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# Heat Shock Gene Expression Is Controlled Primarily at the Translational Level in Carrot Cells and Somatic Embryos

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**We have determined that the synthesis of heat shock proteins is regulated ultimately at the translational level in heat-shocked carrot callus cells and somatic embryos. Polysome analysis revealed that heat-shocked callus cells do not translate most heat shock transcripts, which they abundantly synthesize and accumulate. By contrast, heat-shocked globular embryos accumulate low levels of heat shock mRNA but selectively translate more of the heat shock mRNA molecules compared to callus cells and embryos of later stages. The overall result of these different translational control schemes is that undifferentiated callus cells and globular embryos synthesize comparable levels of heat shock proteins even though they have large differences in heat shock transcript levels.**

## INTRODUCTION

The production of heat shock proteins (HSPs) is a universal response to high temperature exposure (for reviews, see Lindquist, 1986; Neumann et al., 1989). In plants, the predominant HSPs belong to the low molecular weight class that ranges from 16 to 26 kD (Hwang and Zimmerman, 1989; Neumann et al., 1989). There are about 15 to 20 low molecular weight HSPs in plants, whereas there is only one in yeast (Petko and Lindquist, 1986), mouse (Gaestel et al., 1989), and human (Hickey et al., 1986), and only four in Drosophila (Ingolia and Craig, 1982). Because of the predominance of these low molecular weight HSPs in plants, they are suspected to be very important in the establishment of plant thermotolerance.

The synthesis of HSPs is considered to be regulated primarily by transcriptional activation of corresponding heat shock (HS) genes. This regulatory control occurs through the interaction between the "heat shock transcription factor" (HSTF; Parker and Topol, 1984; Wiederrecht et al., 1987; Wu et al., 1987; Sorger and Pelham, 1988) and the *cis*-acting "heat shock elements" (HSEs; Pelham, 1982; Perisic et al., 1989), which are present in multiple, overlapping copies in all systems analyzed so far (for reviews, see Neumann et al., 1989; Gurley and Key, 1991). In addition to transcriptional induction, translational selection of HS transcripts also plays an important role in the preferential production of HSPs. Heat shock is believed to alter the translational machinery of heat-shocked cells (Scharf and Nover, 1982). As a consequence of this translational reprogramming, the translation of HS mRNA is highly favored in comparison with other cellular mRNAs. Implicit in this translational control mechanism is the assumption that if cell internal conditions favor the translation of HS mRNA, then most (if not all) HS mRNAs that are synthesized are fully utilized for HSP synthesis. In other words, the abundance of HS transcripts is believed to serve as a driving force for their translation. In this paper, we demonstrate that this is not the case in cultured carrot cells and embryos. Our results support the idea (Yost et al., 1990) that translation of HS mRNA is neither guaranteed by nor dependent upon vigorous transcription of HS genes during heat shock.

Previously, we showed that as undifferentiated carrot callus cells undergo the process of somatic embryogenesis, the normal heat shock–induced transcription of HS genes does not occur (Zimmerman et al., 1989). Heat-shocked embryos (specifically those at the globular stage) accumulate very low levels of HS mRNA corresponding to the low molecular weight HSPs (Zimmerman et al., 1989) and to HSP70 (Lin et al., 1991; C. H. Hwang and J. L. Zimmerman, unpublished data). In contrast, heat shock induces the transcription of these genes to much higher levels (65-fold over that of globular embryos), and their mRNAs accumulate much more abundantly in callus cells (12-fold over that of globular embryos; Zimmerman et al., 1989). However, in spite of such wide discrepancies in the transcription and accumulation of HS mRNA between callus cells and embryos, heat-shocked globular embryos are fully capable of synthesizing the full complement of all HSPs at levels comparable to those of heat-shocked callus cells (Zimmerman et al., 1989). In this study, we show that callus cells translate only a fraction of the abundant HS mRNA molecules they accumulate. By contrast, globular embryos more selectively translate more of the fewer HS mRNA molecules they contain.

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## RESULTS

### Recruitment of mRNAs into Polysomes Is Very Selective in Globular Embryos

We previously showed that heat-shocked carrot embryos at the globular stage of development accumulate less HS17.7 mRNA than equivalently shocked undifferentiated callus cells and that this low level of accumulation is due to lack of transcriptional induction of the HS17.7 genes (Zimmerman et al., 1989). By contrast, two-dimensional PAGE analysis of labeled HSPs showed that both globular embryos and callus cells contained comparable distributions and amounts of HSPs, including HSP17.7. We proposed two mechanisms that could explain the apparent discrepancy between the level of HS transcripts and the level of synthesized HSPs. One possibility was that heat-shocked globular embryos were capable of translating the relatively few HS transcripts they contained at an enhanced level. The other possibility was that the HS transcripts, which were abundantly synthesized and accumulated in heat-shocked callus cells, were not fully utilized for translation.

To examine the recruitment of mRNAs into polysomes for active translation, we compared the total RNA and polysomal mRNA populations from heat-shocked and non–heat-shocked callus cells and somatic embryos. The results of RNA gel blot analyses are shown in Figure 1. A comparison of the non–heat shock (NHS) and heat shock (HS) panels of Figure 1A (total RNA) demonstrates the induction of HS17.7 RNA accumulation after heat shock. Induction of HS17.7 mRNA in callus cells, and to a lesser extent in plantlet embryos, is due to increased transcription, whereas induction of HS17.7 mRNA in globular embryos is due primarily to posttranscriptional events (i.e., increased transport, stability, etc.; Zimmerman et al., 1989). The relative levels of transcript, normalized to the 5S rRNA hybridization signals, are summarized in Figure 2A.

The level of HS17.7 transcripts in callus cell total RNA is approximately 12-fold greater than that in globular embryo total RNA. Also shown in Figure 1A, for comparison, is the hybridization of two non–heat shock mRNAs, ATP-2 and EF-1α, which encode the mitochondrial ATP-synthase β subunit and the translation elongation factor 1α, respectively. Whereas the abundance of these two mRNAs changes during development in the absence of heat shock (see Figure 2A for quantitation), their abundance in total RNA is relatively constant when the same cell type is compared before and after heat shock. For example, as summarized in Figure 2A, the ATP-2 transcript is approximately fivefold more abundant in plantlet embryo RNA than in globular embryo RNA, regardless of whether NHS or HS samples are compared. This is typical of many NHS mRNAs that remain in the cell during heat shock but that are not translated actively during heat shock (Storti et al., 1980; Lindquist, 1981; also see below). It should be noted, however, that the level of EF-1α transcript decreases slightly in callus cells after heat shock and the band of hybridization of EF-1α is somewhat diffuse in HS callus extracts. This may reflect some degradation of this RNA as a consequence of heat shock, although the other transcripts (e.g., HS17.7 and 5S rRNA) are clearly not degraded.

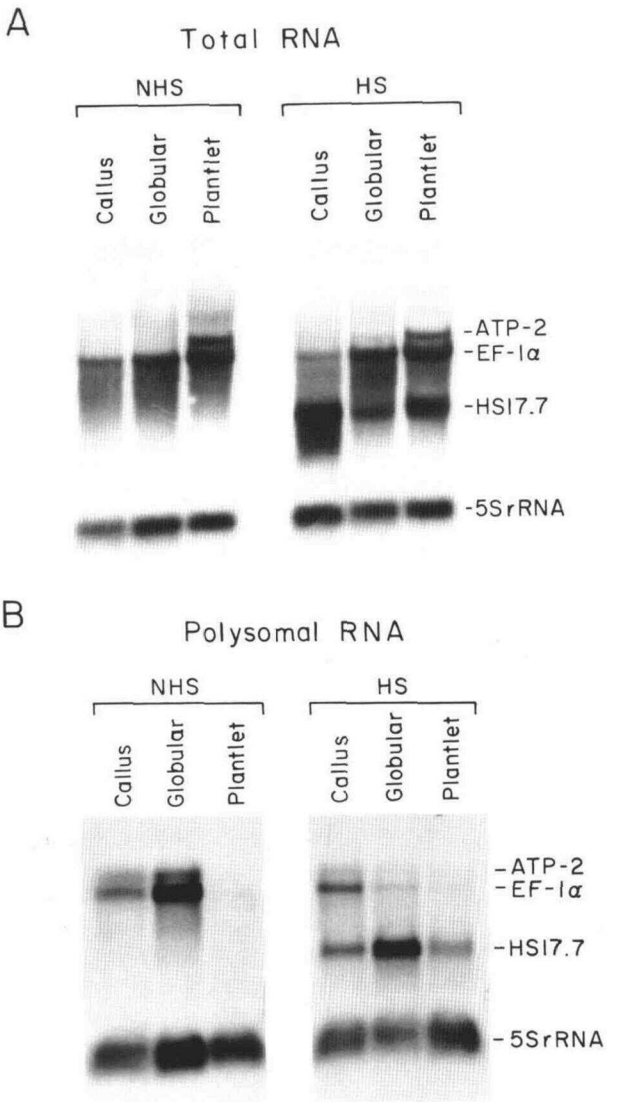
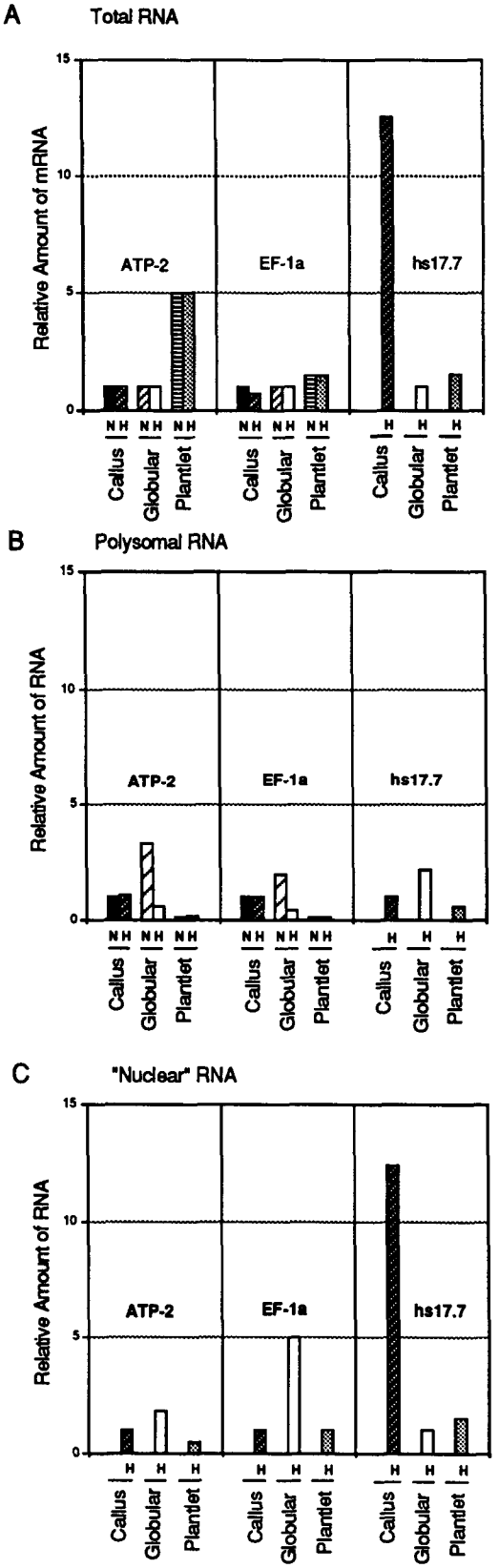



**Figure 1.** RNA Gel Blot Analysis of Total and Polysomal RNA from Non–Heat-Shocked (NHS) and Heat-Shocked (HS) Callus Cells, Globular Embryos, and Plantlet Embryos.

**(A)** Analysis of total RNA. Equivalent amounts of RNA (20 μg of total RNA) were loaded in each lane.

**(B)** Analysis of polysomal RNA. Each lane contained 4 μg of pooled polysomal RNA from each sample.

The blots in both **(A)** and **(B)** were probed successively with HS17.7, ATP-2, and EF-1α plasmid clones. The blots were also probed with a soybean 5S rRNA clone (Quemada et al., 1987) to normalize the loadings of RNA.




A very different hybridization pattern is seen when polysomal RNA samples are compared. As shown in Figure 1B, and quantitated in Figure 2B, the abundance of HS17.7 mRNA in polysomal RNA is highest in HS globular embryos (approximately twofold higher than either HS callus or HS plantlet samples), and there is no detectable hybridization of the HS17.7 probe to the NHS polysomal RNA samples from any cell type. Thus, the relative abundance of HS17.7 mRNA is distinctly different in polysomal RNA than in total RNA from callus, globular embryo, and plantlet embryo. The two NHS mRNAs (EF-1α and ATP-2) also show differences in abundance comparing either total to polysomal RNAs or comparing heat-shocked to non–heat-shocked samples.

It is clear from a comparison of the heat-shocked samples of Figures 1A and 1B that although the EF-1α and ATP-2 mRNAs remain in the heat-shocked cells (total RNA of callus, globular, and plantlet samples), these mRNAs are not equally represented in the polysomal RNA. For example, as summarized in Figure 2A, EF-1α RNA is approximately 1.5-fold more abundant in HS total RNA from globular and plantlet embryos compared to total RNA from HS callus cells, normalizing to the 5S rRNA signal. However, when polysomal RNAs are compared (Figure 2B), the abundance of EF-1α RNA remains relatively constant in callus (before and after heat shock), whereas the level of EF-1α RNA drops dramatically (decreasing by approximately threefold) in the polysomal RNA from heat-shocked globular and plantlet stage embryos compared to the level before heat shock. The ATP-2 mRNA, although significantly less abundant overall, shows a similar trend.

These data show that during heat shock, globular embryos have more HS17.7 mRNA and less EF-1α and ATP-2 mRNA associated with polysomes than equivalently treated callus cells or even plantlet stage embryos. This suggests that the mechanism by which mRNAs are recruited for translation during heat shock is different and more stringent in globular embryos compared to callus cells or more developmentally advanced somatic embryos.

### Polysome Distribution of HS mRNAs Is the Same in Callus Cells and Globular Embryos

To determine whether the twofold difference in HS17.7 mRNA abundance in the polysomes of callus cells and globular

**Figure 2.** Summary of Relative mRNA Abundance in Various Fractions of Callus Cells and Somatic Embryos.

**(A)** Summary of hybridization with total RNA samples.
**(B)** Summary of hybridization with polysomal RNA.
**(C)** Summary of hybridization with nuclear RNA.
In all cases, comparisons between levels of ATP-2 and EF-1α are expressed relative to callus, non–heat shock = 1. Comparisons of the levels of HS17.7 mRNA are expressed relative to globular, heat shock = 1. Note that these are only relative comparisons and are not a reflection of absolute quantity of mRNA in these tissues.

embryos reflected a difference in translation efficiency (i.e., polysome loading), we analyzed the distribution of HS17.7, EF-1α, and ATP-2 mRNAs across a polysome gradient. Polysomes were prepared in such a way as to include the entire cell extract, minus nuclei and cell debris, in the gradient (i.e., there was no preparative presedimentation of the polysomes); this allowed the inclusion of both nonpolysomal and polysome-associated mRNA to be analyzed. The polysome profiles of heat-shocked callus cell and globular embryo extracts displayed in Figure 3 are similar, indicating that the overall distribution of polysome-associated mRNAs is similar in these two cell types after heat shock. RNA was isolated from each fraction across the gradient and was analyzed by blot hybridization.

As shown in Figure 3, hybridization with the HS17.7 probe revealed that, although the mRNA is present in the same fractions of the two gradients (e.g., fractions 8 to 12 for the HS17.7 probe), the intensity of the hybridization signal was significantly higher in the globular embryo polysome sample than in the callus cell extract. This demonstrates that the polysome loading (i.e., the number of ribosomes per mRNA) is the same for the HS17.7 mRNA in callus cells and globular embryos but that the number of HS17.7 mRNAs present on the polysomes is greater in globular embryos. When the hybridization of the two NHS mRNAs, ATP-2 and EF-1α, was compared across the gradients, the distributions were basically the same between callus cell and globular embryo extracts, but the signal intensities were decreased in globular embryos. Finally, there was no evidence of substantial hybridization for any of the probes in the first six fractions of the polysome gradients where free cytoplasmic RNAs would be located. Taken together, these data suggest that those mRNAs (both HS and NHS) that are polysome associated in heat-shocked cells or embryos are equivalently loaded with ribosomes but that the amount of mRNA that is polysome associated is clearly different between callus cells and globular embryos. Indeed, polysomes of heat-shocked globular embryos contain relatively more HS17.7 mRNA molecules and less EF-1α and ATP-2 mRNA molecules than polysomes of callus cells.

### Untranslated RNAs Copurify with Nuclei in Heat-Shocked Cells and Embryos but May Not Be Nuclear Localized

The results shown in Figures 1 to 3 indicate that there is significantly more HS17.7 RNA present in callus total RNA than that observed to be polysome associated. To show whether the remainder of the HS17.7 mRNA was present in the nucleus, we investigated how much HS17.7 RNA could be found in the nuclear fraction and whether or not these transcripts showed a true nuclear localization.

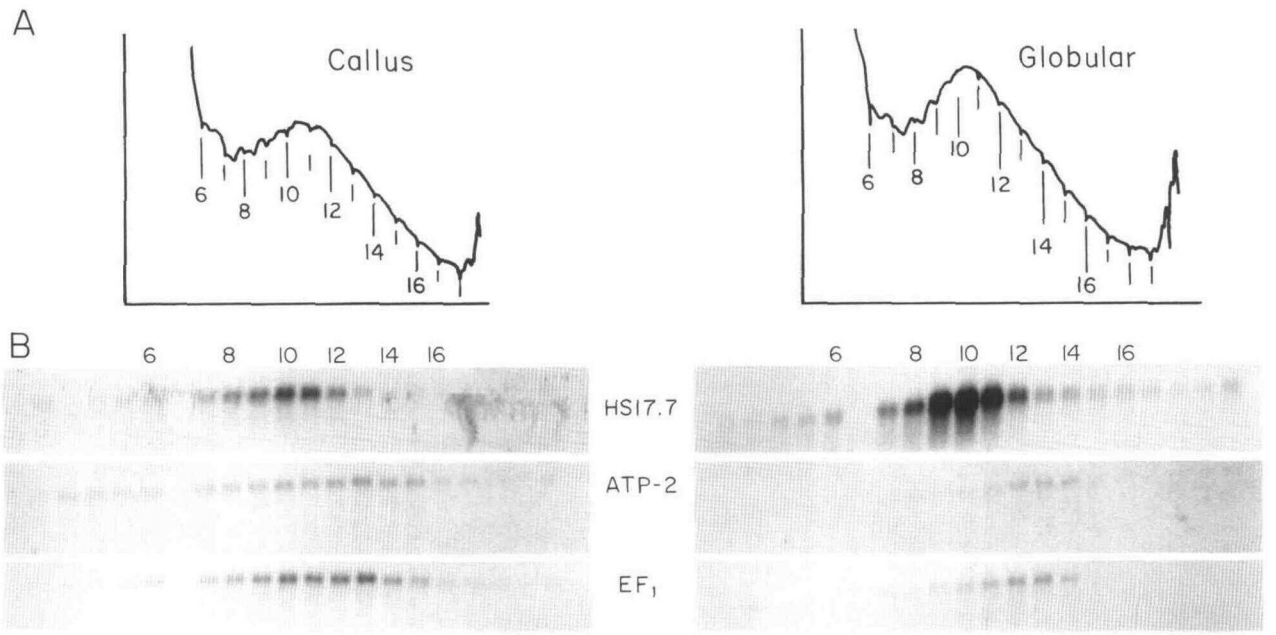



**Figure 3.** Analysis of the Distribution and Abundance of mRNAs in Polysomes from Heat-Shocked Callus Cells and Globular Embryos.

**(A)** Polysome profiles of heat-shocked callus cells and globular embryos. The gradients were monitored and fractionated using an ISCO Fraction Collector.

**(B)** RNA gel blot analysis of RNA isolated from fractions of the polysomes shown in **(A)**.

Equal aliquots (by volume) were analyzed in each fraction. The blots were probed sequentially with clones encoding HS17.7, ATP-2, and EF-1α without any stripping of the membranes between hybridization. Note that the lane between 6 and 7 is empty to allow unambiguous orientation of the autoradiograms.

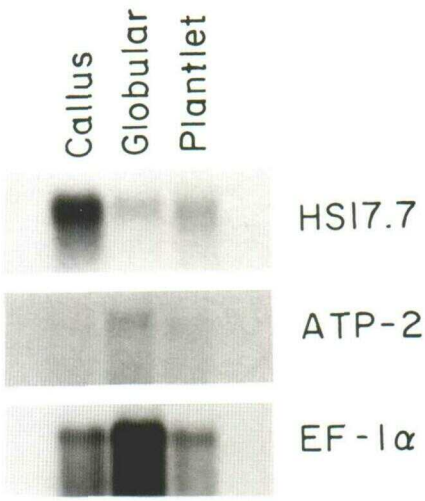



**Figure 4.** RNA Gel Blot Analysis of RNAs Isolated from the Nuclear Extracts of Heat-Shocked Callus, Globular Embryos, and Plantlet Embryos.

Equal amounts of tissue were used in the isolation. Equal amounts of RNA (10 μg) were used in the loading of the gel. The blot was probed with HS17.7, EF-1α, and ATP-2 plasmid clones.

To quantitate how much HS17.7 transcript cofractionated with the nuclear pellet, RNA was isolated from the nuclear fraction of heat-shocked callus cells, globular embryos, and plantlet stage embryos, and RNA gel blot analysis was performed. The results, shown in Figure 4 and quantitated in Figure 2C, show that the nuclear fraction isolated from heat-shocked callus cells contained approximately 12-fold more HS17.7 RNA than the nuclear fraction isolated from heat-shocked embryos. Moreover, hybridization of the same blot using the NHS EF-1α probe showed a very different result; the abundance of EF-1α transcript in the nuclear fraction of globular embryos was approximately fivefold greater than that present in the nuclear fraction of either callus cell or plantlet stage embryos. The ATP-2 probe showed the same trend, although the difference was less dramatic (approximately twofold higher in the globular embryo nuclear fraction compared to the other cell types). Thus, these data suggest that the RNA, both HS and NHS, which is not present in the polysome fraction of heat-shocked callus cells and embryos, can be accounted for by transcripts in the nuclear fraction.

To determine whether the transcripts were localized in the nucleus, or simply copurified with the nuclear fraction, we hybridized an HS17.7 strand-specific probe to paraffin-imbedded sections of heat-shocked callus tissue. The results of in situ hybridization are shown in Figure 5. In a typical field of relatively brief autoradiographic exposure (to avoid covering the entire field with grains), no HS17.7 transcripts were concentrated in the callus cell nuclei. On the contrary, the hybridization signals were dispersed throughout the cells and over the tissue. Although callus tissue is disorganized and highly vacuolated (and hence difficult to visualize in sections), it is clear that if there was 12-fold more hybridization of HS17.7 RNA restricted to the nuclei of these cells, a very different pattern of hybridization would be seen. The simplest interpretation of the results shown in Figures 1 and 5 is that the non–polysome-associated HS17.7 mRNA molecules in heat-shocked callus cells are associated with a cytoplasmic particle that cofractionates with the nuclei.

## DISCUSSION

In this study, we established that heat shock gene expression in carrot cells and somatic embryos is ultimately regulated at the level of translation; indeed, in globular embryos the regulation is almost exclusively translational. There are two interrelated aspects of translational control of HS gene expression that are illustrated by our results. First, the stringency of translational discrimination in favor of HS mRNA and against at least two non-HS mRNAs is developmentally regulated. We have presented evidence that the translational selection is more stringent at the globular stage of somatic embryogenesis. Second, translational selection of HS mRNA is not dictated by a massive synthesis and accumulation of HS mRNA. The results

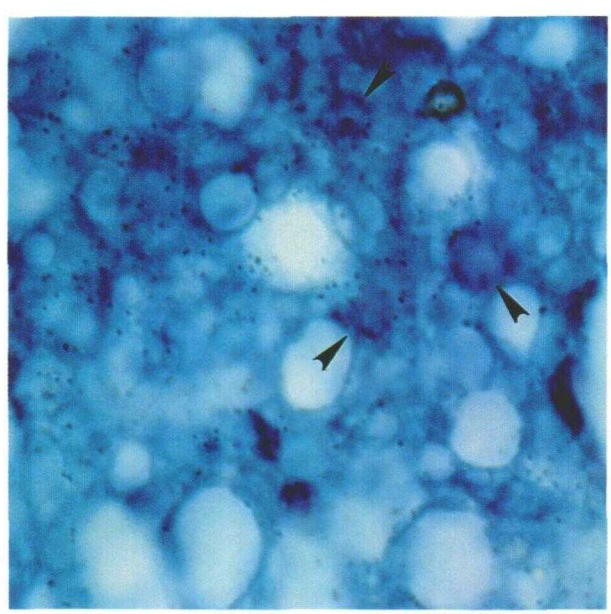


**Figure 5.** In Situ Localization of HS17.7 Transcripts in Heat-Shocked Callus Cells.

Strand-specific RNA probes to the HS17.7 gene were hybridized to paraffin-embedded sections of heat-shocked callus tissue. A few representative nuclei, which stain purple with Giemsa, are indicated by arrowheads. Silver grains can be seen throughout the tissue and on the perimeter of some nuclei but are absent in vacuoles. There is essentially no background outside the tissue section.

we presented are consistent with the idea (Yost et al., 1990) that vigorous transcription during heat shock is neither a guarantee nor a prerequisite for translation.

Translational control has long been recognized as an important component of the heat shock response of higher eukaryotes, and mRNA complexing/sequestration has been theorized to be an important contributor to this control. Studies in Drosophila indicate that during heat shock, many of the non-HS (normal) mRNAs are removed from the polysomes (for review, see Storti et al., 1980; Lindquist, 1981; Yost et al., 1990) and sequestered in an undegraded form somewhere in the cells, but it is not known exactly how these transcripts escape degradation during heat stress. These untranslated normal transcripts are suspected of being pulled into the collapsing intermediate filament network (Biessman et al., 1982; Yost et al., 1990). Under non–heat shock conditions, attachment of NHS mRNA to these cytoskeletal structures is apparently important in determining the translational activity of the mRNA (Cervera et al., 1981). Hence, normal transcripts that were actively translated before the onset of heat stress would already be in proximity to the collapsing intermediate filaments and could be trapped there. In heat-shocked tomato cells, Nover et al. (1983, 1989) showed that NHS (normal) mRNA was primarily associated with HS "granules" that were aggregates of HSPs. Nover et al. (1983, 1989) further suggest that the HS granules are associated with the cytoskeleton in the perinuclear region of the cells and could be the sequestration compartment for non-HS mRNA in the event of heat shock.

The data presented here suggest that mRNA sequestration/availability may be an important component of translational regulation in heat-shocked carrot callus cells and embryos. We propose a model, shown in Figure 6, that presents a possible molecular scenario for the translational control we have observed. Figure 6A considers heat-shocked callus cells. These cells synthesize and accumulate large quantities of HS mRNA (shown in Figure 1A), but most of these transcripts are not associated with polysomes (Figure 1B). We hypothesize that a large fraction of this HS mRNA is complexed with proteins and sequestered (e.g., as HS granules; Nover et al., 1983), and is, therefore, not available for translation. It is assumed that the proteins which form this sequestration complex are present in a finite and ultimately limiting quantity and could be titered out in the presence of large quantities of mRNA (e.g., HS mRNA). If the granule proteins were complexed with HS mRNA, then they could not complex with NHS mRNAs as the NHS mRNAs are released from polysomes during heat shock. The noncomplexed NHS mRNAs would continue to be available for translation, and, hence, discrimination against translating NHS mRNAs would be less stringent in heat-shocked callus cells (consistent with Figure 1B). At the same time, the NHS mRNAs might be more available for enzymatic degradation in this uncomplexed form (consistent with the decreased hybridization signal for EF-1α in HS total RNA; Figure 1A).

Figure 6B considers how the same granule proteins would affect translation in heat-shocked globular embryos. In these cells, HS genes are not transcriptionally induced by heat shock,

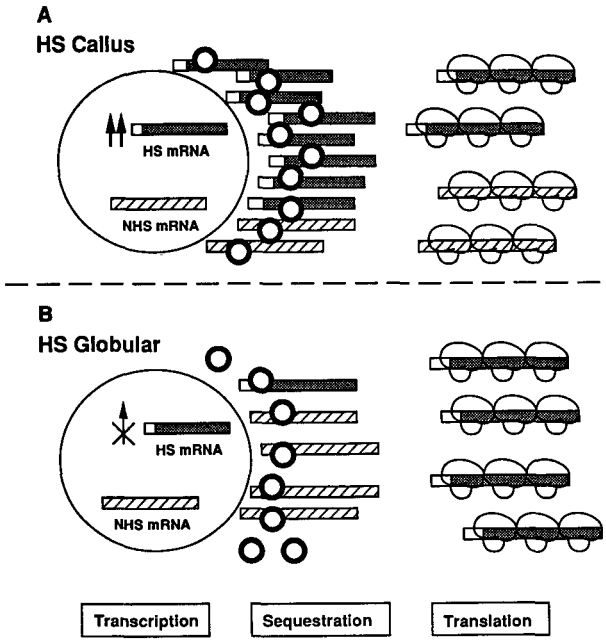



**Figure 6.** Model of Possible mRNA Compartmentalization Strategies of Heat-Shocked Callus Cells and Globular Embryos.

**(A)** Model for heat-shocked callus cells.
**(B)** Model for heat-shocked globular embryos.
In both panels, the small bold circles represent the special compartment (e.g., granules) in the cell. HS mRNA is represented by the shaded bar with an unshaded portion (representing 5′ UTR); NHS mRNA is represented by the striped bar. The arrow beside the HS mRNA in HS callus represents the high level of transcription of the HS genes. The granules are drawn (with the trapped mRNAs) in close proximity to the nucleus to indicate that they are located within the perinuclear region.

and only a low level of HS transcripts accumulate (Zimmerman et al., 1989). We suggest that in the absence of large amounts of HS mRNA the granule proteins are more available to complex with the NHS mRNAs as they are released from the polysomes, resulting in both a more stringent discrimination against translating NHS mRNAs in globular embryos and a greater degree of protection of the mRNA from degradation. Indeed, there could be multiple proteins associated with each NHS mRNA under these conditions.

Superimposed on this sequestration/mRNA availability model, our results also suggest that some form of more active recruitment of HS mRNAs into polyribosomes occurs in globular embryos (as evidenced by their higher abundance in the polysome in spite of their low abundance in the cells). Although we do not know the mechanism by which this is accomplished, there are a number of other possible mechanisms (characterized in other systems) that can contribute to translational control of gene expression. These include specific recognition of elements in the 5′ untranslated region of mRNAs (in HS genes; Klemenz et al., 1985; McGarry and Lindquist, 1985; for review,

see Yost et al., 1990), the length of the poly(A) tail (in stored mRNAs in Xenopus and Spisula; Rosenthal et al., 1983; McGrew et al., 1989), and modification of the translational apparatus (e.g., subunit protein $S_6$ of ribosomes is modified in heat-shocked cells; Glover, 1982; Scharf and Nover, 1982). We do not know the degree to which any of these mechanisms may be critical in our system.

Overall, the results presented in this paper provide evidence that in carrot the establishment of a successful heat shock response is not ultimately controlled at the level of transcription. To the contrary, these results indicate that high levels of transcription and accumulation of HS mRNAs are unnecessary. Moreover, our data suggest that the mechanism of translational recruitment of HS mRNA may be modulated during carrot development, with globular embryos showing particularly stringent discrimination between HS and NHS mRNAs. We have presented a model which predicts that mRNA availability contributes significantly to translational control during heat shock. Our model is heuristic, and further study is needed to establish its validity and to demonstrate the precise mechanism by which this translational control occurs.

## METHODS

### Source of Materials, Maintenance of Cell Culture, and Induction of Embryogenesis

Callus suspension cells were initiated from the hypocotyls of carrot seedlings (cv Danvers Half Long) as described in Zimmerman et al. (1989). They were maintained in Murashige and Skoog's (Murashige and Skoog, 1962) medium supplemented with 5 μg/mL 2,4-D. Somatic embryos were obtained from amplified callus cells following the procedure of Schnall et al. (1988).

### Heat Shock Treatment

Callus cells and embryos were heat shocked at 38°C for 3 hr following the standard procedures established by Hwang and Zimmerman (1989).

### Polysome Preparation and Analysis

Cells were ground to powdered form in a mortar with pestle under liquid nitrogen. Powdered cells were immediately transferred to a tissue homogenizer, and polysome extraction buffer was added (1 mL/g cells). The polysome extraction buffer was modified from that of Jackson and Larkins (1976) and is composed of 0.2 M Tris, pH 9, 0.4 M KCl, 0.1 M sucrose, 35 mM $MgCl_2$, 25 mM EGTA, and 1% Triton X-100. The slurry was homogenized by five to seven strokes of a motor-driven Teflon homogenizer. Nuclei and cell debris were pelleted by centrifugation at 15000 rpm for 10 min (SS-34 rotor; Sorvall Instruments, DuPont). The supernatant (0.5 mL) was loaded onto a 12-mL 15% to 50% linear sucrose gradient. Sucrose solutions were prepared in 40 mM Tris, pH 8.5, 20 mM KCl, and 10 mM $MgCl_2$. The gradients were spun for 1 hr at 39000 rpm (SW-40 rotor; Beckman Instruments) with slow acceleration/slow deceleration. The polysomes from the gradient were fractionated using an ISCO Density Gradient Fractionator at a flow rate of 2 mL/min; 0.6-mL fractions were collected on ice.

### RNA Isolation from Polysomes

RNA was isolated from fractionated polysomes using the procedure of Mechler and Rabbitts (1981) with some modifications. An equal volume of hot SDS buffer (1% SDS, 0.2 M NaCl, 40 mM EDTA, and 20 mM Tris, pH 7.5) was added to the polysome suspension. The mixture was incubated at 100°C for 2 to 5 min and was cooled to approximately 35°C before Proteinase K was added to a final concentration of 0.5 μg/mL. Protein was digested for 10 min at 35°C. SDS was adjusted to a final concentration of 1% before extracting three times with phenol/$CHCl_3$/isoamyl alcohol (25:24:1 v/v/v) and two times with $CHCl_3$/isoamyl alcohol (24:1 v/v). The RNA was precipitated from the aqueous phase by adding 0.1 vol of 3 M Na acetate (pH 5.2) and 2.5 vol of 100% ethanol and incubating overnight at −20°C. The RNA was pelleted by centrifugation.

### RNA Isolation from Nuclear Fraction

The nuclear fraction was isolated following the procedure of Timberlake (1978) using the following nuclei extraction buffer (NEB): 0.5 M sucrose, 4 mM spermidine, 1 mM spermine, 10 mM EDTA, 0.1 M KCl, 10 mM Tris, pH 8, 14 mM β-mercaptoethanol, and 1 mM PMSF. Samples were ground to a fine powder using a mortar with pestle under liquid nitrogen. Powdered cells were transferred to a tissue homogenizer, and NEB was added at 1.5 mL/g cells. The slurry was homogenized by five to seven strokes of a motor-driven Teflon homogenizer. The mixture was filtered through four layers of cheesecloth and one layer of Miracloth. The nuclei from the filtrate were pelleted by centrifugation at 7000 rpm for 10 min (SS-34 rotor; Sorvall Instruments). The nuclear pellet was washed with the extraction buffer (plus 0.5% Nonidet P-40) by gently pipetting several times with a Pasteur pipet. The nuclei were pelleted by centrifugation at 5000 rpm (SS-34 rotor; Sorvall Instruments) for 5 min. RNA from the resulting nuclear pellet was extracted using 2 mL of the following extraction buffer: 0.1 M NaCl, 0.1 M NaOAc, 5 mM $MgCl_2$, and 2% SDS. The mixture was extracted with an equal volume of Tris-saturated phenol. An equal volume of $CHCl_3$/isoamyl alcohol (24:1 v/v) was added after the phenol was thoroughly mixed with the nuclear suspension. The resulting aqueous phase was extracted twice with phenol/$CHCl_3$/isoamyl alcohol (25:24:1 v/v/v) and once with $CHCl_3$/isoamyl alcohol (24:1 v/v) before RNA was precipitated with 2.5 volumes of ethanol. The precipitate was resuspended in 50 μL of 40 mM Tris, pH 7.9, 10 mM NaCl, and 6 mM $MgCl_2$. DNA was digested by adding 10 units of RQ1 DNAse and incubating at 37°C for 2 hr. RNA was precipitated after one phenol/$CHCl_3$ extraction.

### Total RNA Preparation

The total RNA was isolated from carrot tissues using the method of Glisin et al. (1973).

### RNA Gel Blot Analysis

RNAs were displayed on 1.2% agarose-formaldehyde gels following the procedures outlined by Lehrach et al. (1977). Transfer of electrophoresed RNAs to GeneScreen (Du Pont) nylon membrane was done

using the capillary blot method as per the manufacturer's recommendations. Drying, prehybridization, hybridization, and washing of the RNA blots were done following the manufacturer's recommendations.

### DNA Manipulations

Plasmid DNA was isolated following the procedures of Sambrook et al. (1989). Cloned DNA inserts (Darwish et al., 1991) were digested from their plasmid vectors and separated by electrophoresis in 1% low-melting agarose. The DNA inserts were labeled following the random-priming method described by Feinberg and Vogelstein (1983) without purification from the gel. Labeled fragments were immediately denatured and mixed with hybridization mixture for probing.

### In Situ Hybridization

Heat-shocked callus tissue was fixed, paraffin embedded, sectioned, and hybridized as described by Cox and Goldberg (1988). The probe was a $^{35}$S-labeled antisense Riboprobe (Stratagene); neither the sense probe nor an RNAse-treated control gave any detectable hybridization. Slides were coated with nuclear track emulsion (NTB-2; Kodak), and after development they were stained with Giemsa (GIBCO). Photographs were taken on color slide film (Tungsten, Ektachrome 125; Kodak).

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