of Glisin, Crkvenjakov, and Byus (1973). RNA was pelleted through a 5.7 M CsCl cushion, and the pellet was resuspended in a small volume of 0.01 M Tris, 1 μM EDTA, 0.1% SDS and stored as an ethanol precipitate at −20°C.

RNA was analyzed by displaying 10 μg of total RNA per lane through a 1.5% agarose gel containing 10 mM methylmercuric hydroxide followed by transfer to GeneScreen (DuPont-New England Nuclear) according to manufacturer’s instructions. The blotted RNA was hybridized with 32P-labeled probe DNA, clone HS-17.5, which was labeled by the random priming method of Feinberg and Vogelstein (1984).

Hybridization was performed in 50% formamide, 5 × SSCP (0.75 M NaCl, 0.075 M Na citrate, 0.2 M phosphate buffer) and 5 × Denhardt’s solution (GeneScreen Manual) at 42°C for 24 hr. Blots were washed several times in 2 × SSC, 0.1% SDS at 50°C and 0.1 × SSC, 0.1% SDS at room temperature. Following autoradiography, the blot was rehybridized with a 32P-labeled probe for 18S RNA (clone pXlr14a; Sollner-Webb and Reeder, 1979) to standardize the relative RNA loading per lane. When hybridizing with the rRNA probe, a 100-fold mass excess of cold pXlr14a DNA (relative to the total quantity of RNA on the blot) was included in the hybridization mix to ensure probe excess and facilitate quantitation.

**RNA Synthesis by Nuclear Run-Off Transcription**

The quantitation of RNAs that were being transcribed actively at any given time was performed by nuclear run-off analysis in which preinitiated transcripts in isolated nuclei are allowed to continue their synthesis in the presence of a radioactive RNA precursor. Nuclei were isolated from carrot suspension cells or somatic embryos according to Luthe and Quatrano (1980), and run-off transcripts were labeled as described by Marzluff and Huang (1984). Labeled transcripts (5 × 10⁶ cpm of each labeling) were hybridized to slot blots containing 5 μg each of either the heat shock clone (HS-17.5), the rRNA clone (pXlr14a), or pGEM-3Z DNA. Hybridizations and washing were exactly as described for RNA gel blots. Densitometric analysis (using a Bio-Rad 620 video densitometer) was performed on autoradiograms exposed for 3 hr (for the 18S rRNA probe) or 20 hr (for the HS-17.5 probe). The induction of transcription of the HS-17.5 RNA and the inhibition of the 18S rRNA were calculated as the ratio of the peak areas for heat shock and non-heat shock samples for each developmental stage after subtraction of background (determined from pGEM-3Z hybridization).

**Protein Labeling, Isolation, and Analysis**

Proteins were resolved in two dimensions as described by O’Farrell (1975) using 15% (w/v) acrylamide gels for the second dimension. The effective pH range of the isoelectric focusing gels was from pH 6.0 to pH 9.0. Proteins were fixed in 7% (v/v) acetic acid and were visualized by soaking in “Fluorohance” (Research Products International Corp.) followed by drying and exposure to Kodak X-AR film.

The relative incorporation of 35S-methionine per microgram of protein per unit time was quantitated by adding 100 μCi of 35S-methionine to approximately equivalent weights of suspension cells or somatic embryos of progressive stages that had been preincubated for 1 hr at either 23°C or 37°C and allowing the incubation to continue at the preincubation temperature for an additional 2 hr. Samples were washed free of unincorporated 35S-methionine, and proteins were extracted according to Hwang and Zimmerman (1989). From each sample a fraction was used to determine the amount of total protein in the sample by a Bradford colorimetric assay (Bradford, 1976; Bio-Rad protein assay kit) as well as 35S-methionine incorporation into TCA-precipitable counts (insoluble in 5% TCA) that were collected onto glass fiber filters and read in a liquid scintillation counter.

**ACKNOWLEDGMENTS**

We thank Dr. Joe L. Key for providing the soybean heat shock clone, and Dr. Barbara Sollner-Webb for sharing the RNA clone, pXlr14a. We are especially grateful to Dr. Terry Viancour and Brent Holst for their advice and assistance with photomicroscopy. This work was supported by grant 37670 from the National Institutes of Health.

Received September 28, 1989.

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DOI 10.1105/tpc.1.12.1137

This information is current as of October 20, 2017