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Heat Shock Disrupts Cap and Poly(A) Tail Function during Translation and Increases mRNA Stability of Introduced Reporter mRNA[1]

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The effect of heat shock on translational efficiency and message stability of a reporter mRNA was examined in carrot (*Daucus carota*). Heat shock of short duration resulted in an increase in protein yield, whereas repression was observed following extended exposure to the stress. Regardless of the duration of the heat shock, a loss in the function of the 5′ cap [$m^7G(5')ppp(5')N$, where N represents any nucleotide] and the 3′ poly(A) tail, two regulatory elements that work in concert to establish an efficient level of translation, was observed. This apparent paradox was resolved upon examination of the mRNA half-life following thermal stress, in which increases up to 10-fold were observed. Message stability increased as a function of the severity of the heat shock so that following a mild to moderate stress the increase in message stability more than compensated for the reduction in cap and poly(A) tail function. Following a severe heat shock, the increased mRNA half-life was not sufficient to overcome the virtual loss in cap and poly(A) tail function. No stimulation of protein synthesis was observed following a heat shock in Chinese hamster ovary cells, data suggesting that the heat-induced increases in mRNA stability may be unique to the heat-shock response in plants.

The heat-shock response involves dramatic changes in gene expression in eukaryotes. The mechanisms involved in reprogramming transcription following thermal stress have been studied in detail (for recent reviews, see Sorger, 1991; Morimoto et al., 1992). Although the molecular mechanisms by which translation and message stability are altered following heat shock have not been elucidated, significant changes have been observed. As a consequence of thermal stress, translation is reprogrammed so that *hsp* mRNAs are actively translated, whereas normal messages are repressed (reviewed by Lindquist, 1986). In *Drosophila*, a 10-min heat shock is sufficient to release normal cellular mRNAs from polysomes and replace them with heat-shock-specific mRNAs (Storti et al., 1980). Non-*hsp* mRNAs, however, are not destroyed but are maintained in the cell, where they are recruited for translation once the cell has recovered from the effects of thermal stress (Storti et al., 1980). Not all protein synthesis is repressed because *hsp* mRNAs are actively recruited onto polysomes and translated throughout the period of heat shock (Lindquist, 1986). The nature of the regulation, therefore, is not so much the selective translation of *hsp* mRNAs but the global repression of non-*hsp* messages. *hsp* mRNAs merely escape this repression and are, in fact, perfectly competent for translation under physiological conditions.

The reprogramming of translation following thermal stress in animal cells correlates with modifications in their translational machinery. Changes in phosphorylation for several initiation factors in response to heat shock have been observed (Duncan and Hershey, 1989). One of the best-studied examples is the dephosphorylation of eIF-4Fα in HeLa (Duncan et al., 1987) and Ehrlich cells (Lamphear and Panniers, 1991). eIF-4Fα is the cap-binding subunit of the cap-binding complex known as eIF-4F. eIF-4Fα undergoes hypophosphorylation following heat shock resulting in impaired cap-binding activity (Panniers et al., 1985; Lamphear and Panniers, 1991; Zapata et al., 1991). Although the presence of cap analogs represses the translation of capped messages, the failure of cap analogs to repress translation of *hsp* mRNAs in *Drosophila* suggests that their translation may proceed through a cap-independent mechanism during thermal stress (Maroto and Sierra, 1988).

Heat shock can also impact RNA synthesis and turnover. rRNA synthesis is repressed with the onset of thermal stress (Yost et al., 1990). Non-*hsp* mRNAs in soybean and tomato are not rapidly degraded: in vitro translation of mRNA isolated from heat-shocked cells produces the full complement of cellular proteins (Key et al., 1981; Nover et al., 1989). Cellular studies following thermal stress in tomato and maize suggest that non-*hsp* mRNAs are found in HSGs that include two major HSPs, HSP70 and HSP17 (Nover et al., 1983, 1989). The HSGs associate with the cytoskeleton surrounding the nucleus in perinuclear complexes in plants (Nover et al., 1989), vertebrates (Collier and Schlesinger, 1986), and *Drosophila* (Leicht et al., 1986;

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Abbreviations: CHO, Chinese hamster ovary; eIF, eukaryotic initiation factor; HSG, heat-shock granules; HSP, heat-shock protein; *hsp*, heat-shock protein gene; *luc*, luciferase gene.

Arrigo, 1987). Sequestration of normal mRNAs in a cytoplasmic particle associated with the nucleus was also observed in heat-shocked carrot (*Daucus carota*) callus (Apuya and Zimmerman, 1992). The impact that such sequestration has on the rate of mRNA turnover is not known.

mRNAs can be grouped into three classes based on their response to heat shock: messages that are not translated but maintained, messages that continue to be translated and/or are stabilized, and messages that are rapidly degraded (Nover et al., 1989). The bulk of cellular messages in plant and animal species fall into the first category.

In addition to *hsp* mRNAs, expression of storage protein in developing soybean embryos (Altschuler and Mascarenhas, 1982) and some ribosomal protein mRNAs (Scharf and Nover, 1987) are examples of plant mRNAs that remain translationally competent under heat-shock conditions. *Drosophila* and human *hsp70* mRNAs, as well as c-*myc* and c-*fos* mRNAs and *Drosophila* histone H2a, H2b, H3, and H4 mRNAs, are stabilized by heat shock (Farrell-Towt and Sanders, 1984; Andrews et al., 1987; Theodorakis and Morimoto, 1987; Sadis et al., 1988; Petersen and Lindquist, 1989). The translation of avian ferritin mRNA is actually stimulated by heat shock (Atkinson et al., 1990).

In contrast, barley α-amylase mRNA undergoes heat-induced degradation in aleurone tissue. The rapid loss in α-amylase production is paralleled by a loss in α-amylase mRNA, which at ambient temperature is a stable message (Belanger et al., 1986). The mRNAs for two other secreted proteins, a thiol endoprotease and an endochitinase, were also rapidly degraded with the onset of heat shock, although the stability of nonsecretory protein messages, such as actin and β-tubulin mRNAs, remained unaffected (Brodl and Ho, 1991). As heat-shock causes the destruction of the ER in this cell type (Belanger et al., 1986), those messages whose products are cotranslationally transported across the ER, e.g. secretory protein mRNAs, may be targeted for rapid turnover to ensure full repression of this class of protein.

Although changes in the translation and mRNA degradation machinery following heat shock have been observed in a number of species, little has been done to investigate in molecular detail the impact of heat on the translational efficiency and the stability of mRNAs in plants. In this study, we demonstrate that the heat-shock response in plants involves changes in both of these aspects of gene expression. Thermal stress results in the loss in translational competence but increases mRNA stability. We also found that the degree to which these changes take place is a function of the severity of the stress.

MATERIALS AND METHODS

mRNA Constructs and in Vitro Transcription Reaction Conditions

The pT7-*luc* and pT7-*luc*-A_{50} constructs, in which the firefly luciferase-coding region is under the control of the T7 promoter, have been described previously (Gallie et al., 1991). mRNAs were synthesized in vitro following linearization of the template plasmid immediately downstream of the poly(A) tract with the appropriate restriction enzyme. The concentration of the template DNAs was quantitated spectrophotometrically following linearization and brought to a concentration of 0.5 mg/mL. In vitro transcription was carried out as described by Yisraeli and Melton (1989) using 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 100 μg/mL BSA, 0.5 mM each of ATP, CTP, and UTP, plus 160 μM GTP, 1 mM m^7GpppG, 10 mM DTT, 0.3 units/μL RNasin RNase inhibitor (Promega), and 0.5 units/μL T7 RNA polymerase (New England Biolabs). Under our transcription conditions, >95% of the mRNA is capped. The integrity and relative quantity of RNA were determined by formaldehyde-agarose gel electrophoresis as described by Melton et al. (1984).

Preparation and Electroporation of Carrot (*Daucus carota*) Protoplasts and CHO Cells

Protoplasts were isolated from a carrot cell suspension by digestion with 0.25% CELF cellulase (Worthington Biochemicals, Freehold, NJ), 1% Cytolase M103S (Genencor, Rochester, NY), 0.05% Pectolyase Y23 (Seishin Pharmaceutical Co., Tokyo, Japan), 0.5% BSA, and 7 mM β-mercaptoethanol in isolation buffer (12 mM sodium acetate, pH 5.8, 50 mM $CaCl_2$, 0.25 M mannitol) for 75 min. Protoplasts were washed once with isolation buffer, washed once with electroporation buffer (10 mM Hepes, pH 7.2, 130 mM KCl, 10 mM NaCl, 4 mM $CaCl_2$, 0.2 M mannitol), and resuspended in electroporation buffer to a final concentration of 1.0×10^6 cells/mL. Two micrograms of each *luc* mRNA construct was mixed with 0.8 mL of protoplasts immediately before electroporation (500 μF capacitance, 350 V) using an IBI GeneZapper (International Biotechnologies, Inc., New Haven, CT). For time-course experiments, aliquots of protoplasts were taken at the time intervals indicated. For end-point experiments, the protoplasts were incubated overnight.

CHO cells were grown to approximately 80% confluence in Ham's F-12 medium supplemented with 10% fetal calf serum. Cells were collected from flasks by a brief incubation with 4 mM EDTA and washed twice with PBS. Cells (1×10^6) in 0.4 mL were mixed with 1 μg of test mRNA and electroporated in PBS (200 μF capacitance, 400 V). Following electroporation, the cells were incubated for 8 h in Ham's F-12 medium, supplemented with 10% fetal calf serum before assaying.

The expression from exogenously delivered mRNA is linear up to at least 30 μg of input mRNA in carrot protoplasts (Gallie et al., 1989) and CHO cells (D.R. Gallie, unpublished data). For these experiments, we used 2 μg of *luc* mRNA for carrot transformation and 1 μg for the CHO cells to ensure that we were well within the linear dose-response range.

Heat Treatments

For end-point experiments, each mRNA construct was electroporated into triplicate samples of protoplasts, the cells were combined, and equivalent aliquots (approximately 5×10^5 protoplasts per dish) were placed in a total

volume of 4 mL of Murashige-Skoog medium supplemented with 30 g/L Suc, 100 mg/mL inositol, 0.1 mg/mL 2,4-D, and 0.3 M mannitol in plastic Petri dishes. Protoplasts were then subjected to heat shocks by immersing the dishes to a depth of 5 mm in 37, 40, 42, or 45°C water baths for the times indicated. The dishes were gently swirled in the water baths to ensure even heating of the dish. The temperature within a test dish was monitored with a temperature probe. Following the heat shock, the protoplasts were allowed to recover at 24°C for up to 24 h without shaking. At 24°C, translation of *luc* mRNA that is capped and polyadenylated continues up to 2 to 3 h before all of the mRNA is degraded. However, the large increases in message stability in protoplasts subjected to a heat shock necessitated up to a 24-h recovery for the mRNA to be fully translated before the mRNA was finally degraded. The heat-shock treatments used in this study did not significantly affect cell viability as determined by exclusion of Evan's blue dye.

CHO cells were heat treated in a similar fashion and allowed to recover at 37°C in a 5% CO_2 environment. Capped-*luc*-A_{50} mRNA is translationally active for approximately 5 h in CHO cells before the mRNA was finally degraded. Because heat shock does not change *luc* mRNA stability in CHO cells, the cells were harvested 6 h following mRNA delivery.

Luciferase Assay

Cells collected by centrifugation at 100*g* were sonicated for 5 s in 100 mM Tricine, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid, 10% glycerol, and 1% Triton X-100, and the cell debris was pelleted. Aliquots of the extract were added to 100 μL of luciferase assay buffer [20 mM Tricine, pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM CoA, 470 μM luciferin, and 500 μM ATP (Promega)], and the reaction was initiated with the injection of 100 μL of 0.5 mM luciferin in luciferase assay buffer. Photons were counted using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA). Each mRNA construct was assayed in duplicate and the average is reported. Protein concentration was determined by the method described by Bradford (1976).

Physical Half-Life Measurement

Following *luc* mRNA delivery via electroporation and heat treatment, aliquots were taken at time intervals and total RNA was purified as described by Chomczynski and Sacchi (1987). The RNA was displayed on a denaturing formaldehyde-agarose gel, followed by northern transfer, and probed with anti-*luc* mRNA. The region of the membrane representing the full-length form of the *luc* mRNA was cut from the membrane and counted and the $\log_{10}$ of the values was plotted as a function of time. *k*, the slope of the best-fit line through the data points, was used to calculate the half-life ($t_{1/2}$) according to the equation $t_{1/2} = 0.693/k$.

RESULTS

HSP Synthesis Is Induced by Short-Duration Heat Shocks

For our studies of the posttranscriptional response to heat shock, it was necessary to determine whether our use of short-duration heat treatments elicited a heat-shock response from protoplasts. The hallmark of a heat-shock response is the induction of HSP expression. Consistent with the data of previous studies (Pitto et al., 1983; Lin et al., 1984; Hwang and Zimmerman, 1989), we observed that even short-duration heat treatments can elicit the expression of HSPs. We examined the protein profile produced following heat shocks from 0 to 60 min at 37, 40, 42, and 45°C by adding radiolabeled Met to the protoplasts following the heat shock and incubating the cells for a further 2 h at 24°C. Any HSP synthesis detected by this approach

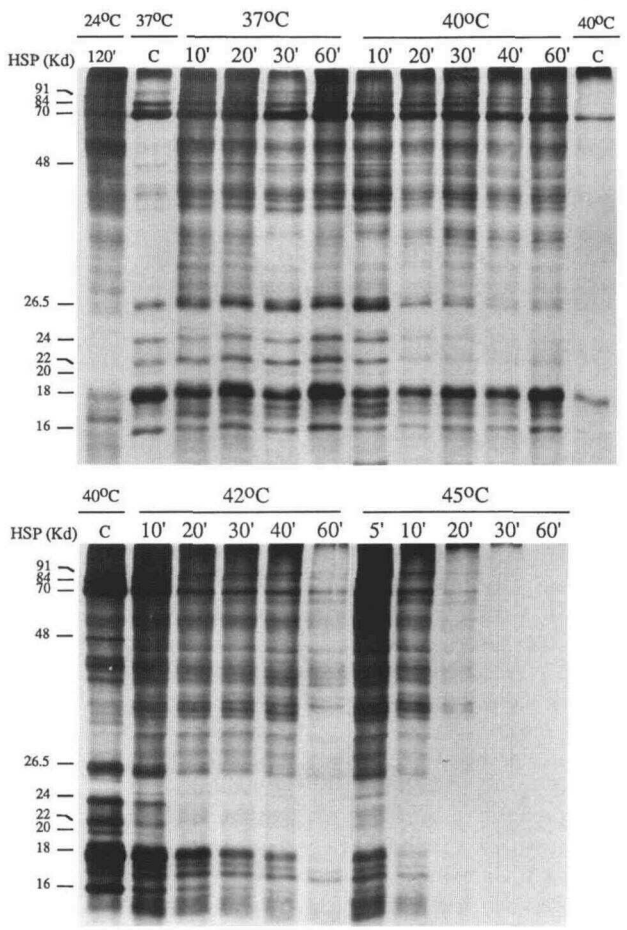


Figure 1. HSP synthesis in carrot protoplasts following heat shock at 37 and 40°C (top) or 42 and 45°C (bottom). Cells were heat shocked for the time and temperature indicated below each lane. Following the heat shock, [^{35}S]Met was added and the cells were incubated at 24°C for a further 2 h. Cell extracts were displayed on a 12% SDS-PAGE gel and subjected to fluorography. For heat-shock controls (labeled C), cells were heat shocked for 60 min and labeled with [^{35}S]Met at the heat-shock temperature for an additional 60 min. For a non-heat-shock control (24°C), cells were labeled for 2 h at 24°C. The molecular masses of the HSPs are indicated to the left of each panel.

would have occurred following the cessation of the thermal stress. As brief as a 10-min heat shock at 37°C resulted in the induction of most of the HSPs (Fig. 1), which have been well characterized in carrot (Pitto et al., 1983; Hwang and Zimmerman, 1989). The profile of HSP synthesis changed with the duration and the temperature of the heat shock. Moreover, the synthesis of non-HSPs observed at moderate heat-shock treatments was reduced with the increase in the severity of the stress. We conclude that even the most moderate heat-shock treatment tested was sufficient to induce HSP synthesis.

Translational Efficiency Is Reduced and mRNA Half-Life Is Increased following Heat Shock

To study the impact of heat shock on the translation and stability of reporter mRNA following delivery by electroporation, we determined how much of the input mRNA is delivered to the cell. One microgram of radiolabeled, capped-*luc*-A_{50} mRNA was electroporated in triplicate into samples of 2×10^6 carrot protoplasts each. Following a 90-min incubation to allow the mRNA to be fully recruited onto polysomes, the cells were harvested and broken in a Dounce homogenizer and the cell debris was pelleted. The resulting cell extract contained 3.6 ± 0.36% of the original input mRNA. The polysomal fraction was isolated from the cell extract by centrifugation through a Suc cushion (Silflow and Key, 1979). Of the input mRNA, 0.067 ± 0.015% was present in the polysomal fraction, representing 1.8% of the delivered mRNA. Based on the size of *luc* mRNA (1800 bases), this meant that 18,000 molecules would have been delivered to each cell and 324 molecules would be recruited onto polysomes.

To focus specifically on changes on translation and mRNA stability, we followed the impact of heat shock on the expression from capped-*luc*-A_{50} mRNA that was delivered to protoplasts immediately before the application of the heat shock at either 37, 40, 42, or 45°C (Fig. 2). In this and all subsequent experiments, mRNA was electroporated into multiple aliquots of protoplasts of 1×10^6 each, which were subsequently combined and divided equally among dishes that represented each time for each heat-shock temperature. This approach ensured that the dishes containing the protoplasts were uniform and equivalent before the application of the heat shock. Following heat treatment, the protoplasts were allowed to recover at room temperature for 24 h before assaying for luciferase activity. Although *luc* mRNA is translationally active for 2 to 3 h in control cells, a 24-h recovery was necessary to accommodate the large increase in *luc* mRNA stability in heat-shocked cells. Because the heat treatments were of short duration, translation of the *luc* mRNA occurred after the application of the heat shock. Therefore, our approach focused on those changes in the translation and RNA degradatory machinery that are a consequence of the heat shock but persist into the recovery phase.

Surprisingly, mild to moderate heat treatments resulted in an increase in protein yield from the reporter mRNA (Fig. 2). Optimal thermostimulation of expression was achieved with a 30-min treatment at 42°C or a 5-min treatment at 45°C. Longer exposure at these temperatures resulted in increased thermorepression. To rule out that the heat treatments were stimulating luciferase enzyme activity directly, we examined the effect of heat on luciferase enzyme activity by heat treating luciferase protein in carrot cell extract or in buffer in the presence or absence of BSA. We also delivered capped-*luc*-A_{50} mRNA to protoplasts and incubated the cells at 24°C for 20 h, at which time all of the reporter mRNA was degraded. The cells were then subjected to a 40 or 45°C heat shock. No thermostimulation of luciferase enzyme activity was observed by any of these approaches (data not shown). These data illustrate that either the translational efficiency or the stability of the introduced mRNA is regulated following a heat shock.

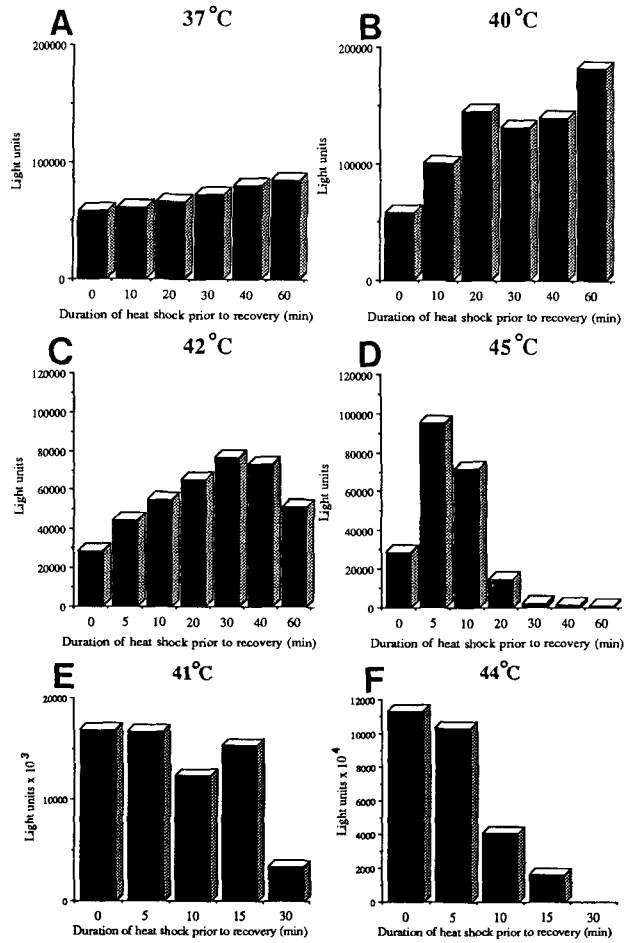
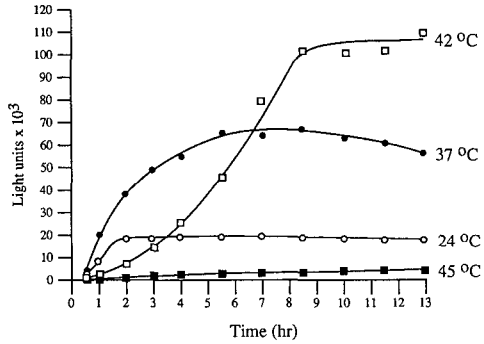


Figure 2. The effect of heat shock on the expression from introduced capped-*luc*-A_{50} in carrot (A–D) and CHO (E and F) cells. Following delivery of the reporter mRNA using electroporation, the carrot protoplasts and CHO cells were incubated in water baths at various temperatures for the times indicated on the *x* axis. The dishes were removed and incubated at 24°C for 24 h (carrot) or 12 h (CHO cells) before assaying for luciferase activity. The data presented in A and B were collected in an experiment separate from that in C and D. Therefore, the absolute levels of expression in A and B can be compared as they can between C and D. The data in E and F were collected in separate experiments. The variability in absolute levels of expression between experiments is primarily due to variation in the efficiency of protoplasting (data not shown).

To distinguish whether the thermal stress caused an increase in the translational efficiency or an increase in mRNA stability, we measured both in control and heat-treated protoplasts. This was accomplished by following the translation of capped-*luc*-A_{50} mRNA over time. As before, we used luciferase activity as a measure of gene expression. With this approach, it was necessary to know the stability of the luciferase protein in control and heat-shocked cells. The stability of the luciferase enzyme was measured in protoplasts that had been electroporated with capped-*luc*-A_{50} and incubated at 24°C for 20 h to ensure that all of the introduced mRNA was fully degraded. The stability of the protein could then be measured by removing aliquots of the protoplasts at time intervals and determining enzyme activity. This was done for both control cells and for cells subjected to a 15-min heat shock at 40 or 45°C, because these were typical heat treatments used in this study. A 6% reduction in luciferase enzyme activity was observed during a 24-h period in both control and heat-shocked cells, data suggesting that luciferase is a stable protein over the time frame used for the experiments in this study and that the stability of the protein during recovery from heat shock is similar to that in control cells (data not shown).

Following delivery of capped-*luc*-A_{50} mRNA and a 15-min heat shock at either 37, 42, or 45°C (control cells were maintained at 24°C), aliquots of cells were taken at time intervals and luciferase assays performed. The kinetics of *luc* mRNA translation were determined by following the appearance of protein as measured by enzyme activity plotted as a function of time. Once the *luc* mRNA has been delivered, the rate of luciferase protein production increases as the *luc* mRNA is recruited onto polysomes. Once the mRNA has been initially loaded onto the polysomes, translation continues at a rate that is dictated by its translational efficiency and translated for a period that is determined by the stability of the mRNA. The eventual degradation of the mRNA results in a decreased rate of protein accumulation. Following degradation of the mRNA, further accumulation of luciferase protein ceases, represented by the plateau of each curve at the later times in Figure 3. Between the loading of the mRNA onto the polysomes and its eventual degradation, there is a phase of steady-state translation in which the rate of luciferase production is both maximal and constant. The translational efficiency of the mRNA is measured during this steady-state phase. The impact that heat shock has on the translation of mRNAs during the subsequent recovery can be determined by comparing the rate of translation for the capped-*luc*-A_{50} following the various heat treatments.

We measured the translational efficiency of capped-*luc*-A_{50} mRNA from the slope of each curve between 30 and 90 min following mRNA delivery, because this was the period of time during which translation of the reporter mRNA at 24°C proceeded at a constant rate. A 15-min heat shock at 37°C increased translational efficiency 2-fold compared to the control cells. In contrast, the rate of luciferase accumulation in the 42°C-treated cells was 2-fold less than the control cells during this same period, data demonstrating



Temp (°C)	Translational efficiency (light units/min)	Functional half life (hr)
24	200	1.1
37	400	1.7
42	98	5.8
45	5.6	9.5

Figure 3. Kinetics analysis of expression from capped-*luc*-A_{50} mRNA in carrot following thermal stress. Following delivery of the reporter mRNA, the protoplasts were heat shocked for 15 min at the temperatures indicated and allowed to recover at 24°C for the duration of the experiment. Aliquots of protoplasts were removed at time intervals and assayed. The resulting luciferase activity was plotted as a function of time. The translational efficiency was determined from the slope of each line between 30 and 90 min following mRNA delivery. The functional mRNA half-life was determined as the amount of time required to complete a 50% decay in the capacity of the *luc* mRNA to synthesize luciferase.

that translational efficiency was impaired by the more severe heat treatment. It should be noted, however, that rate of translation in the 42°C-treated cells continued to increase during the later stages of recovery, suggesting that recovery of the translational machinery was ongoing during this period. The heat-mediated repression of translational efficiency was more pronounced following a 45°C treatment, in which the rate of translation was reduced by 36-fold compared to the control cells at 24°C. This rate did not change during the duration of the experiment.

These observations presented an apparent paradox. Under conditions that resulted in a reduced rate of translation, protein yield was actually stimulated. The paradox is resolved when the effect of heat shock on the stability of *luc* mRNA is examined. We measured the effects of heat treatment on mRNA stability by determining its physical and functional half-life. Following delivery of capped-*luc*-A_{50} mRNA, the protoplasts were divided into dishes that were treated at 24, 37, 42, or 45°C for 15 min. The dishes were further incubated at 24°C, and aliquots of cells were taken at time intervals starting 30 min following mRNA delivery. Total RNA was extracted and probed for the presence of full-length *luc* mRNA using northern analysis (see "Materials and Methods"). The half-life of the capped-*luc*-A_{50} mRNA was 127 min in control cells but increased to 261 min in cells treated for 15 min at 37°C (Fig. 4). The mRNA half-life increased further to 472 min following 15 min at 42°C and 463 min following 15 min at 45°C.

Using the data in Figure 3, we could also measure the functional stability of the capped-*luc*-A_{50} mRNA in the

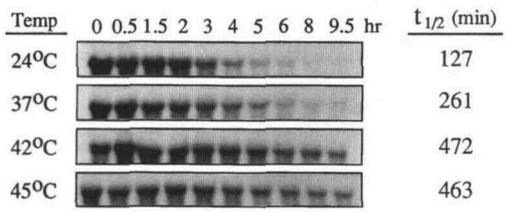


Figure 4. The physical half-life of capped-*luc*-A_{50} mRNA in carrot protoplasts under control temperature or following a 15-min heat shock at 37, 42, or 45°C. The delivery, extraction, and analysis of the mRNA was as described in "Materials and Methods." The time at which time points were taken for the RNA analysis are indicated at the top. The half-life ($t_{1/2}$, min) is indicated to the right.

control and heat-treated cells. The functional half-life of an mRNA is a measure of the integrity of the message as determined by the length of time over which it is translationally active and is defined as the amount of time needed to complete a 50% decay in the capacity of an mRNA to synthesize protein (Kepes, 1963; Pedersen et al., 1978). In contrast, physical half-life follows the physical integrity of a message independently of the translational competence of the mRNA. Because the functional half-life measures the stability of only that mRNA that is being translated, it more accurately describes the stability of the reporter mRNA that is polysome associated than does physical half-life. The half-life of capped-*luc*-A_{50} mRNA in the control cells was 1.1 h, whereas a 15-min heat shock at 37 or 42°C resulted in an increase in the half-life to 1.7 or 5.8 h, respectively. The half-life of the reporter mRNA in 45°C-treated cells was considerably longer than that observed in the 42°C-treated cells but could not be measured in this experiment because the mRNA was still translationally active at 13 h, the last time point taken. Measurements in a subsequent experiment in which time points were taken up to 27 h determined that the half-life was 9.5 h. The linear relationship between the severity of the heat treatment and its effect on mRNA half-life can be seen clearly when the half-life is plotted as a function of the heat-shock temperature (Fig. 5). Extrapolation of the curve suggests that the increase in capped-*luc*-A_{50} mRNA half-life begins between 36 and 37°C. Both the physical and functional half-life measurements illustrate that the stability of mRNA increases following heat shock.

Although the effects of heat stress on translational efficiency, mRNA stability, and protein yield from the reporter mRNA initially appear to be complex, they can be summarized as three observations. First, whereas mild heat shock caused an increase in translational efficiency, moderate to severe heat treatments resulted in a dramatic reduction. Second, reporter mRNA half-life increased with the severity of the heat stress. Third, the interplay between the extent to which the translational efficiency was affected and the extent to which the stability of the mRNA increased following heat treatment determined whether a particular heat-shock treatment stimulated or reduced protein yield. For example, a 15-min exposure at 37°C increased translational efficiency and mRNA stability and, consequently, the protein yield was increased (Fig. 3). With a 15-min exposure at 42°C, there was a reduction in translation efficiency, but the increase in the mRNA half-life more than made up for this lower rate of translation, resulting in a final yield of protein that was higher than that in the control cells. The reduction in the translational efficiency following a 15-min exposure at 45°C, however, was so great that even with the dramatic increase in mRNA stability the protein yield did not reach the level of even the control cells.

Heat Shock Impairs the Function of the Cap and the Poly(A) Tail

The majority of cellular mRNAs use a cap (m^7GpppN) and a poly(A) tail to promote the initiation of translation as well as to stabilize mRNA. We have shown that the cap and poly(A) tail cooperate to form the basis for efficient translation in plants and animals (Gallie, 1991). As a regulator of translation in higher eukaryotes, the poly(A) tail requires the cap for function; for uncapped messages, the translational efficiency of poly(A)$^+$ mRNA is not substantially greater than poly(A)$^-$ mRNA (Gallie, 1991). Moreover, the degree to which a cap stimulates translation is 1 order of magnitude greater for poly(A)$^+$ than it is for poly(A)$^-$ mRNA (Gallie, 1991). Therefore, the cap and poly(A) tail are not functionally separate but work in concert, in conjunction with their associated proteins, to stimulate translation.

To determine whether heat affects the function of the cap or poly(A) tail, we analyzed the regulatory ability of these elements following thermal stress. Although the error associated with RNA delivery via electroporation is no greater than approximately ±15% (Gallie et al., 1991), capped-*luc*-A_{50} mRNA was electroporated into three aliquots of 1×10^6 protoplasts, which were subsequently combined to further lower any variability in mRNA delivery. The protoplasts were subsequently divided equally among dishes that represented times from 0 to 60 min at each heat-shock temperature. This ensured that for a given mRNA all dishes of protoplasts were equivalent before the

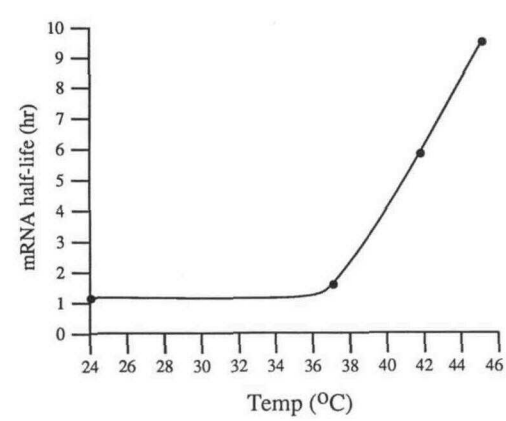


Figure 5. The functional half-life of capped-*luc*-A_{50} mRNA in carrot protoplasts plotted as a function of temperature. The heat treatment at each temperature tested was for 15 min following mRNA delivery. The functional half-life data were taken from the analyses in Figure 3.

application of the thermal stress. Following the heat treatment, the protoplasts were then allowed to recover for 24 h at room temperature. As in the above experiments, translation takes place during the recovery period. The level of luciferase activity is used as a measure of expression, which in this case results from the combined effects of mRNA stability and translational efficiency. Because each temperature was tested in separate experiments, the protein yield (i.e. luciferase activity) resulting from each form of *luc* mRNA can be compared within a given temperature but not between temperatures. As little as 7.5 min of heat shock at 37, 40, 42, or 45°C was sufficient to result in an increase in protein yield for all four forms of the *luc* mRNA (Table I). Therefore thermostimulation of expression occurred regardless of whether a cap or poly(A) tail was present. The uncapped *luc* mRNAs [poly(A)$^-$ and poly(A)$^+$] were thermostimulated to a significantly greater extent than were the capped forms of the mRNA. All forms of the mRNA were subject to thermorepression following long and severe heat-shock treatments, with capped-*luc*-A_{50} mRNA being more repressed than the other forms.

We can measure the degree to which a cap stimulates expression at any given duration of treatment for each temperature in Table I by comparing the luciferase activity resulting from the capped form of the mRNA to that from the uncapped mRNA. The effect of heat shock on the degree to which a cap can increase expression can then be plotted as a function of the duration of exposure to the elevated temperature. In control protoplasts, the addition of a cap to *luc*-A_{50} mRNA increased translation 240-fold, which is defined as 100% cap activity (Fig. 6A). Heat shock impaired the degree to which the cap stimulated expression. The loss in cap activity was a function of both the temperature of the heat treatment and duration of exposure to the stress. Cap function was virtually eliminated following a 40-min exposure to 45°C.

To assess whether the function of a poly(A) tail was also affected by heat shock, we compared the level of expression from capped-*luc*-A_{50} with that from capped *luc* mRNA

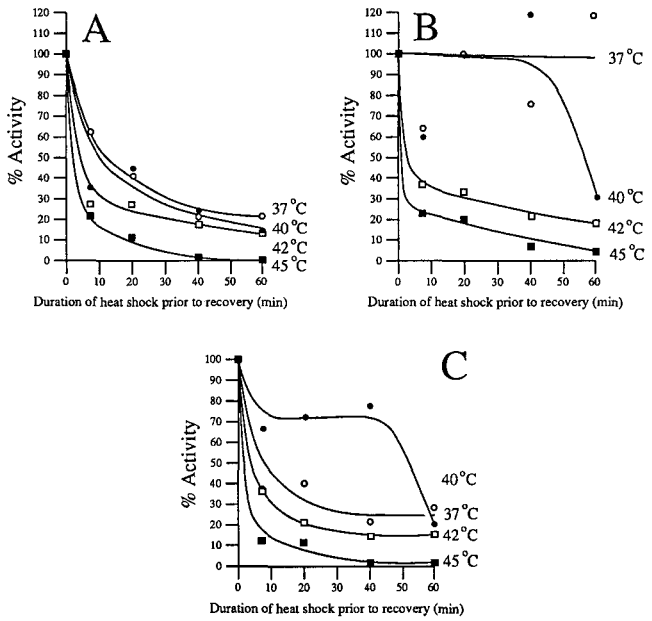


Figure 6. The impact of heat shock on cap and poly(A) tail function in carrot protoplasts. The activity of the cap and poly(A) tail and the synergism between them were calculated from the data presented in Table I. The four forms of *luc* mRNA were synthesized in vitro and delivered to carrot protoplasts immediately before the heat-shock treatment. A, The activity of the cap under non-heat-shock conditions is set at 100% and is determined by the ratio of the expression levels, i.e. capped-*luc*-A_{50}/*luc*-A_{50}. Ratios were determined for the same pair of constructs at each heat-shock temperature and time point from Table I and expressed as a percentage of the control activity. Similar calculations were performed for the poly(A) tail by comparing expression from capped-*luc*-A_{50} to capped-*luc* mRNA (B) and for the cap/poly(A) tail synergy by comparing the ratios of capped-*luc*-A_{50}/uncapped-*luc*-A_{50} mRNA to capped-*luc*/uncapped-*luc* mRNA (C). The data were then plotted as a function of the duration of the heat shock to which the protoplasts were subjected before being allowed to recover.

Table I. *The impact of heat shock on translation during recovery in carrot*

Temp (°C)	mRNA	Luciferase Activity 24 h following a Heat Shock of Duration				
		0 min	7.5 min	20 min	40 min	60 min
				light units/mg protein		
37	*luc*	1,016	1,745	2,494	2,255	3,338
	luc-A_{50}	1,039	3,041	6,270	8,501	13,815
	capped-*luc*	20,256	57,724	48,469	49,627	48,097
	capped-*luc*-A_{50}	247,556	447,672	599,725	453,202	732,860
40	*luc*	478	3,681	4,093	9,582	7,395
	luc-A_{50}	931	6,285	11,003	29,709	22,813
	capped-*luc*	16,349	63,334	86,936	109,074	175,068
	capped-*luc*-A_{50}	216,894	500,069	1,161,884	1,792,341	812,377
42	*luc*	837	4,922	6,852	11,282	5,598
	luc-A_{50}	1,660	9,840	23,818	34,087	13,530
	capped-*luc*	17,318	72,682	198,384	298,788	119,113
	capped-*luc*-A_{50}	356,256	572,727	1,443,116	1,497,585	555,791
45	*luc*	1,194	8,180	6,711	1,851	2,006
	luc-A_{50}	1,471	21,383	15,806	9,956	3,526
	capped-*luc*	14,424	189,414	84,973	21,615	4,931
	capped-*luc*-A_{50}	321,183	993,990	423,905	29,627	4,456

for each length of heat treatment at each temperature in Table I. As with cap activity, the poly(A) tail activity can be plotted as the function of the duration of the heat treatment. Although heat shocks at either 37 or 40°C had only a small effect on poly(A) tail function, a substantial reduction in its activity occurred following a 42 or 45°C treatment (Fig. 6B).

The synergism between the cap and poly(A) tail is measured as the ratio of the impact that a cap makes on the translation of poly(A)$^+$ versus poly(A)$^-$ mRNA. For instance, at the control temperature, the addition of a cap to poly(A)$^-$ *luc* mRNA stimulates expression 20-fold, but the addition of a cap to poly(A)$^+$ *luc* mRNA stimulates expression 240-fold (Table I). This represents a 12-fold level of synergism (i.e. 240 divided by 20 = 12-fold) and is designated as 100% synergy in Figure 6C. The effect of heat shock on the cap and poly(A) tail synergy can be determined by the same analysis at each heat-shock temperature for each length of treatment. With increasing temperature and increasing exposure to the elevated temperatures, the synergy decreased accordingly. Heat shock, therefore, had an inhibitory effect on the synergy between the cap and poly(A) tail. This observation is further supported by examining to what extent the function of a cap or a poly(A) tail was affected by heat shock when either was present in the absence of the other element. No decrease in function was observed for either element following heat shock (Table I). One explanation of these observations is that heat shock decreases cap and poly(A) tail function by disrupting the functional interaction between these two regulatory elements.

No thermostimulation of reporter mRNA translation was observed in CHO cells (Table II). Because CHO cells are normally grown at 37°C, the temperatures used for a mild and severe heat shock were 41 and 44°C, respectively. The repression of translation occurred with as little as 5 min of heat treatment. Translation of reporter mRNA was repressed regardless of whether the mRNA was capped or polyadenylated. Because uncapped-*luc*-A_{50} mRNA was subject to greater repression following the heat shock than was capped-*luc*-A_{50} mRNA, translation became increasingly cap dependent (Fig. 7A). A loss in the function of the poly(A) tail was observed at both heat-shock temperatures (Fig. 7B), although the synergy between the cap and poly(A) tail decreased only following the 44°C heat treatment (Fig. 7C). This contrasted with the observations made in carrot in which cap activity was quite sensitive to heat shock, data suggesting that the heat-shock response in plants may differ significantly from animal cells.

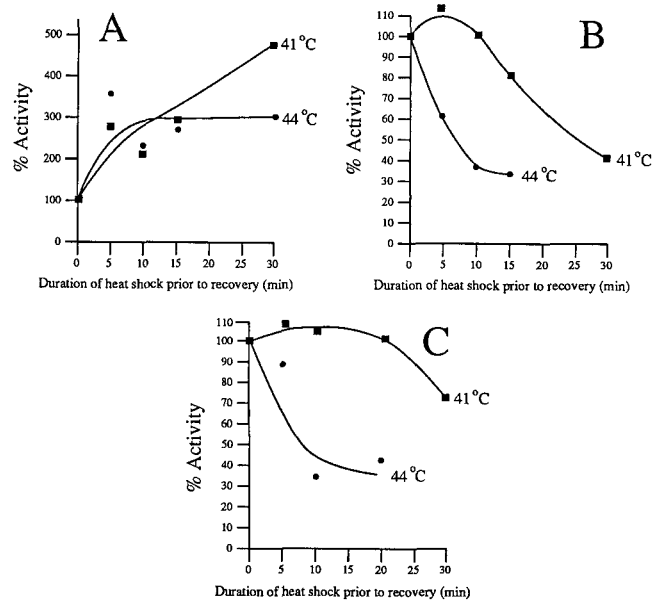


Figure 7. The impact of heat shock on cap and poly(A) tail function in CHO cells. The activity of the cap and poly(A) tail and the synergism between them were calculated from the data presented in Table II. The four forms of *luc* mRNA were synthesized in vitro and delivered to CHO cells immediately before the heat-shock treatment. A, The activity of the cap under non-heat-shock conditions is set at 100% and is determined by the ratio of the expression levels, i.e. capped-*luc*-A_{50}/*luc*-A_{50}. Ratios were determined for the same pair of constructs at each heat-shock temperature and time point from Table II and expressed as a percentage of the control activity. Similar calculations were performed for the poly(A) tail by comparing expression from capped-*luc*-A_{50} to capped-*luc* mRNA (B) and for the cap/poly(A) tail synergy by comparing the ratios of capped-*luc*-A_{50}/uncapped-*luc*-A_{50} mRNA to capped-*luc*/uncapped-*luc* mRNA (C). The data were then plotted as a function of the duration of the heat shock to which the CHO cells were subjected before being allowed to recover.

The Translational Efficiency of Uncapped Messages Is Preferentially Stimulated following Heat Shock

The rate-limiting step in the assembly of the translational initiation complex is the binding of the cap-binding factor,

Table II. *The impact of heat shock on translation during recovery in CHO cells*

Temp (°C)	mRNA	Luciferase Activity 6 h following a Heat Shock of Duration				
		0 min	5 min	10 min	15 min	30 min
		light units/mg protein				
41	*luc*	140,366	47,656	50,479	54,401	10,887
	luc-A_{50}	512,066	184,565	178,641	156,391	21,719
	capped-*luc*	174,493	148,720	123,378	195,438	86,303
	capped-*luc*-A_{50}	16,895,945	16,780,078	12,397,055	15,358,216	3,414,069
44	*luc*	724,700	266,677	126,468	44,201	1,895
	luc-A_{50}	3,478,684	872,174	544,847	181,397	2,159
	capped-*luc*	1,016,672	1,513,349	1,166,038	394,246	2,539
	capped-*luc*-A_{50}	113,126,490	103,170,360	41,186,965	16,155,179	212,021

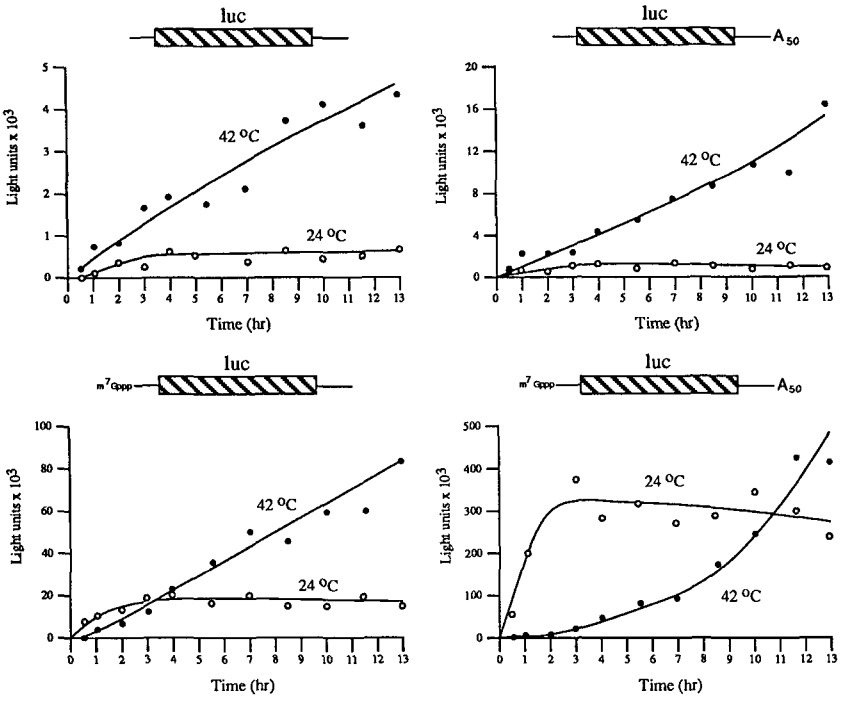


Figure 8. The effect of heat shock on the translation of mRNAs that lack a cap or poly(A) tail in carrot protoplasts. *luc* mRNA was synthesized as poly(A)$^-$ or poly(A)$^+$ with or without a cap. Each form of mRNA was electroporated into protoplasts and one-half of the cells was immediately subjected to a 15-min heat shock at 42°C before being allowed to recover at 24°C. The other half of the cells was maintained at 24°C to serve as a control. Aliquots of protoplasts were taken at times up to 13 h following mRNA delivery and assayed for luciferase activity. Enzyme activity was used as a measure of the extent of translation and plotted as a function of time. The form of *luc* mRNA delivered is shown at the top of each graph.

eIF-4F. Capped messages, therefore, possess a competitive advantage for initiation factor binding. Following heat shock, we have observed that the translational machinery loses its ability to discriminate between uncapped and capped messages (Table II; Fig. 6A). In other words, the translational efficiency of uncapped mRNAs and capped mRNAs approaches a similar level. This can be achieved two ways: either the translational efficiency of capped mRNA decreases to the rate normally observed for uncapped mRNA or the translational efficiency of uncapped mRNA increases up to that observed for capped mRNA. These two possibilities are not necessarily mutually exclusive.

To determine the effect of heat shock on the translational efficiency of uncapped and capped messages, the translational efficiency of the four forms of the *luc* mRNA were measured following mRNA delivery and a 15-min 42°C heat treatment in carrot protoplasts. The stability of the reporter mRNA increased significantly following a heat shock regardless of whether the transcript contained a cap or poly(A) tail (Fig. 8). The heat treatment resulted in a preferential increase in the translational efficiency (measured from the slope of each line) of the uncapped forms of the mRNA [i.e. either with or without a poly(A) tail]. The translational efficiency of the capped mRNAs, especially for capped, poly(A)$^+$ mRNA, was substantially repressed following heat shock. These data demonstrate that under conditions in which the translation from capped messages is subject to increasing repression by heat shock the translational efficiency of uncapped mRNAs actually improves. Moreover, messages that are both capped and polyadenylated are far more sensitive to heat-mediated translational inactivation than is any other form of mRNA.

DISCUSSION

Although the reprogramming of translation following heat shock has been documented in many species, little is known about the mechanism responsible for the change. Such investigations have been complicated by the profound changes that occur at the level of transcription following a heat shock (Sorger, 1991; Morimoto et al., 1992). One means by which to focus on heat-induced changes in translational efficiency and mRNA stability is to follow the fate of introduced mRNA. This approach does require that expression from introduced mRNA be subject to the same changes following heat shock that are observed for an endogenous mRNA. We know that regulatory elements defined for endogenous mRNAs [e.g. a cap and poly(A) tail] also act to regulate expression of introduced mRNA, that the presence of a *hsp70* 5′-leader sequence in an introduced reporter mRNA confers translational competence during heat shock (Pitto et al., 1992), and that introduced *luc* mRNA is released from polysomes following a heat shock (D.R. Gallie, unpublished observations), as has been observed for endogenous mRNAs (Key et al., 1981), and co-fractionates with the organellar fraction, consistent with the observation that nonheat-shock mRNAs move into nuclear-associated HSGs following heat shock (Nover et al., 1983, 1989). We conclude that, at first approximation, exogenously introduced mRNA can behave similarly to endogenous mRNAs following heat shock. It remains to be determined whether there are respects in which they may differ.

We have observed that significant changes occur in both the translational efficiency and mRNA stability of reporter mRNA as a consequence of thermal stress. The degree to

which each of these two processes is affected determines whether the final protein yield will be higher or lower than that achieved at the control temperature. A mild heat shock caused a small increase in both the translational efficiency and message stability that resulted in increased protein yield. A moderate heat shock reduced translation but increased the stability of the mRNA to such an extent that the final protein yield still exceeded that observed at the control temperature. Although a severe heat shock caused a dramatic 9-fold increase in mRNA stability relative to that in control cells, it was not sufficient to overcome the 36-fold reduction in translational efficiency and, consequently, protein yield was reduced. Therefore, the changes in protein yield following a heat shock is a consequence of the combined impact that the stress makes on translation and mRNA stability.

The changes in translational efficiency and mRNA stability of the reporter mRNA were rapid in two respects. First, a heat shock of very short duration (as little as 5 min) was sufficient to cause changes in translation and mRNA stability. We interpret this as meaning that these processes are extremely sensitive to elevated temperature. Second, the changes in translation and mRNA stability were observed immediately following the heat shock, i.e. the changes had already occurred by the first time point taken in any of the kinetics analyses. This suggests that, in addition to the response being sensitive to even short periods of heat stress, it is also extremely rapid. This is in agreement with observations made in *Drosophila*, in which non-*hsp* mRNAs were released from polysomes within 10 min of thermal stress (Lindquist McKenzie et al., 1975). Such rapidity suggests that the changes in the translation and RNA degradatory machinery occur as a result of alterations in the existing machinery at the time of the stress.

What then are the mechanisms underlying the changes in translation and mRNA stability following heat stress? Because our analyses focused on events that take place following the cessation of the thermal stress, our observations focus on those changes in the translational and RNA degradatory machinery that are a consequence of the heat shock but persist into the recovery phase. We observed that there was a collapse in the function of the cap for poly(A)$^+$ reporter mRNA following heat shock. Studies in animals cells have established that hypophosphorylation of the cap-binding complex, eIF-4F, occurs as a consequence of heat shock (Panniers et al., 1985; Lamphear and Panniers, 1991; Zapata et al., 1991). Because the phosphorylation of the cap-binding subunit, eIF-4Fα, is required for eIF-4F activity, hypophosphorylation of eIF-4Fα results in a reduced rate of translation. Although it remains to be established whether similar modifications of eIF-4F occur in plants subject to heat shock, this could constitute the basis for the selective loss in translational efficiency of capped mRNA following thermal stress. The loss of cap function as a consequence of thermal stress was observed only when the transcript contained a poly(A) tail. Our explanation for this is that heat shock disrupts not cap function per se but its interaction with the poly(A) tail. The loss in the synergy between these two regulatory elements supports this hypothesis. A loss in poly(A) tail function of capped mRNAs was also observed. Little is known about the effect of heat stress on the function of the poly(A)-binding protein, the protein responsible for mediating the regulation associated with the poly(A) tail. Multiple isoforms of poly(A)-binding protein have been observed in yeast and sea urchin (Drawbridge et al., 1990), suggesting that this protein, like eIF-4F, may be subject to phosphorylation. Whether poly(A)-binding protein activity is regulated by modification and/or heat shock remains to be determined. However, because of the co-dependent interaction between the cap and poly(A) tail (Gallie, 1991), any alteration in the activity of one element would be expected to affect the synergy between both regulatory elements.

We also observed an inverse correlation between the effect on translation and mRNA stability following heat shocks of increasing severity. Because translation of capped mRNA was subject to increasing repression, there was a concomitant increase in the half-life of the message. Increased stability was observed for mRNA regardless of whether it was capped or polyadenylated, data suggesting that, unlike the alterations in the translational machinery that specifically affected capped messages, the changes in the RNA degradatory machinery affected all classes of mRNA.

There are two possible mechanisms by which an increase in mRNA stability can occur. There could be a reduction in the activity of those RNases responsible for mRNA turnover or there could be increased protection from the mRNA degradatory apparatus. These are not mutually exclusive possibilities. The observations that non-*hsp* mRNAs are sequestered in HSGs (Nover et al., 1989; Apuya and Zimmerman, 1992) suggests that mRNAs might be protected from RNase attack. During recovery, the mRNA may be released from the HSGs at a constant rate for subsequent recruitment onto polysomes or the translational machinery may access the mRNA contained in the HSGs in a way that the degradatory machinery cannot. Either mechanism would result in an apparent increase in mRNA half-life. Only if the RNA degradatory machinery itself were to be impaired following heat shock would there be a real increase in message stability. Examination of RNase activity before and after heat shock will be necessary to distinguish between these possibilities.

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