CELL REGULATION, Vol. 1, 135–149, November 1989

Regulation of HSP70 synthesis by messenger RNA degradation

Robert B. Petersen and Susan Lindquist
Howard Hughes Medical Institute
Department of Molecular Genetics and Cell Biology
University of Chicago
Chicago, Illinois 60637

When *Drosophila* cells are heat shocked, hsp70 messenger RNA (mRNA) is stable and is translated at high efficiencies. During recovery from heat shock, hsp70 synthesis is repressed and its messenger RNA (mRNA) is degraded in a highly regulated fashion. Dramatic differences in the timing of repression and degradation are observed after heat treatments of different severities. The 3′ untranslated region (UTR) of the hsp70 mRNA was sufficient to transfer this regulated degradation to heterologous mRNAs. Altering the translational efficiency of the message or changing its natural translation-termination site did not alter its pattern of regulation, although in some cases it changed the absolute rate of degradation. We have previously shown that hsp70 mRNA is very unstable when it is expressed at normal growth temperatures (from a metallothionein promoter). We report here that the 3′ untranslated region of the hsp70 mRNA is responsible for this instability as well. We postulate that a mechanism for degrading hsp70 mRNA pre-exists in *Drosophila* cells, that it is inactivated by heat shock and that it is the reactivation of this mechanism that is responsible for hsp70 repression during recovery. This degradation system may be the same as that used by other unstable mRNAs.

Introduction

The induction of heat-shock proteins (or stress proteins) is the most highly conserved genetic induction known. Experimentally, the proteins are most commonly induced by an increase in temperature, but they can also be induced by a wide variety of other stresses, including anoxia and exposure to heavy metal ions. In all organisms investigated, the response is highly regulated and varies with the severity of the stress (reviewed in Craig, 1985; Lindquist, 1986).

The heat-shock response of *Drosophila melanogaster,* the organism in which the response was discovered, is the most well characterized among higher eukaryotes. The intensity of the *Drosophila* response is particularly striking and provides one of the best examples of a reversible, global redirection of macromolecular synthesis (Lewis *et al.,* 1975; Chomyn *et al.,* 1979; DiDomenico *et al.,* 1982b). Immediately after a shift from 25°C (the normal growing temperature for *Drosophila* tissue culture cells) to 37°C (a heat-shock inducing temperature) transcription is redirected from the synthesis of normal 25°C mRNAs to the synthesis of heat-shock mRNAs, the most abundant of which is hsp70 mRNA (Ashburner, 1970; Tissieres *et al.,* 1974; McKenzie *et al.,* 1975; Spradling *et al.,* 1977; McKenzie and Meselson, 1977). At the same time, preexisting mRNAs are translationally repressed while newly transcribed heat-shock mRNAs are translated at very high rates (Mirault *et al.,* 1978; Lindquist, 1980a,b; Storti *et al.,* 1980; Kruger and Benecke, 1981; Petersen and Mitchell, 1981; Scott and Pardue, 1981). This translational pattern persists as long as the temperature remains elevated. When the cells are returned to 25°C, heat-shock protein synthesis is repressed and normal protein synthesis is restored (Lewis *et al.,* 1975; Chomyn *et al.,* 1979; DiDomenico *et al.,* 1982a).

The repression of heat-shock protein synthesis during recovery is a highly regulated process that differs in several respects from the repression of 25°C protein synthesis during heat shock. First, during heat shock, most 25°C proteins are repressed synchronously. During recovery, heat-shock proteins are repressed asynchronously (DiDomenico *et al.,* 1982a). Hsp70 is invariably the first heat-shock protein to be repressed. Depending upon the intensity and duration of the heat treatment, the synthesis of other heat-shock proteins may still be increasing at a point when synthesis of hsp70 has already been substantially reduced (Lindquist and DiDomenico, 1985). Second, during heat shock, normal protein synthesis is repressed very rapidly, usually within 10 min of the shift to high temperature. During recovery, heat-shock proteins are repressed more gradually, often requiring several hours for repression to be complete (DiDomenico *et al.,* 1982a). Third, during heat shock the messenger RNAs for 25°C

proteins are very stable and are returned to translation during recovery. In contrast, during recovery, messenger RNAs for the heat-shock proteins are degraded as they are repressed (DiDomenico *et al.,* 1982b). The repression of hsp70 synthesis and the degradation of its message is the focus of this study.

The stability of the hsp70 message varies dramatically with the physiological state of the cell. When cells are maintained at elevated temperatures, hsp70 mRNA is very stable, with no detectable turnover for at least 5 h. When cells are returned to 25°C after a mild heat shock, hsp70 mRNA may be degraded within 30 min (Petersen and Lindquist, 1988). When hsp70 mRNA is expressed at normal temperatures (from a metallothionein promoter), it is extremely unstable, with a half-life of ~15–30 min. However, when cells are shifted to 36°C after induction, preexisting mt/hsp70 mRNA is stabilized and persists for several hours (Petersen and Lindquist, 1988).

The change in hsp70 mRNA stability is regulated and is not just a function of the change in temperature per se. This is most apparent from the biphasic character of the heat-shock recovery process. In the first phase of recovery at 25°C, cells continue to produce heat-shock proteins, almost exclusively, at a very high rate. The length of this phase varies with the severity of the preceding heat treatment but may last for several hours. The second phase of recovery begins with the repression of hsp70 and the degradation of its message, followed by the repression of other hsps.

Several lines of evidence suggest that heat-shock proteins themselves may play a role in regulating the repression of heat-shock mRNAs. First, although the timing of heat-shock mRNA degradation varies over a wide range, for any given treatment it is very reproducible, with a specific quantity of heat-shock protein always being produced before degradation is initiated (DiDomenico *et al.,* 1982b). Second, cells that are given a mild preheat treatment (which preinduces synthesis of heat-shock proteins) repress heat-shock protein synthesis much more rapidly after a severe heat shock than do cells that are not given a pretreatment. Third, when the rate of heat-shock protein synthesis is reduced, by limiting the concentration of heat-shock messages, the repression of heat-shock protein synthesis is delayed in proportion (DiDomenico *et al.,* 1982b). Fourth, if the synthesis of heat-shock proteins is blocked by cycloheximide, heat-shock mRNAs remain stable indefinitely at 25°C (DiDomenico *et al.,* 1982b). The messages are not stabilized when the drug is added after heat-shock proteins have been produced. Although the evidence suggests the heat-shock proteins affect regulation of the response, it is not known whether they affect the translational machinery directly or indirectly, by repairing some other heat-induced lesion.

Limited information exists about the features of the hsp70 mRNA that govern its stability. Previous studies in our laboratory with *HSP70* genes that carry deletions in the protein coding region suggest that sequences within the coding region itself play no direct role in message degradation (McGarry and Lindquist, 1985; J. Rossi, T. McGarry, and R. Petersen, unpublished results). Simcox *et al.* (1985) examined the stability of chimeric hsp70 mRNAs produced by an X-ray induced *HSP70* deletion which replaced the 3′ half of the gene with an unknown sequence. RNAs produced by this mutation were much more stable than wild-type hsp70 messages during recovery from heat treatment. Although it was not clear whether stabilization of the mRNA was due to the removal of an intrinsic destabilizing sequence from hsp70 mRNA or to the addition of an extraneous, stabilizing sequence from some other source, the results suggested that the 3′ half of the hsp70 message might be involved in its degradation.

To examine the features of the hsp70 mRNA required for regulated turnover, we have constructed a series of *HSP70* genes containing a variety of sequence modifications and substitutions, transformed them into *Drosophila* tissue culture cells, and monitored the stability of their mRNAs during recovery from heat shocks of different severity. The questions we have addressed are 1) does the 3′ untranslated region play a major role in destabilizing the hsp70 message during recovery from heat shock or in reducing constitutive expression during growth at normal temperatures, 2) does the efficiency of translation affect the stability of the hsp70 message during recovery, 3) does premature termination of translation alter degradation of the hsp70 mRNA, and 4) does translation beyond the normal termination site interrupt regulation?

Results

Repression of hsp70 synthesis and the degradation of its message

To investigate features of the hsp70 message that regulate its repression, cells were transformed with a variety of genes which contain different elements of the hsp70 message. Repression of the messages derived from these genes was compared with that of the wild-type hsp70 message during recovery from heat shock. Actino-

mycin D (actD) was added during recovery to eliminate effects of transcription on hsp70 expression (Lindquist, 1980a).

For the experiment shown in Figure 1, cells were transformed with an *HSP70* gene carrying a deletion in the middle of the protein coding sequence that produces a 44 kDa protein. (The construct is shown in Figure 2, designated pDM420). The cells were heat shocked at either 36 or 38°C for 30 min and then returned to 25°C for recovery. Proteins were pulse-labeled with ^{3}H-leucine for 10 min beginning immediately after return to 25°C and at hourly intervals thereafter.

After the 36°C heat shock, repression of hsp70 synthesis began between 1 and 2 h after the return to 25°C. As hsp70 synthesis was repressed, normal protein synthesis was restored. Recovery was nearly complete within 4 h. After the more severe 38°C heat shock, no repression of hsp70 synthesis and no resumption of normal protein synthesis was apparent during this period. (In other, more extended recovery experiments, repression of hsp70 synthesis was initiated between 4 and 5 h after the 38°C treatment; data not shown).

These results demonstrate that repression of hsp70 is not simply a function of the return to 25°C, but varies with the severity of the preceding heat treatment and the physiological state of the cell. Since recovery took place in the presence of actinomycin D, this repression of hsp70 synthesis is regulated at the posttranscriptional level. The results also demonstrate that deletion of a major portion of the *HSP70* coding region does not change its pattern of regulation. The repression of the 44 kDa deletion protein closely paralleled the repression of hsp70 in these cells, occurring much more rapidly after the mild (36°C) heat shock than after the severe (38°C) heat shock.

We have previously shown that repression of hsp70 protein synthesis is paralleled by the degradation of its message (DiDomenico *et al.*, 1982b; Petersen and Lindquist, 1988). During recovery from a 36°C heat shock, degradation of hsp70 mRNA begins ~60 min after the cells are returned to 25°C. After the 38°C heat shock,

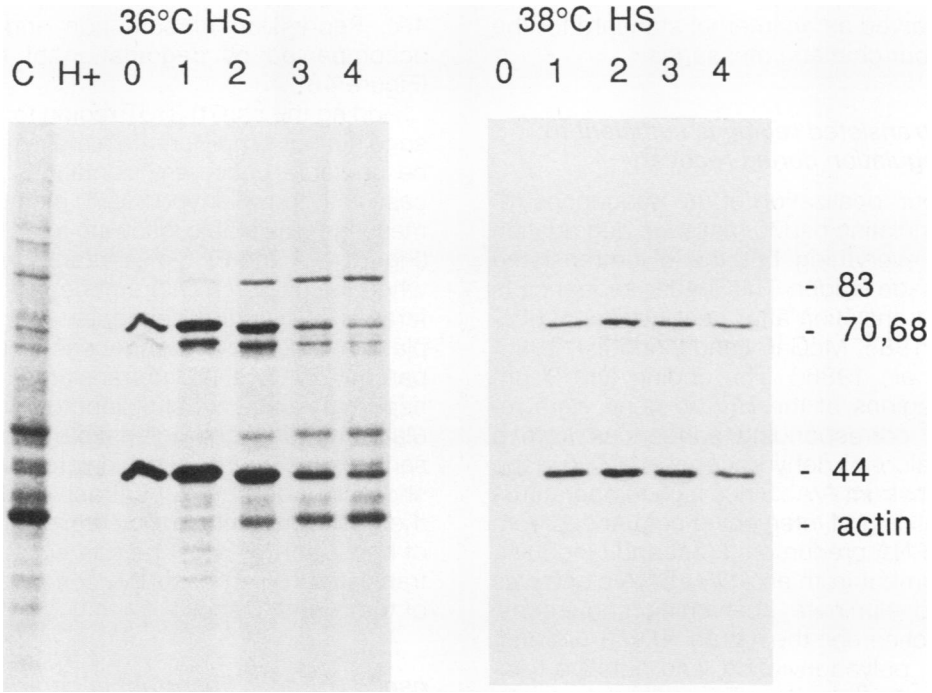

Figure 1. **Repression of hsp70 and an hsp70-derived protein-coding deletion mutant (44 kDa) during recovery from heat shocks of different severity.** Cells transformed with the plasmid p70/70 were heat-shocked at 36 or 38°C for 30 min and transferred to 25°C for recovery. Individual aliquots were pulse-labeled with ^{3}H-leu for 10 min at 1 h intervals. Numbers above the lanes refer to the time in hours after the return to 25°C when labeling was initiated. Fifty-five minutes after return to 25°C, act D was added (1 μg/ml) to all remaining samples. C, cells maintained at 25°C before labeling; H+, cells to which act D was added 5 min before a 36°C heat shock; the absence of heat-shock protein synthesis in these cells demonstrates that the drug was effective in blocking transcription. Samples C and H+ were labeled at the same time as the 0 recovery time point. Proteins were extracted, separated by SDS-PAGE, and visualized by fluorography. The positions of the major heat-shock proteins, the hsp70 deletion product hsp44, and actin are indicated on the right.

hsp70 mRNA is stable throughout the 4-h recovery period. In the present case, degradation of the message produced by the 44 kDa deletion mutation paralleled degradation of the wild-type hsp70 message after both a 36 and a 38°C heat shock (data not shown).

In other experiments reported here the timing of recovery varied somewhat from transformant to transformant, possibly because the production of aberrant proteins can exert an influence on the heat-shock response. Hence, in all cases we monitored the general pattern of protein synthesis in these cells, in addition to the pattern of messenger RNA degradation, to provide an assessment of the general physiological state of the cell. Although the precise timing of events varied, in all cell lines hsp70 was repressed much more rapidly following a mild 36°C heat shock than following a severe 38°C heat shock. To ensure that we were focusing on physiologically regulated changes in messenger RNA stability, we took advantage of the difference in the stability of the hsp70 message after a 36 and a 38°C heat shock. This difference provided an assay for features of the message that are responsible for its regulated degradation. The behavior of endogenous hsp70 messages served as an internal standard for the behavior of our chimeric messages.

hsp70 3′ untranslated region is sufficient to confer co-regulation during recovery

We began our localization of the sequences required for regulating hsp70 message degradation by deleting everything but the 5′ untranslated hsp70 message leader. This leader sequence is required for translation after heat treatment (Klemenz *et al.,* 1985; McGarry and Lindquist, 1985; Hultmark *et al.,* 1986.). The coding and 3′ untranslated regions of the *HSP70* gene were replaced with corresponding sequences from a *Drosophila* alcohol dehydrogenase (*ADH*) gene (Figure 2, construct A/A). Since high temperatures block the splicing of intervening sequences from messenger RNA precursors (Yost and Lindquist, 1986), a segment from an *ADH* cDNA clone was employed to eliminate intervening sequences. The construct carried the natural *ADH* 3′ untranslated region, polyadenylation, and putative transcription termination sites. This gene was transformed into *Drosophila* tissue culture cells and regulation of the encoded transcript was monitored after heat shock.

During recovery from a 36°C heat shock (Figure 3, A and B) adh was synthesized at a constant rate and its message was maintained at a constant level. In the same cells, endogenous hsp70 synthesis declined sharply and the concentration of its message was proportionately reduced (Figure 3, A and B). During recovery from a 38°C heat treatment, synthesis of both adh and hsp70 was uninterrupted and their transcripts were stable (Figure 3, A and B). Thus, replacing the *HSP70* coding region and 3′ untranslated region with sequences from the *ADH* gene produces a stable message, which is not subject to the selective degradation characteristic of the hsp70 message after a mild heat shock.

In other experiments in our laboratory, hsp70 messages carrying deletions in the protein coding region coregulated with the endogenous hsp70 messages (McGarry and Lindquist, 1985; Rossi *et al.,* manuscript in preparation). The deletions analyzed covered, in different constructs, the entire *HSP70* coding region. Since coding sequences in the message did not appear to be critical in controlling message degradation, we transfered the 3′ untranslated region of the *HSP70* gene to the *ADH* construct (Figure 2, A/70). In sharp contrast with the previous experiment, in cells transformed with this gene adh synthesis was repressed together with hsp70 synthesis during recovery from a 36°C heat shock (Figure 4A). Repression of both adh and hsp70 was accompanied by degradation of their mRNAs (Figure 4B).

Adding the hsp70 3′ UT region to the adh message did not simply create a message that would be unstable under any conditions. As was the case with the wild type hsp70 message, this adh message was stable following a 38°C heat shock (Figure 4, A and B). Similar results were obtained when the bacterial chloramphenicol acetyl transferase (CAT) coding sequences were used to replace hsp70 coding sequences (see below). Apparently, the 5′ and 3′ untranslated regions of the hsp70 message are sufficient to transfer the regulated turnover characteristic of the hsp70 message to other messages during recovery from heat shock. Since the 5′ untranslated sequences themselves are not sufficient, regulated turnover of hsp70 mRNA must be due either to the 3′ untranslated region or to the combined properties of the 5′ and 3′ ends.

hsp70 3′ End is important in controlling constitutive levels of expression

When the hsp70 message is artificially induced at normal temperatures, it is extremely unstable. This was demonstrated by placing the *HSP70* gene under the control of a metallothionein promoter, so that its transcription could be induced at 25°C with copper (Petersen and Lindquist,

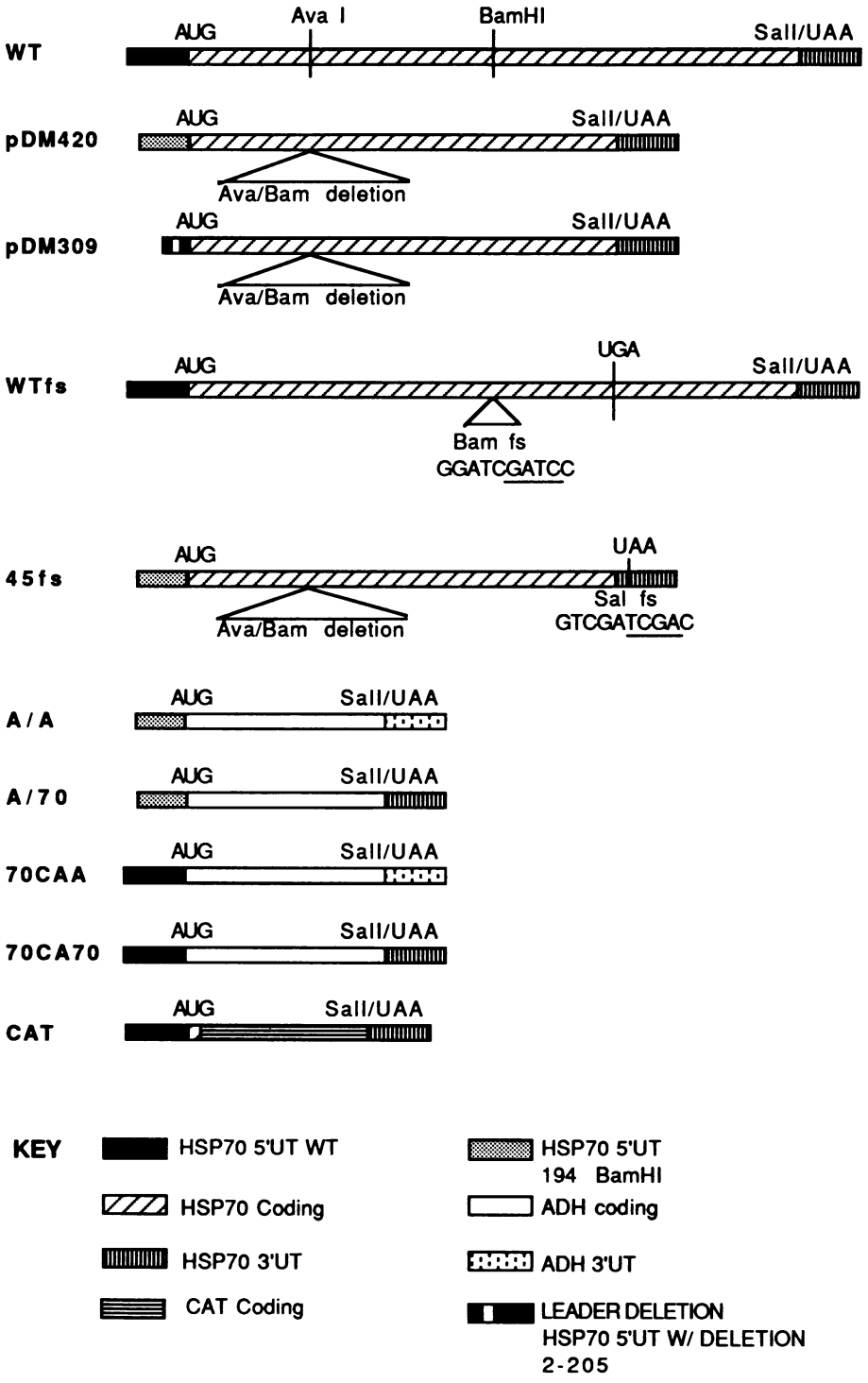

Figure 2. **Maps of the DNA constructs used in this study.** The wild-type *HSP70* gene from the genomic clone G3 (Ingolia *et al.*, 1980), designated WT at the top of the figure, was the starting point for all the remaining constructs. Plasmids were generated by standard recombinant-DNA techniques as described in materials and methods. All constructs retained the *HSP70* promoter from the *Bgl* II site at position −1125 to the start site of transcription.

1988). When induced cells were maintained at 25°C, the hsp70 message had a half-life of <15 min. However, when the same cells were shifted to 37°C, the message had a half-life of >4 h. This instability of the hsp70 message at normal temperatures might play an important role in reducing constitutive levels of hsp70 expression by ensuring that any messages that leak through the normal transcriptional regulatory pathway are rapidly degraded. The very fact that hsp70 message

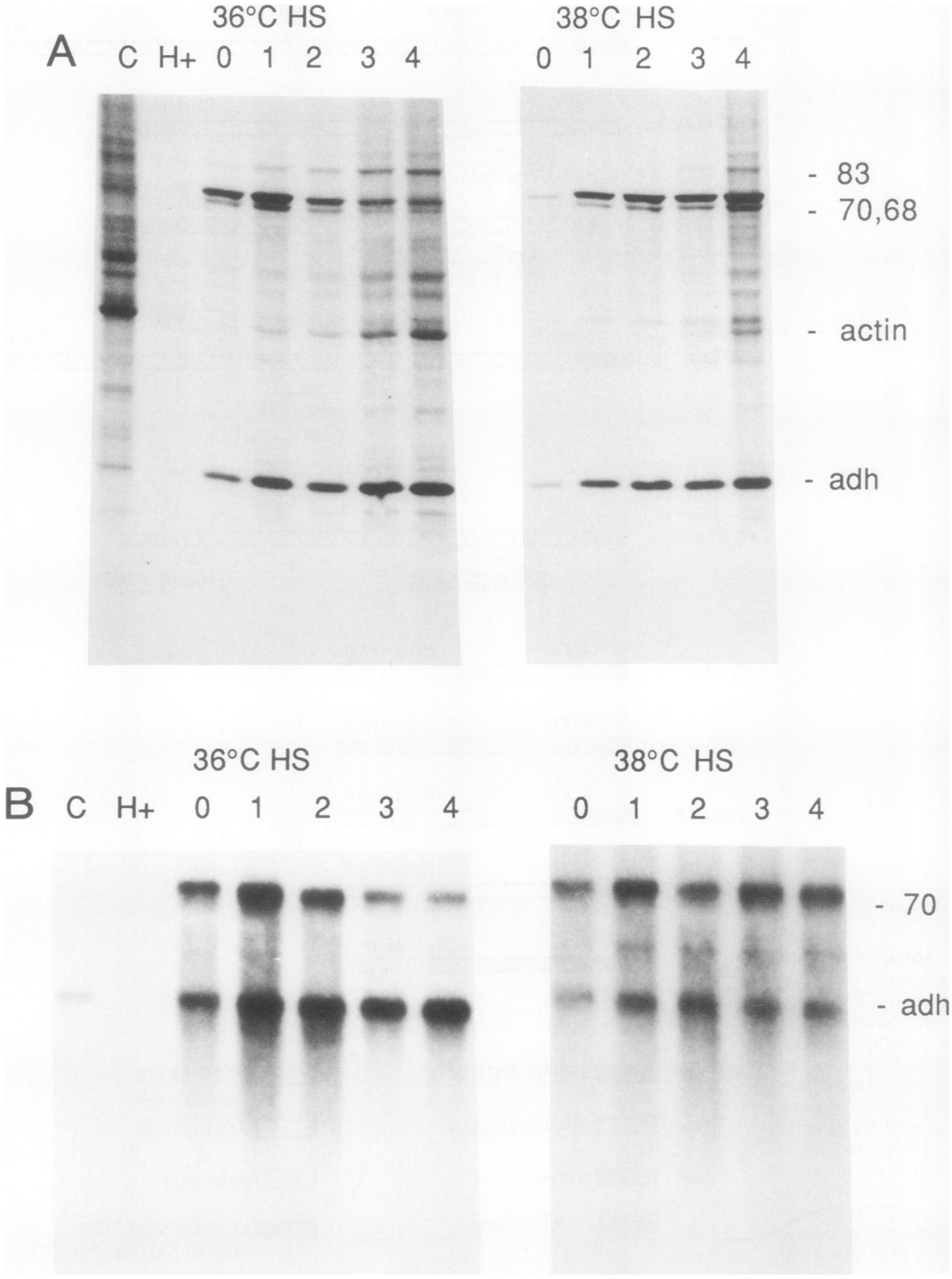

Figure 3. **Adh messages expressed from the *HSP70* promoter and containing the hsp70 5′ UTR do not coregulate with the endogenous hsp70 message.** (A) Protein synthesis in cells transformed with the plasmid pA/A, analyzed as in Figure 1A. (B) Total nucleic acids were extracted from duplicate samples at the midpoint of the labeling period in panel A, glyoxalated, fractionated on a 1.5% agarose gel, and transferred to Hybond N. The blot was hybridized with a 1:1 mixture, by radioactivity, of nick-translated, ^{32}P-labeled DNA fragments from the *HSP70* and *ADH* coding regions (the *Bam*HI/*Sal* I fragment from pDM420*Sal and the *Bgl* II/*Sal* I fragment of pAdh B/S).

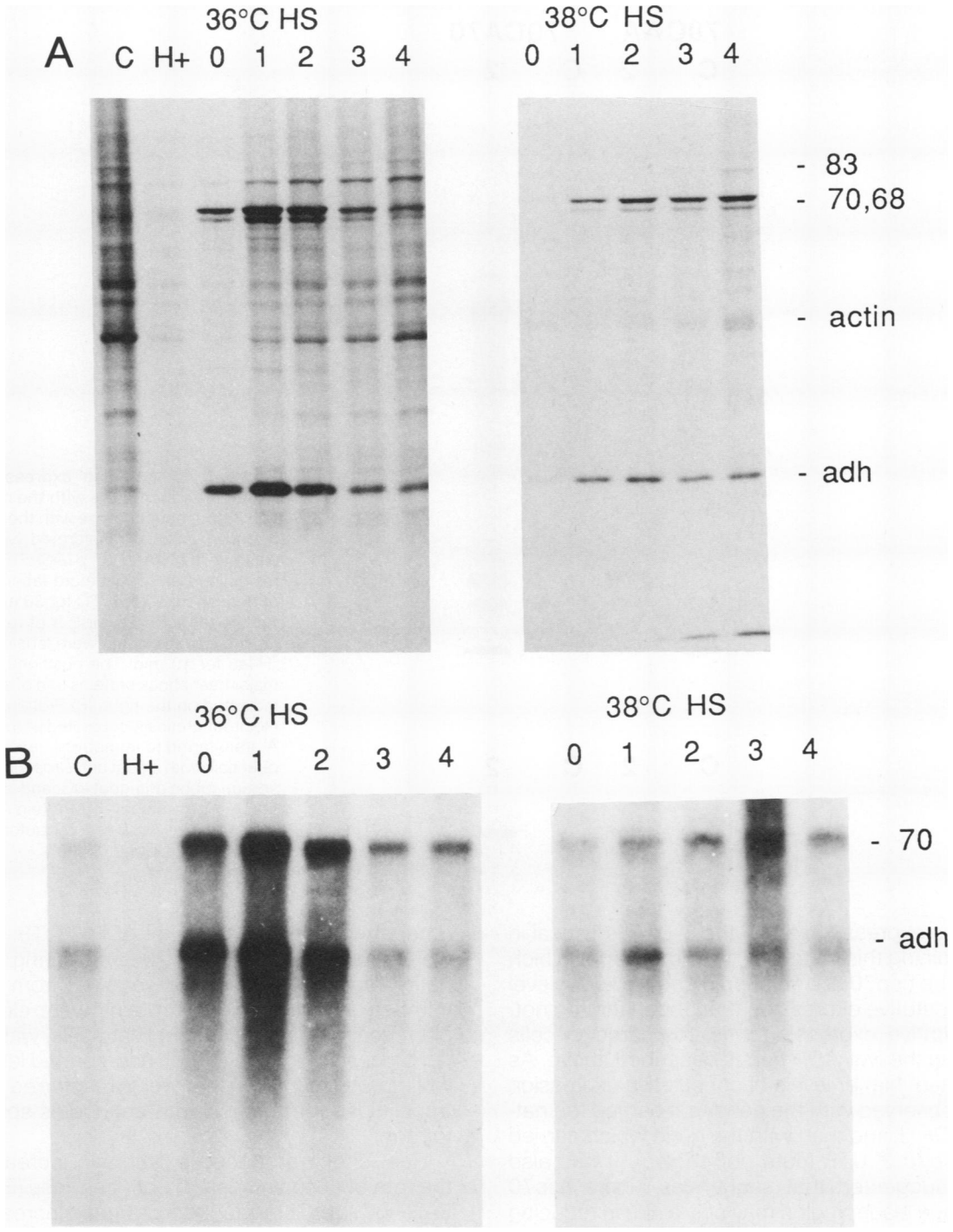

Figure 4. **Adh mRNAs containing the hsp70 3′ end are coregulated with endogenous hsp70 mRNAs.** (A) Protein synthesis in cells transformed with the plasmid pA/70, analyzed as in Figure 1A. (B) Total nucleic acids, analyzed as in Figure 3B using the same ^{32}P-labeled, mixed DNA probe.

concentrations increase by >1000-fold after heat shock (Velasquez *et al.*, 1983) suggests that such a mechanism is operating. With *HSP70* promoter elements alone, the differences between constitutive and heat-inducible expression are usually of a much lower magnitude.

If instability of the hsp70 message at normal temperatures does play a role in reducing con-

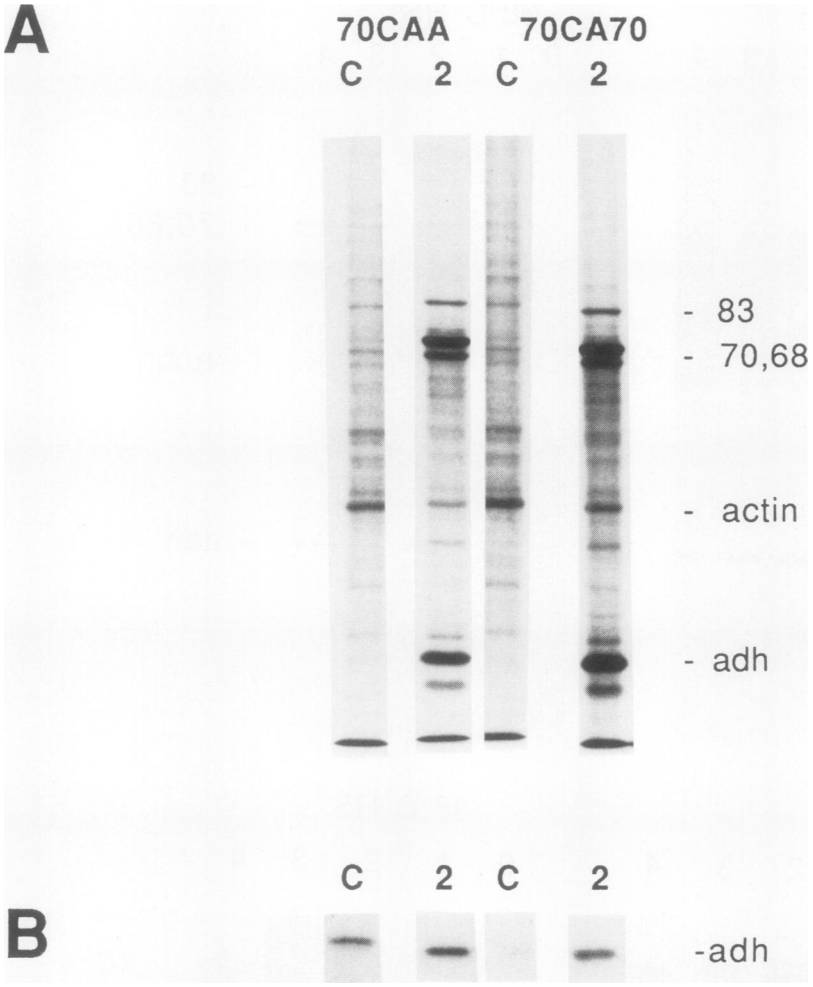

Figure 5. **Constitutive expression is higher for adh mRNAs with the natural adh 3′ end than for those with the hsp70 3′ end.** (A) Cells transformed with the plasmid p70CAA or p70CA70 were maintained at 25°C before labeling (C) or heat shocked at 37°C for 30 min and transferred to 25°C for 2 h of recovery (2). Individual aliquots were labeled with ^{3}H-leu for 10 min. The positions of the major heat-shock proteins and of adh are indicated on the right. (B) Proteins were electrophoretically separated as for panel A, transferred to Immobilon, reacted sequentially with a goat anti-*Drosophila* adh serum, rabbit anti-goat IgG, and ^{125}I-Protein A. The antibody-^{125}I-Protein A complexes were visualized by autoradiography.

stitutive expression, and if the 3′ UTR is critical in determining this instability, then messages which carry the hsp70 3′ UTR should show a lower level of constitutive expression than those that do not. Constitutive expression was compared in cells carrying the two *ADH* genes described above. As predicted, higher levels of constitutive expression were observed with the gene that carried the natural *ADH* 3′ end than with the gene which carried the hsp70 3′ UTR (data not shown). It has also been suggested that sequences in the hsp70 message leader region may play a role in reducing constitutive expression of hsp70, since engaged RNA polymerases appear to be blocked there at normal temperatures (Gilmore and Lis, 1986; Rougvie and Lis, 1988). To create a matched set of *ADH* genes which retained this entire region, we used site-directed mutagenesis to introduce a restriction site just in front of the initiating AUG codon of the *HSP70* gene. Again, one gene was constructed to carry the *HSP70* 3′ UTR, while the other gene carried the natural *ADH* 3′ UTR. Cells were pulse-labeled with ^{3}H-leucine during incubation at 25°C or after 2 h of recovery from a 30-min heat shock at 36°C. Proteins were electrophoretically separated on duplicate gels, with one gel processed for analysis of incorporated leucine while proteins in the other were transferred to nitrocellulose for reaction with antibodies specific for adh.

Heat shock produced a dramatic increase in the rate of adh synthesis in both cell lines (Figure 5A). However, it produced a dramatic increase in adh accumulation only in transformants that carried the *ADH* gene with the HSP70 3′ end. In transformants that carried the *ADH* gene with the *ADH* 3′ end, protein concentrations were high initially and they increased only a few fold with heat shock (Figure 5B). Thus, the 3′ UTR of the hsp70 message can produce a dramatic reduction in constitutive levels of gene expression in *Drosophila* cells. The biological importance of this

mechanism is suggested by the fact that it has proven extremely difficult to obtain cell lines which constitutively overexpress hsp70 in this laboratory (J. Rossi, R. Petersen, T. McGarry, K. Golic, and S. Lindquist, unpublished data.)

Effect of translational efficiency on mRNA stability

Heat shock messenger RNAs are normally translated at very high efficiencies during heat shock and during recovery (Lindquist, 1980b). To determine whether high rates of translation are required for regulated degradation of heat-induced messages, we altered the translational efficiency of the message. Two very different methods were employed to alter translational efficiencies. In one case, we took advantage of the difference in codon bias between *Drosophila* and *E. coli* messages to reduce the rate of ribosome transit along the message. The *HSP70* coding region was replaced with that of the bacterial chloramphenicol acetyl transferase (*CAT*) gene, (Figure 2, CAT). The construct contained the entire *HSP70* 5′ and 3′ UTRs required for selective translation during heat-shock and for regulated degradation during recovery. As expected, the translational efficiency of the CAT mRNA was very low. (Compare the quantities of CAT and adh protein produced in Figures 4A and 6A with the quantities of their mRNAs, relative to hsp70. Since the proteins were pulse-labeled for only 10 min and since all three proteins are relatively stable in *Drosophila* cells these differences in labeling were not due to differences in protein turnover.) Although the CAT mRNA had a much lower translational efficiency than the wild-type hsp70 mRNA, it was subject to the same pattern of regulated degradation. After a 36°C heat treatment, degradation of both the CAT mRNA and the hsp70 mRNA was initiated between 1 and 2 h after the cells were returned to 25°C (Figure 6B); after a 38°C heat treatment both mRNAs were stable throughout the 4 h recovery period (Figure 6B). (Note that the CAT message shows a discrete reduction in size as it is degraded. A similar reduction in size is observed for the endogenous hsp70 message when analyzed on longer gels and its cause is currently under investigation. It is more obvious for the CAT message in this figure simply because the message is smaller and the change in size is relatively greater.) Although CAT mRNA showed the same pattern of degradation as hsp70 mRNA, its rate of turnover exceeded that of hsp70 mRNA after a 36°C heat shock (Figure 6B). Apparently, altering the ribosome transit rate for a heat-induced mRNA may affect its absolute rate of degradation, without affecting the manner in which it is regulated.

In another case, we took advantage of an *HSP70* mutant (described previously) which carries a deletion of the 5′ untranslated region, from nucleotide +2 to 205 (McGarry and Lindquist, 1985). All heat-shock messages in *Drosophila* have long untranslated 5′ leader sequences that share a number of structural features. Messages carrying large leader deletions are very inefficiently translated after a severe heat shock. They can be translated during recovery, but their translation is short lived. As may be seen in Figure 7, the leader deletion mRNA is rapidly degraded, together with endogenous hsp70 mRNA, after a 36°C heat shock. Again, both messages were stable after a 38°C heat shock.

Effect of changing the translation termination site

Since the hsp70 3′ untranslated region is of critical importance in regulating the turnover of the message during recovery from heat shock, we asked whether changing the relationship between this sequence and translational termination would disrupt this regulation. Two mutations which affect termination were constructed: in one, translation terminates before reaching the normal hsp70 termination site, and in the other, translation proceeds beyond the normal hsp70 termination site. To produce a message with premature termination, a frameshift was introduced in the middle of the *HSP70* coding region, (Figure 2, WT fs). This mRNA, although the same size as the endogenous hsp70 mRNA, produces a 42 kDa protein. Approximately one-half the mRNA has become part of the 3′ untranslated region. The protein produced by this gene was repressed even more rapidly than hsp70 mRNA during recovery from a 36°C heat shock (Figure 8; note in particular the difference between the 0 and 1 h time points). Nevertheless, expression of the 42 kDa protein was stabilized by a 38°C heat shock, as was expression of hsp70 itself (Figure 8). Apparently, premature termination of translation may affect the rate at which a message is degraded during recovery, but it does not affect the basic pattern of regulation.

To produce a message in which ribosomes would proceed beyond the normal translation termination site a frameshift was introduced in the coding sequence immediately preceding the normal termination codon. This mutation allows translation to proceed an additional 72 nucleotides beyond the normal termination site (Figure 2, 45 fs). To allow us to compare the effects of

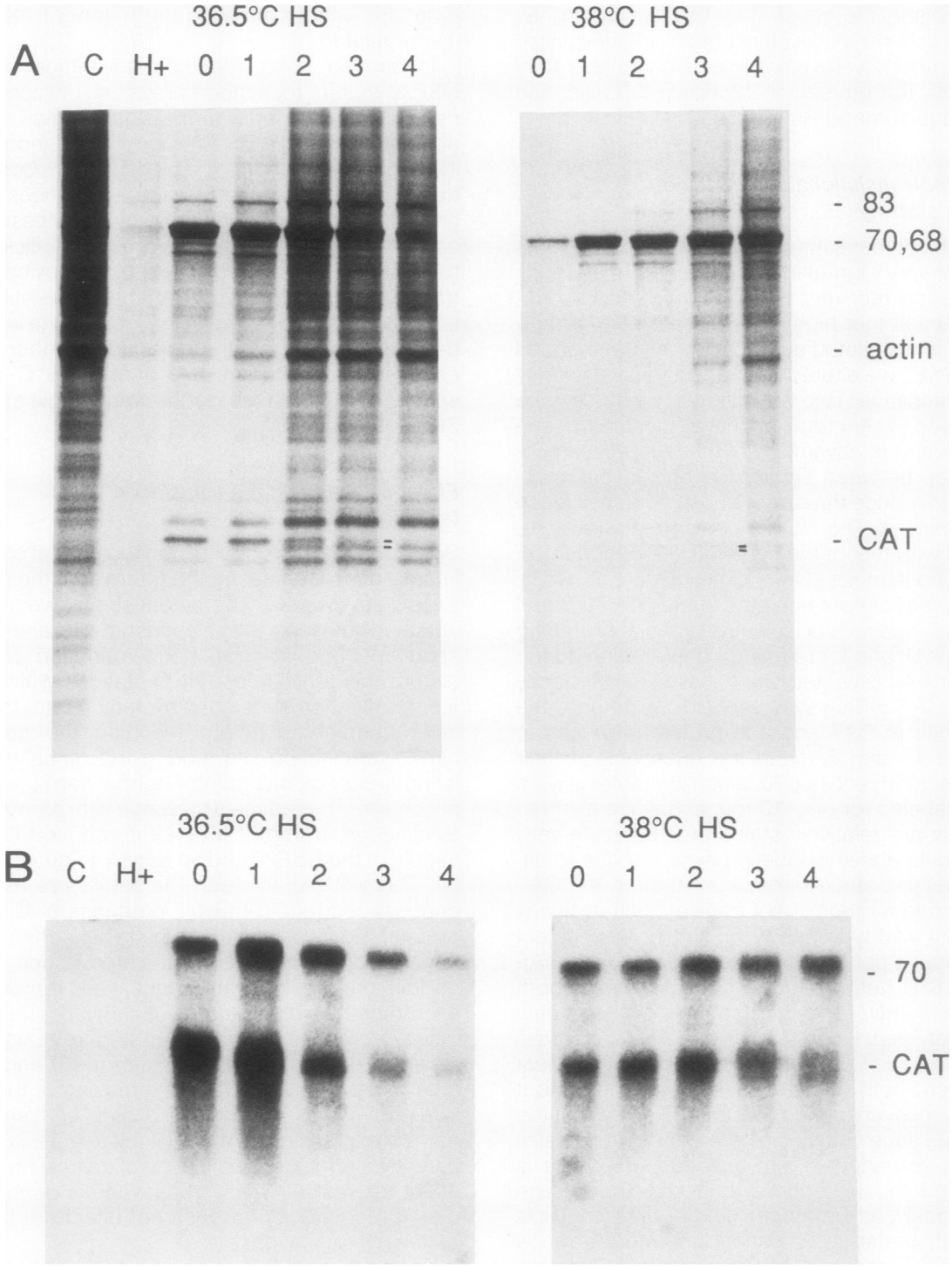

Figure 6. **Heat shock regulation of mRNA turnover is maintained when the translational efficiency of the mRNA is low because of codon bias.** (A) Protein synthesis in cells transformed with the plasmid *CAT* analyzed as in Figure 1A. The two lines between lanes 3 and 4 indicate the positions of CAT (upper) and hsp23 (lower). (B) Total nucleic acids, analyzed as in Figure 3B except that the ^{32}P-labeled probe consisted of a mixture of nick-translated, radiolabeled *HSP70* and *CAT* DNAs (the *Bam*HI/*Sal* I fragment from pDM420 and the *Eco*RI fragment from pCAT).

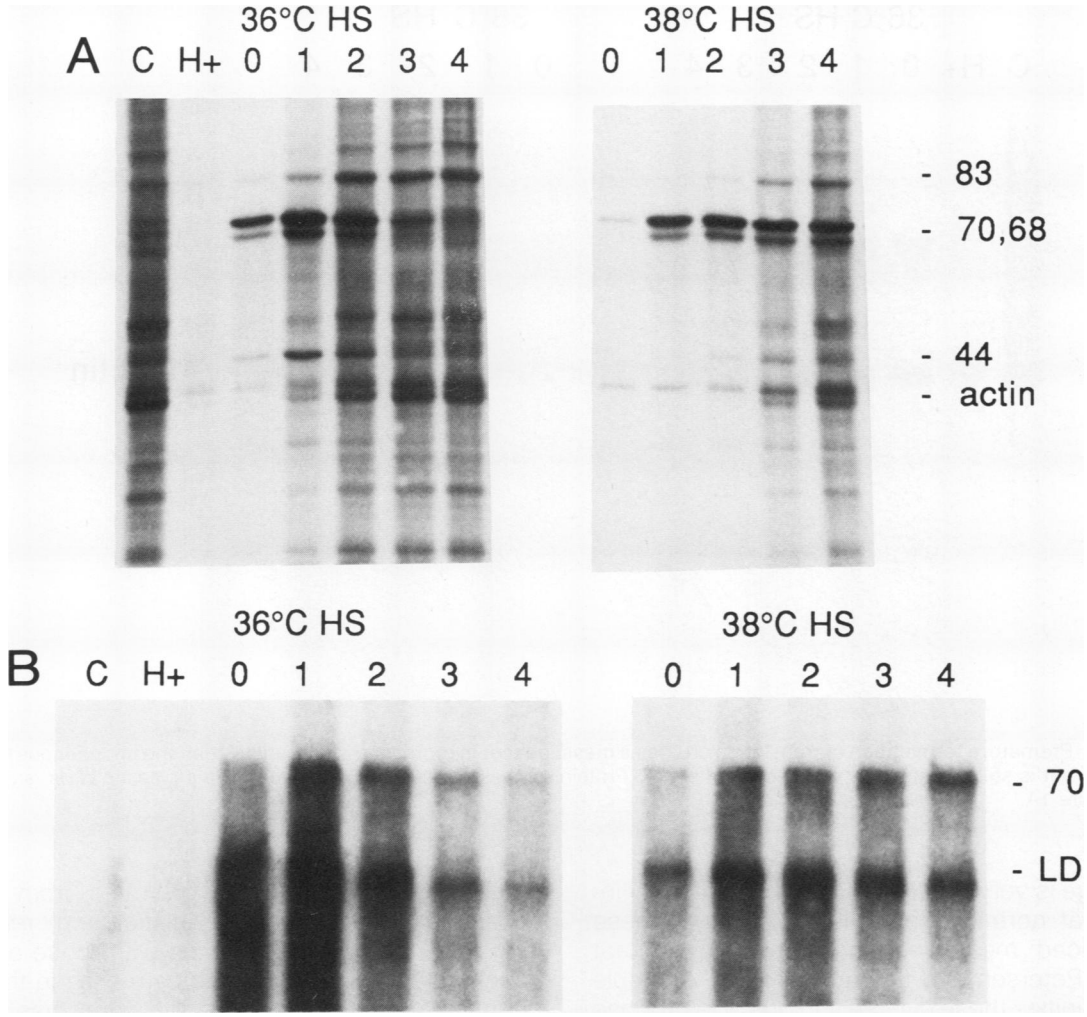

Figure 7. **Heat shock regulation of mRNA turnover is retained when translation of a message is reduced due to a deletion in the hsp70 5′ UTR.** (A) Protein synthesis in cells transformed with the leader deletion plasmid pDM309, analyzed as in Figure 1A. (B) Total nucleic acids analyzed as in Figure 3B.

this mutation on expression of the message with that of the wild-type hsp70 message, the frameshift was produced on the protein coding deletion mutation analyzed in Figure 1. Synthesis of the 45 kDa protein produced by this message paralleled the synthesis of hsp70 (Figure 9). Both the 45 kDa protein and hsp70 were repressed rapidly during recovery from a 36°C heat treatment, and both were stable after a 38°C heat treatment.

Discussion

The repression of hsp70 synthesis during recovery from heat shock is regulated according to the severity of the preceeding heat treatment and is accompanied by selective degradation of the hsp70 message. We have shown that sequences in the 3′ untranslated region of the hsp70 message play a critical role in this regulation. When both *HSP70* coding sequences and 3′ untranslated sequences are replaced with corresponding sequences from the *ADH* gene (retaining only the *HSP70* 5′ untranslated region) regulation was lost. The message produced by this gene was stable after both mild and severe heat shocks. However, when *HSP70* 3′ untranslated sequences were used to replace the *ADH* 3′ untranslated sequences, regulation was restored. The message was rapidly degraded after a mild heat shock but was stable for several hours after a severe heat shock, closely mimicking regulation of the endogenous hsp70 message.

ADH genes that carried the *HSP70* 3′ end also showed a much lower level of constitutive expression than those which carried the *ADH* 3′ end. We have previously shown that the hsp70

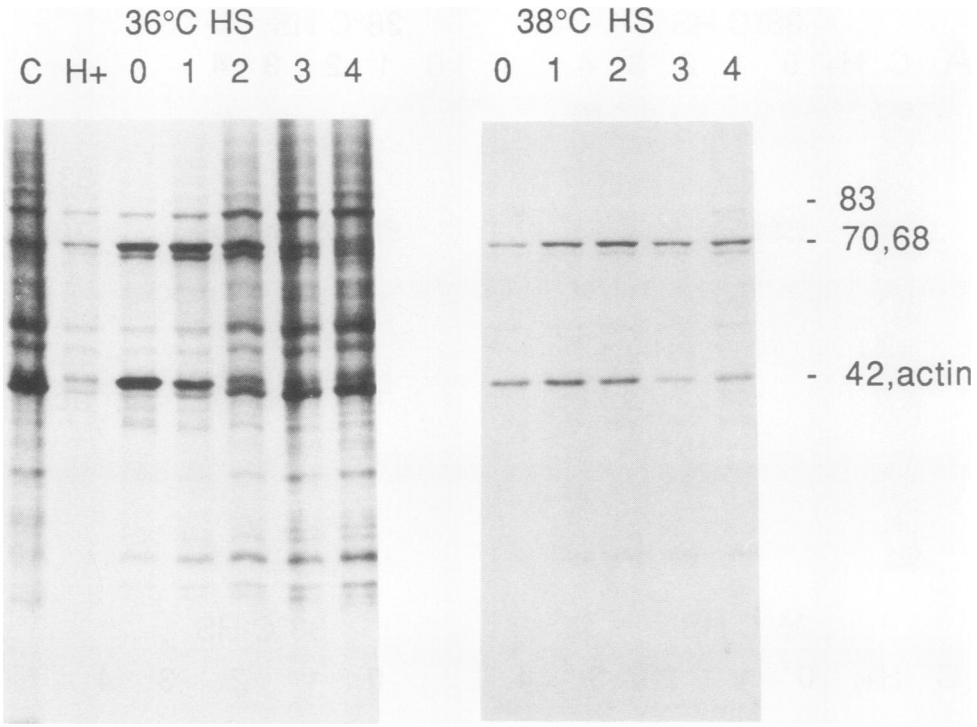

Figure 8. **Premature termination of translation creates a message that is repressed more rapidly than the endogenous hsp70 message but is still stabilized by a severe heat shock.** Protein synthesis in cells transformed with the plasmid WTfs, analyzed as in Figure 1A.

message is very unstable when it is artificially induced at normal temperatures and that these preinduced messages are stabilized by heat shock (Petersen and Lindquist, 1988). A simple model unifies these diverse findings. We propose that *Drosophila* cells have a mechanism for the rapid degradation of hsp70 messages at normal temperatures, which operates through recognition of the hsp70 3′ UTR. This mechanism is inactivated by heat shock, allowing a rapid and massive increase in protein synthesis to follow the transcriptional induction of the message. During recovery, the mechanism is reactivated, with a timing that depends upon the severity of the preceeding heat treatment. Thus, heat shock stabilizes the naturally unstable hsp70 message and the recovery process simply restores it to its natural state.

In separate experiments we examined other features of the hsp70 message that might affect its regulation. Certain of the changes we introduced resulted in the production of messages with turnover rates that were slightly faster than that of the endogenous hsp70 message. However, by comparing the turnover rate of the same message after a mild heat shock and after a severe heat shock, we were able to distinguish general changes in degradation rates from true changes in the pattern of regulation; e.g., replacing *HSP70* coding sequences with those of the bacterial *CAT* gene produces a message that is translated very poorly, due to codon bias. The turnover of this message is slightly increased relative to the endogenous hsp70 message, but it is still repressed much more rapidly after a mild heat shock than after a severe heat shock. Moreover, it shows the same pattern of size reduction during degradation that we have observed for the hsp70 message itself. Thus, completely replacing protein coding sequences and changing the translational efficiency of the hsp70 message does not alter its unique pattern of regulated degradation.

We also examined the degradation of a message that carries a deletion in the 5′ leader sequence, required for efficient translation during heat shock. This message is degraded exactly as is the endogenous hsp70 message. This confirms that regulation of hsp70 message degradation does not require high rates of translation and also demonstrates that it does not require the hsp70 5′ UTR.

Finally, we examined the influence of the natural translational termination site on the degradation

of the hsp70 message. Translation beyond the natural termination site did not change the regulation of degradation. Premature termination of translation produced a message that was degraded more rapidly than the endogenous hsp70 mRNA, but retained the basic pattern of regulation. That is, it was much more stable after a severe heat shock than after a mild heat shock. This contrasts with the well-studied regulation of histone message degradation (Graves *et al.,* 1987). The histone message must be translated to its natural termination site for it to be subject to rapid turnover in the absence of DNA synthesis. Our results also contrast with those of another well-characterized case of selective message degradation, the β-tubulin message. Degradation of this message depends upon coding sequences in the amino terminus (Gay *et al.,* 1987). Degradation of hsp70 message does not depend on coding sequences.

Since the hsp70 message is degraded during recovery from heat shock while the vast majority of other cellular messages are stable, it would appear that this degradation mechanism has a strict specificity for hsp70. A different picture emerges, however, when the hsp70 message is compared with certain other unstable messages. Several mammalian messages, such as the messages for c-fos, c-myc, interferon, and the lymphokine GM-CSF, contain AU-rich sequences at their 3′ ends that play a critical role in targeting them for rapid turnover (Shaw and Kamen, 1986; Brawerman, 1987). The *HSP70* 3′ UTR is 75% AU and contains 2 close matches to the putative mammalian consensus sequence. At least two of these mammalian messages, c-myc and c-fos, are stabilized by heat shock (Andrews *et al.,* 1987; Sadis *et al.,* 1988), as is the mammalian hsp70 message (Theodorakis and Morimoto, 1987). The stabilization of these messages in mammalian cells is not as dramatic as that of the hsp70 message in *Drosophila* cells, but the response of mammalian cells to heat shock in general is not as dramatic as that of *Drosophila* cells.

We suggest that the mechanism which regulates the degradation of the *Drosophila* hsp70 message is highly conserved and is common to other unstable messages. We suspect that certain other *Drosophila* messages are degraded by the same mechanism, but that these are expressed at such low levels in tissue culture cells that their regulation is not obvious in the analysis undertaken here. Since hsp70 messages are produced in great abundance and their degradation is so readily subject to experimental manipulation, they may provide an ideal system for investigating the factors that are responsible for the turnover of highly unstable messenger RNAs.

Materials and methods

Plasmid constructions

The plasmid pDM420 was created by linker-scanning mutagenesis with *Bam*HI linkers as previously described (McGarry,

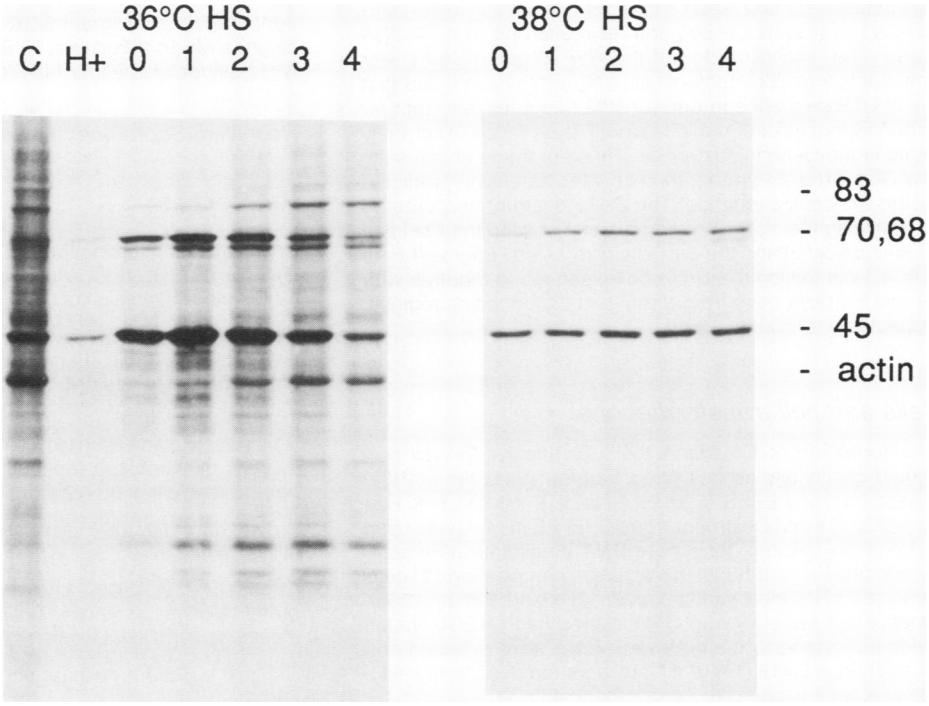

Figure 9. **Termination of translation beyond the normal site does not affect the regulation of repression.** Protein synthesis in cells transformed with the plasmid 45fs, analyzed as in Figure 1A.

1986). The linker insertion was accompanied by deletion of bases +194 to +231, which does not affect translation or regulation of the message (McGarry and Lindquist, 1986). The first 194 bases of the 5′ UTR are sufficient to confer heat-shock translation to other mRNAs (McGarry, 1986). The related plasmid, pDM420*Sal, was created by removing the *Sal* I site in the pUC13 polylinker by partially digesting with *Sal* I and filling in the overhanging ends with Klenow. This allowed us to use the *Sal* I site that is in the sequence encoding the last two amino acids of the *HSP70* gene to exchange 3′ UTRs. The *ADH* derivatives were made using oligonucleotide-directed site-specific mutagenesis to put *Bgl* II and *Sal* I restriction sites at the 5′ and 3′ ends of the coding region of an *ADH* cDNA generously provided by William Sofer. Mutagenesis was performed using the BioRad Mutagene kit according to the manufacturer's protocol. The sequence AGTCACCATGT was changed to AGATCTCCATGT at the 5′ end and the sequence CTAAGAAGTGAT was changed to CTAAGAAGTCGAC at the 3′ end resulting in the plasmid pAdhB/S. The mutations were confirmed by DNA sequence analysis. To make pA/A a *Bgl* II/*Xba* I fragment from pAdhB/S was inserted into *Bam*HI/*Eco*RI cut pDM420*Sal, resulting in the replacement of the *HSP70* coding and 3′ UTR with the *ADH* coding and 3′ UTR (the *Xba* I and *Eco*RI sites were filled in prior to ligation). The plasmid pA/70 was made by replacing the *ADH* 3′ UTR in pA/A with the *HSP70* 3′ UTR, by substituting the *Sal* I/*Eco*RI fragment of pA/A with the *Sal* I/*Eco*RI fragment of pDM420*Sal. The plasmids p70CAA and p70CA70 are the same as pA/A and pA/70 except that they contain the entire *HSP70* 5′ UTR. To construct them a wild type *HSP70* gene was mutated to change the sequence CAATGCCTGC to CAGGATCCTGC, which replaces the *HSP70* AUG codon with a *Bam*HI restriction site. Construction of the leader deletion plasmid, which lacks nucleotides +2 to +205 in the *HSP70* 5′ UTR, was previously described (McGarry and Lindquist, 1985). The *HSP70* frame-shift mutation WTfs was made by cutting the wild type *HSP70* gene in pDM300, (McGarry and Lindquist, 1985), at the *Bam* site, filling in the overhanging ends with Klenow, and recircularizing with T4 DNA ligase. The frame-shift mutation 45fs was made by cutting pDM420*Sal with *Sal* I, filling in the overhanging ends with Klenow, and recircularizing with T4 DNA ligase. The original source of the *HSP70* gene used in our constructs was G3 (Ingolia *et al.*, 1980). We have found that the 3′ UTR sequence in our constructs agrees with that reported by Ingolia *et al.* rather than with the sequence currently found in the Genbank database. Thus our frame-shift results in a 24aa extension rather than an 11aa extension predicted by the Genbank sequence. The *CAT* construct was created by replacing the *HSP70* coding region between the *Cla* I site (at amino acid 6) and the *Sal* I site with a *Taq* I fragment from pDV801 containing the *CAT* coding sequence together with 29 and 84 base pairs from the 5′ and 3′ noncoding regions, respectively (McGarry, 1986).

Cells and cell transformations

Transformation of *Drosophila* cells (Schneider 2) was performed essentially as described (Bourouis and Jarry, 1983) except: 1) 2× HEBES (270 mM NaCl, 9 mM KCl, 3 mM Na_2HPO_4, 11 mM Dextrose, 42 mM HEPES pH 7.1) was used to form the calcium phosphate DNA precipitate as recommended by Rio and Rubin (personal communication); 2) cells were cotransformed with pcopneo (Rio and Rubin, 1985), which yields resistance to G418 rather than pHGC0, which yields resistance to methotrexate. Cells were selected and maintained in G418 (1 mg/ml), but were removed from selection at least 1 wk before use in experiments.

Protein and RNA analysis

Proteins were pulse labeled with ^{3}H-leucine and analyzed as previously described (DiDomenico *et al.*, 1982b) except that the labeling period was 10 min and the proteins were analyzed on 10% polyacrylamide gels (Laemmli, 1970). For analysis of adh accumulation, protein samples were separated on polyacrylamide gels and electrophoretically transferred to Immobilon nylon membrane. After blocking with 5% dry milk in 1× PBS the blot was reacted with 1) goat anti-*Drosophila* adh antiserum (provided by William Sofer), 2) rabbit anti-goat IgG, and 3) with 3 μCi of ^{125}I-Protein A. The blot was then exposed to Kodak XAR-5 film. Protein labeling and RNA isolation were performed at the same time on duplicate aliquots of cells. RNAs were extracted at the midpoint of the protein-labeling period as described (McGarry and Lindquist, 1985), except that after the cells were washed in saline they were resuspended directly in lysis buffer (50 mM Tris pH 7.0, 0.25% SDS, 100 mg/ml proteinase K). RNA samples were denatured with glyoxal as described (McMaster and Carmichael, 1977), separated on 1.5% agarose gels in 1× TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA pH 7.0), blotted onto the UV-crosslinkable nylon membrane Hybond N (Amersham), UV crosslinked, and baked to remove the glyoxal. Hybridization with ^{32}P-labeled nick-translated DNA was performed in 1 M NaCl, 1% SDS, 100 mg/ml sheared salmon sperm DNA at 65°C as described (Hardy *et al.*, 1985). After hybridization and washing, the blots were wrapped in Saran wrap and exposed to Kodak XAR-5 film with a Kodak Lightning Plus intensifying screen.

Acknowledgments

We thank Tom McGarry for providing pDM300, pDM420, and pCAT and for sharing preliminary data; Jan Rossi for sharing unpublished data; Kent Golic and Julie Feder for helpful discussions; Jonathon Solomon and Michael Welte for critical reading of the manuscript; and Janet Meredith-Patla for typing the manuscript. We also thank William Sofer for providing the ADH cDNA and the goat anti-adh antiserum. This work was supported by NSF grant DCB-8617147 and by the Howard Hughes Medical Institute. R. B. Petersen was supported by NIH postdoctoral fellowship F32 GM11794.

Received: September 1, 1989.

References

Andrews, G.K., Harding, M.A., Calvet, J.P., and Adamson, E.D. (1987). The heat shock response in HeLa cells is accompanied by elevated expression of the c-fos proto-oncogene. Mol. Cell. Biol. *7,* 3452–3458.

Ashburner, M. (1970). Patterns of puffing activity in the salivary gland chromosomes of *Drosophila.* V. Responses to environmental treatments. Chromosoma *31,* 356–376.

Bourouis, M., and Bruno, J. (1983). Vectors containing a prokaryotic dihydrofolate reductase gene transform *Drosophila* cells to methotrexate-resistance. EMBO J. *2,* 1099–1104.

Brawerman, G. (1987). Determinants of messenger RNA stability. Cell *48,* 5–6.

Chomyn, A., Moller, G., and Mitchell, H.K. (1979). Patterns of protein synthesis following heat shock in pupae of *Drosophila melanogaster.* Dev. Genet. *1,* 77–95.

Craig, E. (1985). The heat shock response. CRC *18,* 239–280.

DiDomenico, B.J., Bugaisky, G., and Lindquist, S.L. (1982a). Heat shock and recovery are mediated by different translational mechanisms. Proc. Natl. Acad. Sci. USA *78,* 3531–3535.

DiDomenico, B.J., Bugaisky, G., and Lindquist, S.L. (1982b). The heat shock response is self-regulated at both the transcriptional and post-transcriptional levels. Cell *31,* 593–603.

Gay, D.A., Yen, T.J., Lau, J.T.Y., and Cleveland, D.W. (1987). Sequences that confer beta-tubulin autoregulation through modulated mRNA stability reside within exon 1 of a beta-tubulin mRNA. Cell *50,* 671–679.

Gilmour, D.S., and Lis, J. (1986). RNA polymerase II interacts with the promoter region of the noninduced *HSP70* gene in *Drosophila melanogaster cells.* Mol. Cell Biol. *6,* 3984–2989.

Graves, R.A., Pandey, N.B., Chodchoy, N., and Marzluff, W.F. (1987). Translation is required for regulation of histone mRNA degradation. Cell *48,* 615–626.

Hardy, K.J., Peterlin, B.M., Atchison, R.E., and Stobo, J.D. (1985). Regulation of expression of the human interferon gamma gene. Proc. Natl. Acad. Sci. USA *82,* 8173–8177.

Hultmark, D., Klemenz, R., and Gehring, W.J. (1986). Translational and transcriptional control elements in the untranslated leader of the heat shock gene hsp22. Cell *44,* 429–438.

Ingolia, T.D., Craig, E.A., and McCarthy, B.J. (1980). Sequence of three copies of the gene for the major *Drosophila* heat shock induced protein and their flanking regions. Cell *21,* 669–679.

Klemenz, R., Hultmark, D., and Gehring, W.J. (1985). Selective translation of heat shock mRNA in *Drosophila melanogaster* depends on sequence information in the leader. EMBO J. *4,* 2053–2060.

Kruger, C., and Benecke, B.-J. (1981). In vitro translation of *Drosophila* heat-shock and non-heat-shock mRNAs in heterologous and homologous cell-free systems. Cell *23,* 595–603.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature *227,* 680–685.

Lewis, M., Helmsing, P.J., and Ashburner, M. (1975). Parallel changes in puffing activity and patterns of protein synthesis in salivary glands of *Drosophila.* Proc. Natl. Acad. Sci. USA *72,* 3604–3608.

Lindquist, S. (1980a). Varying patterns of protein synthesis in *Drosophila* during heat shock: implications for regulation. Dev. Biol. *77,* 463–479.

Lindquist, S. (1980b). Translational efficiency of heat induced messages in *Drosophila melanogaster* cells. J. Mol. Biol. *137,* 151–158.

Lindquist, S., and DiDomenico, B. (1985). Coordinate and Noncoordinate Gene Expression During Heat Shock: A Model for Regulation. New York: Academic Press.

Lindquist, S.L. (1986). The heat-shock response. Annu. Rev. Biochem. *55,* 1151–1191.

McGarry, T. (1986). Genetic analysis of heat shock protein synthesis. Ph.D. thesis, University of Chicago, Chicago, IL.

McGarry, T., and Lindquist, S. (1986). Translational control of heat-shock proteins in *Drosophila.* In: The Translational Control of Protein Synthesis, ed. M. Matthews, Cold Spring Harbor, NY: Cold Spring Harbor Press, 86–90.

McGarry, T.J., and Lindquist, S.L. (1985). The preferential translation of *Drosophila* hsp70 mRNA requires sequences in the untranslated leader. Cell *42,* 903–911.

McKenzie, S., and Meselsohn, M. (1977). Translation in vitro of *Drosophila* heat shock messages. J. Mol. Biol. *117,* 279–283.

McKenzie, S.L., Henikoff, S., and Meselson, M. (1975). Localization of RNA from heat-induced polysomes at puff sites in *Drosophila melanogaster.* Proc. Natl. Acad. Sci. USA *72,* 1117–1121.

McMaster, G.K., and Carmichael, G.G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA *74,* 4835–4838.

Petersen, N., and Mitchell, H.K. (1981). Recovery of protein synthesis following heat shock; pre-heat treatment affects mRNA translation. Proc. Natl. Acad. Sci. USA *78,* 1708–1711.

Petersen, R., and Lindquist, S. (1988). The *Drosophila* hsp70 message is rapidly degraded at normal temperatures and stabilized by heat shock. Gene *72,* 161–168.

Rio, D.C., and Rubin, G.M. (1985). Transformation of cultured *Drosophila melanogaster* cells with a dominant selectable marker. Mol. Cell. Biol. *5,* 1833–1838.

Rougvie, A.E., and Lis, J.T. (1988). The RNA polymerase II molecule at the 5′ end of the uninduced hsp70 gene of *D. melanogaster* is transcriptionally engaged. Cell *54,* 795–804.

Sadis, S., Hickey, E., and Weber, L.A. (1988). Effect of heat shock on RNA metabolism in HeLa cells. J. Cell. Physiol. *135,* 377–386.

Scott, M.P., and Pardue, M.L. (1981). Translational control in lysates of *Drosophila melanogaster* cells. Proc. Natl. Acad. Sci. USA *78,* 3353–3357.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell *46,* 659–667.

Simcox, A.A., Cheney, C.M., Hoffman, E.P., and Shearn, A. (1985). A deletion of the 3′ end of the *Drosophila melanogaster HSP70* gene increases stability of mutant mRNA during recovery from heat shock. Mol. Cell Biol. *5,* 3397–3402.

Spradling, A. Pardue, M.L., and Penman, S. (1977). Messenger RNA in heat-shocked *Drosophila* cells. J. Mol. Biol. *109,* 559–587.

Storti, R.V., Scott, M.P., Rich, A., and Pardue, M.L. (1980). Translational control of protein synthesis in response to heat shock in *D. melanogaster* cells. Cell *22,* 825–834.

Theodorakis, N.G., and Morimoto, R.I. (1987). Posttranscriptional regulation of hsp70 expression in human cells: effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. Mol. Cell. Biol. *7,* 4357–4368.

Tissieres, A., Mitchell, H.K., and Tracy, U.M. (1974). Protein synthesis in salivary glands of *Drosophila melanogaster:* relation to chromosome puffs. J. Mol. Biol. *84,* 389–398.

Velasquez, J., Sonoda, S., Bugaisky, G.E., and Lindquist, S. (1983). Is the major *Drosophila* heat shock protein present in cells that have not been heat shocked? J. Cell Biol. *96,* 286–290.

Yost, J.G., and Lindquist, S.L. (1986). RNA splicing is interrupted by heat shock and rescued by heat shock protein synthesis. Cell *45,* 185–193.