Heat Shock Causes Selective Destabilization of Secretory Protein mRNAs in Barley Aleurone Cells[1]

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ABSTRACT

The aleurone layer of GA$_3$-stimulated barley (*Hordeum vulgare* L., cv Himalaya) grains is normally devoted to the synthesis and secretion of hydrolytic enzymes. Heat shock, however, suppresses the synthesis of the main hydrolytic enzyme, α-amylase, by destabilizing its otherwise highly stable mRNA (FC Belanger, MR Brodl, T-hD Ho [1986] Proc Natl Acad Sci USA 83: 1354–1358). In this paper we document that heat shock causes the suppression of the synthesis of some normal cellular proteins, while the synthesis of other normal cellular proteins is unaffected by heat shock. There are two major isozymic forms of α-amylase encoded by distinct mRNAs. The mRNA levels for both isozymic forms and the mRNA levels of two other secretory proteins, a protease and an endochitinase, were markedly reduced during heat shock. However, the levels of actin and β-tubulin mRNAs, both nonsecretory proteins, were not diminished during heat shock. In addition, the levels of three other mRNA species detected by a set of unidentified cDNA clones (the sequence of one shows that it lacks a signal sequence) remained unchanged during heat shock. These data indicate that there are two classes of normal cellular protein mRNAs with regard to the effect of heat shock upon their persistence in the cell, and suggest that the distinction between them is whether or not they encode secretory proteins.

Heat shock is, quite simply, a transient elevation in temperature, usually about 10 to 15°C above ambient temperature. This simple manipulation produces remarkable changes in gene expression. In every organism tested to date heat shock induces the synthesis of a characteristic set of proteins, the so-called hsps[3] (for review see ref. 1). At the same time, the synthesis of several or all of the proteins synthesized prior to heat shock (normal cellular proteins) is interrupted, presumably allowing the cell to redirect its energy toward responding to heat shock.

The mechanisms by which cells accomplish the shift in gene expression from normal cellular protein synthesis to hsp synthesis involves a variety of mechanisms. Heat-shocked *Drosophila* cells accomplish this shift at the translational level.

There is a translational bias established during heat shock that favors the translation of the newly transcribed hsp mRNAs (18). The mRNAs encoding normal cellular proteins persist in the cytoplasm; however, they are not translated during heat shock. When returned to normal temperatures, the synthesis of normal cellular proteins resumes even in the presence of actinomycin D, indicating that these mRNAs that had been quiescent during heat shock are reactivated during recovery (9).

In yeast, the shift in gene expression is controlled at the transcriptional level. Heat shock induces the transcription of genes encoding hsps but represses the transcription of genes encoding normal cellular proteins. The normal cellular protein mRNAs already present in the cytoplasm continue to be translated (along with hsp mRNAs) until they are turned over at their normal, fairly rapid rates (13). In soybean a similar transcriptional regulation of normal cellular protein synthesis appears to be operating. Vierling and Key (20) have demonstrated that the mRNA for the small subunit of ribulose-1,5-phosphate carboxylase decreases during heat shock, presumably as it is turned over at its normal rate.

We have previously investigated the heat-shock response in the cells of barley aleurone layers (2). α-Amylases are the principal enzymes synthesized and secreted by these cells when they are incubated with the hormone GA$_3$ at normal temperature. During heat shock, the synthesis of α-amylase is suspended (2). In addition, heat shock causes a rapid degradation of low-pI (one of the isozymic groups) α-amylase mRNAs. However, this response differs significantly from that of yeast and soybean in that α-amylase mRNA normally has a half-life of at least 100 h (5). Therefore, normal mRNA turnover cannot account for the decrease in α-amylase mRNA levels. Heat shock also results in the disruption of the lamellar network of ER in these cells (2). We have proposed that in the absence of intact ER lamellae α-amylase mRNA, which is translated on ER-bound polysomes, is no longer stable and is therefore selectively degraded during heat shock (2). The data presented here document that heat shock selectively reduces the levels of several secretory protein mRNAs whereas the mRNA levels for several nonsecretory proteins remain unaffected by heat shock.

MATERIALS AND METHODS

Plant Materials

Embryos were dissected from barley grains (*Hordeum vulgare* L. cv Himalaya, 1981 harvest) obtained from Washing-

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[3] Abbreviations: hsp(s), heat shock protein(s); pI, isoelectric point; SSC, saline-sodium citrate.

ton State University (Pullman, WA). The resulting "embryo-less half seeds" were surface sterilized and imbibed aseptically for 4 d in Petri dishes on filter paper overlaying vermiculite, which was soaked with 20 mM Na succinate, pH 5.0, 20 mM $CaCl_2$ (2). The aleurone layers were peeled away from the softened endosperm under aseptic conditions and incubated under the appropriate experimental conditions.

Protein Analysis

For experiments examining protein synthesis, each sample constituted 10 aleurone layers in 2 mL of incubation buffer (20 mM Na succinate, pH 5.0, 20 mM $CaCl_2$) in a 25 mL flask. These samples were incubated for 16 h at 25°C in the presence or absence of 1 μM GA_3 in a reciprocal shaker at 120 cycles/min. Heat-shocked aleurone layers were incubated for an additional 3 h at 40°C, whereas nonheat-shocked controls were incubated for an additional 3 h at 25°C. Samples were labeled with 50 μCi [^{35}S]Met (specific activity >1000 Ci/mmol, New England Nuclear, Boston, MA) in 1 mL of fresh incubation buffer during the last hour of incubation. At the end of the labeling, samples were rinsed with 1 mM nonradioactive Met and homogenized in a chilled mortar and pestle with sterile sand and 10 μL of 100 μM leupeptin (a thioprotease inhibitor). The homogenates were mixed with 300 μL of extraction buffer (0.01 M Tris-Cl, pH 6.8; 1.4 M 2-mercaptoethanol; 0.1% bromphenol blue; 30% glycerol, 1% SDS).

Samples of homogenized tissues were diluted in an appropriate volume of 4 × gel loading buffer (0.025 M Tris, pH 6.8; 0.282 M 2-mercaptoethanol; 0.04% bromphenol blue; 80% glycerol, 10% SDS) and analyzed by one-dimensional 11% SDS-PAGE according to the procedures of Laemmli (12).

RNA Analysis

For RNA analysis, 100 aleurone layers were incubated for 16 h at 25°C in 20 mL incubation buffer with or without 1 μM GA_3 in 250 mL flasks in a reciprocal shaker at 120 cycles/min. Samples for heat shock were then subjected to a series of 40°C treatments (0.25, 0.5, 1, 2, 3, and 4 h) or to 40°C for 3 h followed by a series of 25°C recovery treatments (2, 4, 8, and 16 h), while controls were retained at 25°C.

RNA was isolated using the guanidine·HCl extraction procedure of Chirgwin et al. (7) adapted by Belanger et al. (2). RNA was electrophoretically separated on formaldehyde-containing 1% agarose gels. The electrophoresed RNA was transferred to GeneScreen Plus membranes (New England Nuclear) according to manufacturer's instructions. With the exception of hybridizations using the Volvox β-tubulin clone (where stringency was significantly reduced), the membrane was prehybridized, hybridized, and washed according to the procedures of Church and Gilbert (8). Prehybridization and hybridization with reduced stringency (for the β-tubulin clone) were done in a solution containing 50% formamide, 5 × SSC (0.75 M NaCl, 0.075 M Na citrate, pH 7.0) and 100 μg/mL sheared salmon sperm DNA at 42°C. Under reduced stringency conditions, the hybridized membranes were washed (10 min each) three times in 2 × SSC, 0.1% SDS at room temperature; once in 2 × SSC, 0.1% SDS at 60°C, and once in 0.2 × SSC, 0.1% SDS at 60°C.

Probes were made by nick-translating cloned cDNAs for the high-pI isozyme of α-amylase (pM/C) (14), the low-pI isozyme of α-amylase (clone E) (16), actin (pMAC-1) (17), endochitinase (19), protease (11), β-tubulin (10), and three unidentified clones (nonGA₃-inducible from barley aleurone layers) (L-S Lin, G Heck, T-hD Ho, unpublished data) in the presence of α-[^{32}P]dCTP (New England Nuclear, specific activity >500 Ci/mmol).

RESULTS

Effect of Heat Shock on Protein Synthesis

To investigate the effect of heat shock on protein synthesis, we performed labeling experiments in vivo with GA_3-treated aleurone layers heat shocked for 3 h. When barley aleurone layers are incubated in the presence of 1 μM GA_3, α-amylases are synthesized de novo (for review see ref. 5) (Fig. 1, cf. lanes 1 and 2). Results from SDS-PAGE analysis of [^{35}S]Met-labeled proteins indicated that after 3 h of heat shock at 40°C, the synthesis of α-amylase had decreased to barely detectable levels and the synthesis of hsps was induced (Fig. 1, cf. lanes 2 and 4). The synthesis of several other proteins normally synthesized at 25°C continued during exposure to heat shock. These results demonstrate that there are three classes of proteins in heat shocked barley aleurone cells: those whose synthesis is induced, those whose synthesis is suppressed, and those whose synthesis is unaffected by heat shock.

Timing of the Response

In barley aleurone cells, α-amylase is composed of two sets of isozymes with differing pIs (6). These isozymes are categorized into a high-pI group and a low-pI group (6) and are encoded by two distinct genetic loci located on separate chromosomes (4). It is not known whether heat shock affects the mRNA levels of both isozymic forms equally. For this reason, we investigated the timing of the decrease in mRNA levels for the two isozyme groups of α-amylase using Northern blot analyses. As shown in Figure 2, changes in the levels of mRNA for both pI groups paralleled each other over the course of the heat shock and subsequent recovery. Within 30 min of heat shock the levels of both high-pI α-amylase mRNA and low-pI α-amylase mRNA were less than 50% of nonheat-shocked tissues (Fig. 2, lane 3). Time points earlier than 30 min indicated little change in the mRNA levels of either isozyme from nonheat-shocked tissues (Fig. 2, cf. lanes 1 and 2 with lane 3). After 3 h of heat shock, mRNAs from both isozymes were present at levels less than 10% of nonheat-shocked tissues (Fig. 2, lane 6). Significant recovery of α-amylase mRNA for both isozymes was observed by 8 h, and total recovery by 16 h (Fig. 2, cf. lanes 10 and 11 with lane 1).

Heat Shock Reduces mRNA Levels for Other Secreted Proteins but Does Not Affect mRNA Levels of Nonsecreted Proteins

Because heat shock has such a marked effect on α-amylase mRNAs, we were curious as to whether heat shock affected the synthesis of GA_3-induced hydrolases in general. Thus, we
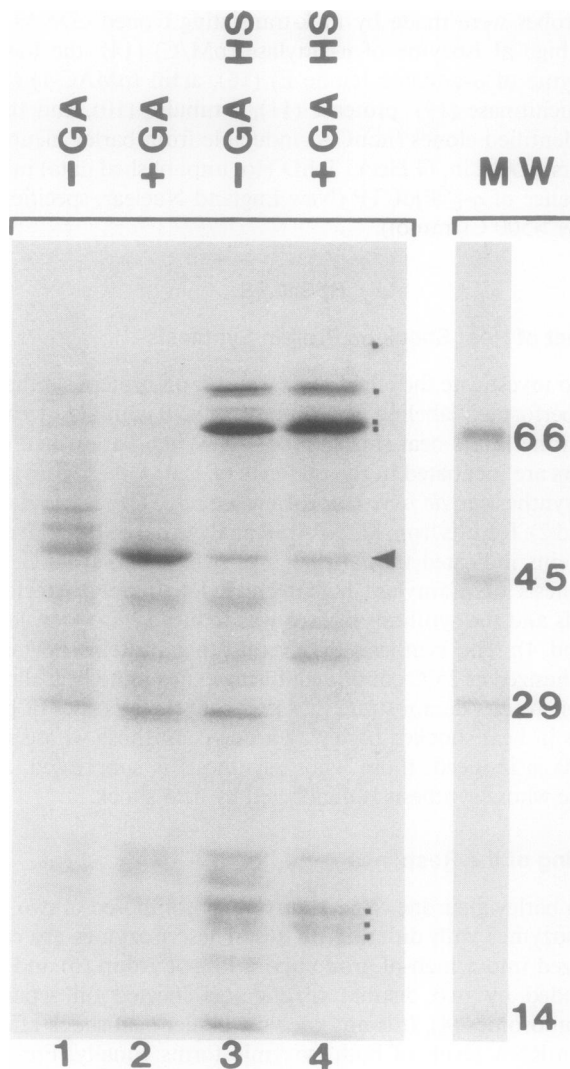
Figure 1. The pattern of protein synthesis in GA₃-stimulated and heat-shocked barley aleurone cells. Aleurone layers were incubated at 25°C in the absence (−GA) or presence (+GA) of 1 μM GA₃ for 18 h. The final 2 h of this incubation took place at either 25°C (−HS) or 40°C (+HS). Labeling with 50 μCi of [³⁵S]methionine per mL of buffer took place in the final hour of incubation. Protein synthesis was analyzed by 11% SDS-PAGE followed by fluorography. Equal amounts of acid-precipitable radioactivity were intended for each lane. Arrowhead indicates the position of α-amylase; dots indicate the positions of hsps. MW and numbers indicate the positions and sizes of molecular mass markers in kD.

have extended our studies to mRNAs encoding a secreted thiol endoprotease (11) and an endochitinase (19). By Northern blot analysis it was apparent that the levels of protease mRNA were markedly increased by the presence of GA₃ (Fig. 3, cf. lanes 1 and 2). A 3-h heat shock caused at least an 80% reduction in the amount of protease mRNA (Fig. 3, lane 3). Although endochitinase mRNA levels do not increase in the presence of GA₃, heat shock caused a marked decrease in the levels of this mRNA in GA₃-treated cells (Fig. 3, cf. lanes 1 and 2 with lane 3).

The level of expression of many of the proteins synthesized by barley aleurone layers is not affected by heat shock (Fig. 1). Because many of these proteins are also synthesized by aleurone cells incubated in the absence of GA₃ and the effect of GA₃ is primarily the induction of secretory protein synthesis, we speculated that these proteins were nonsecretory. To test this assertion, we isolated total RNA from heat-shocked aleurone layers and analyzed it on Northern blots probed with a maize genomic clone for actin (pMAC-1) (17) and a *Volvox* clone for β-tubulin (10). The mRNA levels of both actin and β-tubulin (the high background is due to the reduced stringency required for hybridization of the highly heterologous probe) were not affected by GA₃, as shown in Figure 4 (cf. lanes 1 and 2). Notably, heat shock did not affect the level of either mRNA (Fig. 4, lane 3). In addition, the levels of mRNA encoding three unidentified, non-GA₃-inducible transcripts (Fig. 5, cf. lanes 1 and 2) were also unaffected by heat shock (Fig. 5, lane 3). Although the identities of these mRNAs are not known, their behavior during heat shock indicates that the effect of heat shock on α-amylase and protease mRNAs is not the result of a generalized destabilization of normal cellular protein mRNAs. One of these clones, pHvGS-4, lacks a signal sequence (G Heck, T-hD Ho, unpublished data), indicating that this mRNA species encodes a protein that is not secreted.

DISCUSSION

Exposure of GA₃-stimulated barley aleurone cells to heat shock has a pronounced impact upon their pattern of protein synthesis. We show that in addition to inducing the synthesis of hsps, heat shock also repressed the expression of several normal cellular proteins, but had no effect on the expression of others. We provide evidence that the selectivity in the heat shock-induced suppression of normal cellular protein synthesis is drawn upon the distinction of secretory versus nonsecretory proteins. Northern blot analysis demonstrated heat
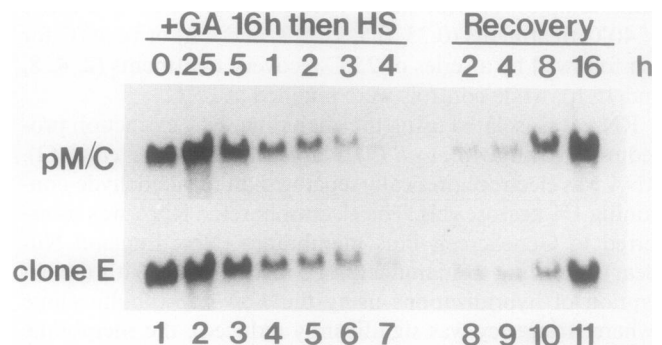

Figure 2. Time course of α-amylase mRNA levels during heat shock and recovery. RNA was isolated from aleurone layers incubated for 16 h at 25°C then heat shocked for progressively longer periods of time at 40°C and from aleurone layers incubated for 16 h at 25°C then recovering from a 3-h incubation at 40°C for progressively longer periods of time. All incubations were in the presence of 1 μM GA₃. Total RNA was electrophoresed in formaldehyde-containing agarose gels, transferred to GeneScreen Plus membranes, probed with cDNA clones specific for high-pI (pM/C) and low-pI (clone E) α-amylases, and visualized by fluorography.
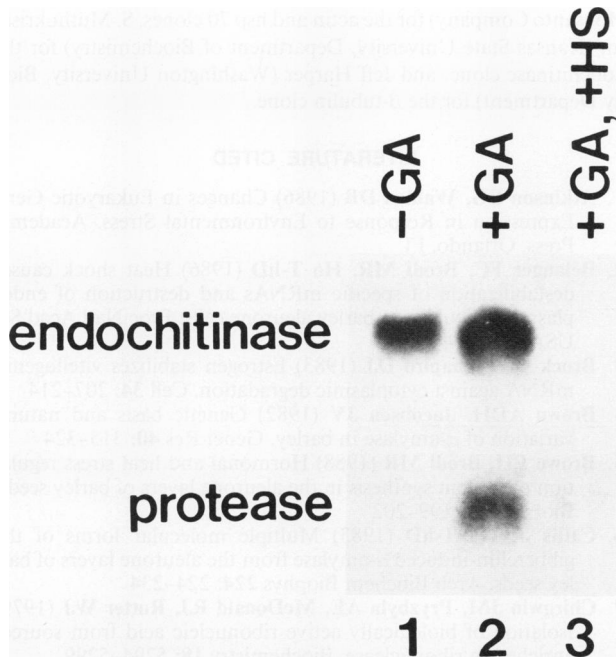
Figure 3. mRNA levels of other secretory proteins in heat-shocked aleurone tissues. RNA was isolated from aleurone cells incubated for 19 h at 25°C in the absence (−GA) or presence (+GA) of 1 μM GA₃ and from aleurone cells incubated at 25°C for 16 h then at 40°C for 3 h in the absence (−GA, +HS) or presence of 1 μM GA₃(+GA, +HS). Total RNA was electrophoresed in formaldehyde-containing agarose gels, transferred to GeneScreen Plus membranes, probed with cDNA clones specific for endochitinase and protease. The distribution of radioactivity on transfers was visualized by fluorography.

shock-induced reductions in the mRNA levels for protease, endochitinase, and both isozymic forms of α-amylase. All of these mRNAs encode secretory proteins. However, the levels of mRNAs encoding actin and tubulin (nonsecretory protein mRNAs) were not perturbed by heat shock. In addition, heat shock did not affect the levels of three other mRNAs not induced by GA₃ (one of these cDNA clones lacks a signal sequence).

Although both isozymic forms of α-amylase are induced by GA₃, the timing of their expression differs. The increase in high-pI isozyme mRNA levels begins 5 to 7 h after GA₃ induction, and these levels diminish 25 h after GA₃ induction (15). The increase in low-pI isozyme mRNA levels is initiated roughly 3 h earlier and is maintained for roughly 15 h longer than the increase in high pI isozyme mRNA levels (15). Addition of ABA midcourse in the induction of α-amylase synthesis by GA₃ results in the selective reduction of high-pI isozyme mRNA levels (15). These observations indicate that there are mechanisms normally in place to differentially regulate the stability of these mRNAs. Belanger et al. (2) had previously documented that heat shock caused the destabilization of α-amylase mRNA. However, that study examined α-amylase mRNA levels using dot blots probed at rather low stringency; distinctions among α-amylase isozymes in their response to heat shock would not be apparent. In this report we have shown that the timing of the decrease in the levels of

the mRNAs encoding both isozymic forms of α-amylase was parallel. This indicates that the mechanism for the destabilization of high- and low-pI α-amylase mRNAs during heat shock is probably different from the mechanisms usually operating to regulate α-amylase mRNA turnover.

Germane to the results presented in this paper are the observations of the effect of heat shock on the ER lamellae of barley aleurone cells. Heat shock causes a loss of ER lamellar structures (2). As proposed by Belanger et al. (2), the loss of ER lamellar structures could cause the destabilization of mRNAs normally translated there, resulting in the selective suppression of secretory protein synthesis. The observation that the levels of mRNAs for nonsecretory proteins are not affected by heat shock is consistent with this hypothesis. Because nonsecretory protein mRNAs are translated on free ribosomes, these proteins would represent those normal cellular proteins whose synthesis continues during heat shock.

The selective discontinuation of secretory protein synthesis during heat shock has been observed in other systems as well. We (MR Brodl, T-hD Ho, unpublished data) have also investigated the heat shock response of wounded carrot root tissues. In this system, mechanical wounding induces the synthesis and secretion of hydroxyproline-rich cell wall glycoproteins (notably extensin). Northern blot analyses using genomic clones for cell wall proteins and actin suggest that there is a
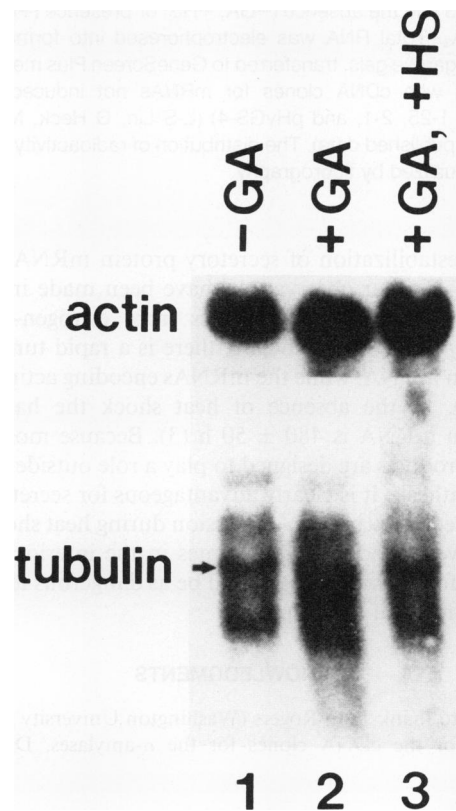

Figure 4. Levels of mRNA encoding nonsecretory proteins in heat-shocked aleurone cells. Northern gel transfers prepared as indicated in the legend of Figure 3 were probed with a genomic clone specific for actin (pMAC-1) and a cDNA clone for β-tubulin. The distribution of radioactivity on transfers was visualized by fluorography.
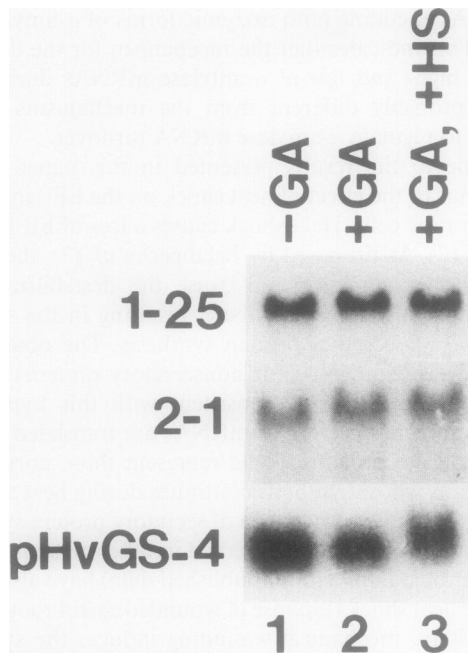
Figure 5. Levels of mRNA encoding proteins not stimulated by GA₃ in heat-shocked aleurone cells. RNA was isolated from aleurone cells incubated for 19 h at 25°C in the absence (−GA) or presence (+GA) of 1 µM GA₃ and from aleurone cells incubated at 25°C for 16 h then at 40°C for 3 h in the absence (−GA, +HS) or presence (+GA, +HS) of 1 µM GA₃. Total RNA was electrophoresed into formaldehyde-containing agarose gels, transferred to GeneScreen Plus membranes, and probed with cDNA clones for mRNAs not induced by GA₃ (designated 1-25, 2-1, and pHvGS-4) (L-S Lin, G Heck, MR Brodl, T-hD Ho, unpublished data). The distribution of radioactivity on transfers was visualized by fluorography.

selective destabilization of secretory protein mRNAs during heat shock. Similar observations have been made in animal secretory cells. When hepatocytes from estrogen-injected, male *Xenopus* are heat shocked, there is a rapid turnover of vitellogenin mRNA, while the mRNAs encoding actin remain stable (21). In the absence of heat shock the half-life of vitellogenin mRNA is 480 ± 50 h (3). Because most of the secretory products are designed to play a role outside the cells of their synthesis, it is clearly advantageous for secretory cells to repress secretory protein expression during heat shock. The release of vesicle-contained enzymes in the interior of aleurone cells, for example, might well be as dangerous to the cell as high temperature exposure.

ACKNOWLEDGMENTS

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