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The heat shock response has been studied in rainbow trout to investigate the possibility that accumulation of the heat shock protein, hsp70, may be useful as a biomarker for environmental stress due to metal contaminated water. The heat shock response of rainbow trout has been characterized, and the rainbow trout hsp70 gene sequenced. A polyclonal antibody has been generated which is very specific for trout hsp70. Hps70 does accumulate in juvenile trout tissues including gill, liver, in response to metal (Cd^{++}, Cu^{++}, Pb^{++}, Zn^{++}) contaminated water and diet. Hsp70 levels in juvenile rainbow trout do not increase significantly when live rainbow trout tissues are exposed singly to environmentally relevant Cd^{++} or Cr^{++} levels.

Experiments done in Drosophila to determine the basis for the rapid turnover of hsc70 following heat shock indicate that the rate of turnover of hsc70 is determined by the temperature at which it is synthesized, suggesting the conformation of the protein is critical in the regulation of its turnover. Possible differences in the folding environment which could account for this are the temperature itself and the association of nascent hsc70 with nascent hsp70. This turnover does not depend on the ubiquitin pathway for proteolysis.

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Turnover of the Stress Induced Protein, HSP70.

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Objectives

The synthesis of heat shock protein, hsp70, appears to be essential for recovery from heat and chemical stress. In *Drosophila*, hsp70 labeled at 35°C decays rapidly during recovery and chase at 25°C. However, when tissues are incubated at 35°C during the chase rather than 25°C, the protein decays much more slowly. It is important to understand the mechanism regulating this turnover both because of the role of hsp70 in cellular recovery from stress and because of the possibility of using levels of hsp70 synthesis or accumulation as a measurement of cellular response to stress. This will be done using the Drosophila system which has already been characterized.

In order to determine whether the accumulation of hsp70 in rainbow trout would be a useful biomarker of environmental stress, the rainbow trout heat shock response has been characterized and the cloning and sequencing rainbow trout heat shock genes initiated. Antibodies to trout hsp70 will be generated and used to measure the accumulation of hsp70 in rainbow trout tissues exposed to heavy metals in collaboration with the H. Bergman laboratory.

Summary of Accomplishments

The results from this grant have been published in one thesis and two papers as referenced below. The results of the basic research on the turnover of hsp70 are about to be submitted for publication, but since they are not yet in print, a preprint of the paper is included with this report. A brief description of the major conclusions is included below.

The heat shock response has been characterized in trout tissues including gill, liver, kidney, and brain. The rainbow trout heat shock response is different from other vertebrates in that the most abundant constitutive trout hsp70 family member is also the synthesized most rapidly in response to heat. This protein turns over with a half life 21 to 43 hours depending on the water temperature, being most unstable at lower temperatures. A polyclonal antibody has been generated which is very specific for trout hsp70. In collaboration with the Harold Bergman laboratory, it has been shown that hsp70 levels in gill and liver do not increase significantly when live rainbow trout tissues are exposed to environmentally relevant Cd^{++} or Cr^{++} levels.

Experiments done in Drosophila to determine the basis for the rapid turnover of hsp70 following heat shock indicate that the rate of turnover of hsc70 is determined by the temperature at which it is synthesized, suggesting the conformation of the protein is critical in the regulation of its turnover. Possible differences in the folding environment which could account for this are the temperature itself and the association of nascent hsc70 with nascent hsp70. This turnover does not depend on the ubiquitin pathway for proteolysis.

Publications:

(1995) Jeanne Williams Ph.D. Thesis: Characterization of HSP70: Expression and Stability in Selected Organisms. (available at the University of Wyoming Libraries)

(1996) Jeanne H. Williams, Aida M. Farag, Mark A. Stansbury, Patricia A. Young, Harold L. Bergman and Nancy S. Petersen Accumulation of Hsp70 in Juvenile and Adult Rainbow Trout Gill Exposed to Metal Contaminated Water and/or Diet. Journal of Environmental Toxicology and Chemistry 15: 1324-1328.

(1996) Jeanne H. Williams, Gary E. Moss, Lew K. Hunnicutt and Nancy S. Petersen, Induction of the Heat Shock Response and Translational Thermotolerance in Day 15 Ovine Trophectoderm. submitted to Biology of Reproduction. (started earlier and completed by Jeanne during the tenure of the AF grant)

Petersen, N., Williams, J., and Qin, Haiying Stability of the major cytosolic hsc70 protein in *Drosophila melanogaster* is determined by its temperature of synthesis. Manuscript in preparation.

ABSTRACTS

(1996) Petersen, N.S., Williams, J., and Young, P. HSC70 Synthesized at 35°C in Drosophila is Unstable. Cold Spring Harbor Meeting Abstracts for "Molecular Chaperones and the Heat Shock Response", May 1-5,1996 Cold Spring Harbor, NY.

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The stability of the *Drosophila* hsp70 family member, hsc4, depends on the temperature at which it is synthesized.

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INTRODUCTION

Members of the hsp70 family of proteins are chaperonins involved in protein folding and transport in cells (Becker and Craig, 1994). Certain members of the family also appear to play a role in recovery from heat and chemical stress. The heat induced member of the family, hsp70, was first studied because of the rapid induction of hsp70 gene expression following heat shock at 37°C in Drosophila (Tisseres et al., 1974; Schlessinger et. al., 1982). Both the rapidity of the transcriptional activation of the hsp70 gene and the selective translation of its mRNA suggest that regulation of the amount of hsp70 is important to all cells. As might be expected of a protein whose levels are regulated in the cell, hsp70 is unstable following a mild heat shock (35°C) in Drosophila (Mitchell et al., 1985; Palter et al., 1986; Petersen et al., 1992). However, the normally expressed member of the family, hsc70, has been reported to be quite stable in several organisms (Li and Lazlo, 1985). In order to better understand the regulation of the turnover of hsp70, we decided to compare the stability of hsp70 and hsc70.

There are at least five hsp70 homologues expressed at 25°C in Drosophila (Rubin et al., 1993). The most abundant of these is hsc4, referred to here as hsc70. This protein has 74% amino acid homology to hsp70 and is present in all normal cells (Rubin et al., 1993). Of particular interest is the abundance of this protein in oocytes and embryos. At these stages hsc70 is more abundant than actin (Palter, 1986). The role of hsc70 as a chaperonin for nascent polypeptide chains could explain this abundance. Hsc70 has also been shown to have clatherin uncoating activity and to play a role in protein transport across membranes (Chappell et al., 1986; Deshaies et al., 1988; Chirico et al., 1988; Becker and Craig, 1994)

In comparing two cytoplasmic heat shock proteins one of which appears to be stable and the other unstable, we hoped to be able to identify the sequences responsible for the differences in stability. Instead, we found that hsc70 is stable when made at 25°C, but it is just as unstable as hsp70 when synthesized at 35°C. This difference in stability is not due to post-translational modification that can be detected on a two dimensional gel. The most likely explaination of

this phenomenon is that nascent hsc70 folding is affected in the presence of newly synthesized hps70. Furthermore, the stability of both hsp70 and hsc70 synthesized at 35°C can be greatly increased by a 30 minute heat shock at 40°C. The temperature dependence of the stability of these proteins indicates that their turnover is regulated in a complicated manner.

MATERIALS AND METHODS

Drosophila Stocks

The Oregon-R wild-type stock of *Drosophila melanogaster* originally obtained from the stock center at Caltech was used in these experiments. Flies were grown in mass culture at 25°C and approximately 60% humidity (Mitchell and Mitchell, 1964).

Tissue Preparation and Labeling

White pre-pupae were collected (time zero) and maintained at 25°C until dissection. At specific times (19, 42, 72, and 90 hours), pupae were removed from their pupal cases; wings were dissected, and collected in Mops buffer (10mM Mops buffer-pH 7.0, 80mM NaCl, 10mM KCl, 1mM $CaCl_2$ 0.2mM $MgCl_2$) as previously described (Petersen and Mitchell, 1982). Wings were pulse-labeled in 10µl Mops buffer containing a radioactive amino acid: 10µCi ^{35}S-methionine (1175 Ci/mmole) or 25µCi ^{3}H-leucine (144 Ci/mmole) or 25µCi ^{3}H-lysine (110 Ci/mmole) or 1 µCi ^{14}C-leucine (325 mCi/mmole). After labeling for 40 minutes at the appropriate temperature, the labeling media was removed and replaced with Mops buffer containing greater than 1000 fold excess unlabeled amino acid (about 5mM). This amount completely prevented further incorporation of the labeled amino acids in controls.

The chase was stopped by removal of the chase media and addition of 25µl of sample buffer (126mM Tris-pH6.8, 13 mM dithiothreitol, pyronin Y, 2%SDS, 25% glycerol). Samples were then frozen in liquid nitrogen and stored at -80°C. Before running gels each sample was thawed, ground in its tube using a glass pestle, placed in a boiling water bath for 10 minutes, vortexed, and centrifuged at room temperature for 10 minutes at 15,000 Xg.

One-dimensional SDS-Polyacrylamide Gel Electrophoresis

A volume of sample buffer which contained solubilized proteins from three pairs of wings was loaded on a 10 to 18% gradient sodium dodecyl sulfate-poly-acrylamide gel, or SDS-PAGE gel, (1:76 w/w bis-acrylamide:acrylamide) with a 7.5% acryamide

stacking gel according to the procedure of Laemmli (1970) modified as described previously by Mitchell and Petersen (1981).

Two dimensional Gel Electrophoresis

Proteins were separated in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE. Two-dimensional gel electrophoresis was carried out using a protocol modified from Buzin and Petersen (1982) which was modified from O'Farrell (1975). Isoelectric focusing gels were prepared in cylindrical tubes with 5.7% w/v Duracryl (30% acrylamide/0.8% bis solution from Millipore), 9M urea, 3% triton X-100, and 3% ampholytes (Pharmacia). The ampholytes were used in a ratio of 2 parts pH 5-8 to 1 part pH 2.5-5. The resulting PH range in the gel was from 4 to 7.5. The electrode solutions were: 0.10M degassed NaOH with a small amount of solid $Ca(OH)_2$ in the lower reservoir (cathode), and 0.05 M H_3PO_4 in the upper reservoir (anode). Sample buffer equivalent to three wing pairs was mixed with 100µl of loading buffer [9M urea, 3% triton X-100, 3% ampholytes (5:2, pH 5-8:pH 2.5-5)] and loaded on the acidic end of the gel under 100µl of overlay buffer (loading buffer diluted 1:1 with water). The samples were electrophoresed at 300 volts for 22 hours followed by 500 volts for 2 hours. After electrophoresis, the gels were extruded into equilibration buffer (126 mM Tris, 2% SDS, 10mM DTT, and bromphenol blue) and equilibrated for 30 minutes. The isoelectric focusing gels were sealed across the top of one dimensional gradient gels with agarose in 126mM Tris and 3%SDS (to minimize streaking). The second dimension gels were prepared and run as described for one dimensional gels.

Staining, Autoradiography, and Quantitation

One dimensional gels were fixed for 30 minutes in 5% acetic acid and 30% methanol, stained for 1 hour in quantitative CBB stain (0.25% Coomassie brilliant blue, 5% acetic acid, 45% methanol) and destained in 5% acetic acid and 30% methanol. The gels were then dried onto filter paper and autoradiography was done using Fuji

RX/GCU film. Two dimensional gels were fixed, but not stained prior to autoradiography.

An LKB Ultroscan XL Laser Densitometer was used to quantitate the densities on the autoradiograms using LKB2400 Gel scan XL software. Autoradiograms representing several exposures were scanned for each gel to ensure that all band and spot intensities were within the linear range of the film.

RESULTS

The rapid turnover at 25°C of hsp70 synthesized at 35°C, has been described previously for whole Drosophila larvae and adults (Mitchell et al., 1985; Palter et al., 1986). In order to study this phenomenon more easily, we looked at the turnover of hsp70 made during a 35°C treatment in larval salivary glands and pupal wings. In both cases the half-life of hsp70 made during 30 minutes at 35°C and chased at 25°C is about 3 hours (Petersen et al., 1992). This is in good agreement with previously published results using injection of ^{35}S-methionine into intact larvae, and coomassie staining of proteins from adults. We chose wings rather than salivary glands for further study because they have a protective cuticle layer so that they survive longer (at least eight hours) under our culture conditions (Petersen and Young, 1989). Figure 1 shows the results of pulse chase experiments done with wings from pupae of different ages, 19 hours, 42 hours, and 90 hours. The decay of hsp70 does not appear to depend on the developmental stage of the wings. In each case, the hsp70 labeled during the 35°C incubation decays with a half-life of about 3 hours during the chase at 25°C. Since hsp70 stability is not dependent on developmental stage, most of the following experiments were done using wings from 72 hour pupae. Seventy two hours wings are relatively easy to stage and dissect, and they survive well in culture.

The heat shock cognate hsc70 has been reported to be stable in Drosophila and other systems. Figure 2 shows that hsc70 synthesized and labeled at 25°C is in fact stable in wings under our culture conditions during a 25°C chase for at least six hours. This is in agreement with previously published reports. In order to directly compare the stability of hsp70 and hsc70 we labeled wings with ^{35}S-methionine during a 40 minute incubation at 35°C and chased the label at 25°C for various lengths of time. The labeled proteins were run on two-dimensional isoelectric focusing/ SDS-PAGE gels in order to separate hsc70 from the hsp70s. To our surprise, when hsc70 is synthesized at 35°C it is just as unstable as hsp70 (Figure 3).

The instability of hsp70 and hsc70 is in dramatic contrast to the majority of proteins which appear to be quite stable.

The instability of hsc70 synthesized at 35°C raises the question of whether the heat itself activates proteases which in turn degrade the excess hsp70 made at this temperature. If this were the case, hsc70 made at 25°C and shifted to 35°C transiently during the chase period should be degraded due to the synthesis of excess hsp70. Figure 4 shows that this is not the case. Hsc70 made at 25°C is quite stable during a 35° heat shock as well as during the remainder of the chase period at 25°C. These results lead to the conclusion that there is a difference in the hsc70 protein made at the different temperatures which determines its stability. It is not just that newly synthesized hsc70 is more susceptible to turnover, because hsc70 made at 35°C continues to turnover for at least six hours during a 25°C chase. Furthermore, hsc70 is not degraded because some general protein turnover mechanism is activated at 35°C. If this were the case, hsc70 synthesized at 25°C would turn over when it is exposed to a 35° heat shock during the chase period; but it does not. Also, other proteins including actin and the heat shock protein hsp83 are stable under these conditions. This result leads us to the conclusion that the hsc70 made at 25°C must be different in some way from hsc70 made at 35°C. Altered post-translational modification, different secondary, tertiary or quaternary structure, or changes in cellular localization of the protein could be responsible for this effect.

Another question raised by these experiments is how exposure to temperatures higher and lower than 35°C affect the stability of hsc70 and hsp70. Figure 5 shows that at temperatures between 30°C and 37°C hsp70 decays with a half life of 2-4 hours. This was also true for hsc70 at 31°C and 33°C as well as 35°C where 2D gels were run (data not shown). At 38°C the stability of hsp70 increases somewhat, and hsp70 synthesized at 40°C is stable for at least four hours (Figure 6). The transition from stable to unstable at the lower temperatures seems to correlate with induction of heat shock protein

synthesis. The transition to stability which occurs at the higher end of the temperature range correlates with temperatures at which protein synthesis is inhibited. Overall, hsp70 and hsc70 are unstable at temperatures where cells are actively synthesizing heat shock proteins.

DISCUSSION

The turnover of the hsp70 and hsc70 proteins is dependent on temperature, and appears to be regulated in Drosophila. This should not come as a surprise since the synthesis, translation, and stability of the messages encoding these proteins is clearly very carefully regulated, indicating the importance to the cell of regulating the amounts of these proteins present under different conditions.

The most interesting and unexpected conclusion from these experiments is that there is a difference in the stability of hsc70 depending on the temperature at which it is synthesized. The fact that hsc70 synthesized at 25°C can co-exist in a cell with hsc70 (and hsp70) synthesized at 35°C, but that these proteins have very different turnover rates demonstrates that they are different in some important way. The difference is probably not due to post translational modifications because the molecular weight and the isoelectric point of the protein synthesized at the two temperatures is identical. Although a post-translational modification involving a relatively small molecule which does not affect the charge of the protein is still a possibility.

Other possibilities which could account for the changes in stability are differences in cellular localization or in protein folding and/or the association with other cellular proteins. There are no major differences in the localization of hsp70 as detected by immunofluoresence in wing cells at 25°C and 35°C (data not shown). The most likely explanation of these results seems to be that translation at the different temperatures results in different secondary or tertiary structure, which in turn leads to associations with other proteins which determine the stability. A simple example would be that hsp70 may be a stable multimer at 25°C, but may be an unstable monomer when synthesized at 35°C.

Since hsp70 is not made in significant amounts at 25°C, we do not know whether it would be as stable as hsc70. However, it is clear that hsp70 made at 40°C is much more stable than hsp70 made at 35°C. This heat treatment does not kill the cells, but does inhibit the synthesis of all proteins except for the synthesis of a small

amount of hsp70 several hours following the heat shock (Petersen and Mitchell, 1981). From a regulatory point of view it is advantageous to the cell for hsp70 to remain stable under these conditions where the cell is accumulating hsp70. Alternatively, it may just be that the protein turnover mechanism has been inactivated by heat or requires normal protein synthesis for activity.

The temperature profile for the decay of the heat shock seventy proteins indicates a sharp rather than a gradual transition from the stable condition to the unstable condition. The temperatures at which the unstable form is made correspond to the temperatures at which the synthesis of hps70 is induced. The meaning of this correspondence is unclear, however, it may reflect changes in the translational machinery which are associated with heat shock. Further experiments examining the stability of hsp70 and hsc70 induced by chemical stress may help to clarify this issue.

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FIGURE LEGENDS

Figure 1. Hsp70 synthesized following a heat shock at different times during wing development decays with a half-life of about 3 hours.
Wings (6 per sample) were dissected from pupae of the age specified and incubated for 30 minutes at 35°C in buffered saline (MOPS) containing 5 mCi/ml ^{35}S-methionine. After incubation the wings were washed 5 times in MOPS and incubated at 25°C for the times indicated. The proteins were solubilized, run on one dimensional SDS-PAGE gels and the autoradiograms were analyzed as described in Materials and Methods. The amount of hsp70 is represented as the percent remaining and data from each sample was normalized to the amount of hsp68, which does not decay under these conditions (Mitchell, et al., 198 5)

Figure 2. Hsc70 labeled at 25°C and chased at 25°C is stable.
Autoradiograph of a one dimensional gel showing that hsc70 made at 25°C is stable for at least 6 hours under our culture conditions when compared to actin or hsp83. Wings (3 pair) were dissected from 72 hour and incubated for 40 minutes at 25°C in MOPS buffer containing 1 mCi/ml ^{35}S-methionine. The wings were washed 3 times and incubated in MOPS containing 1 mg/ml methionine at 25°C for 0, 3 or 6 hours as indicated below each lane.

Figure 3. Two-dimensional gel autoradiograph showing that hsp70 and hsc70 both decay when made at 35°C and chased at 25°C. Two-dimensional gel autoradiographs of 72 hour pupal wings heat-shocked at 35°C for 40 minutes in the presence of ^{35}S-Methionine and chased with unlabeled methionine for 0 hours (A) and 3.5 hours (B). Hsp70s and hsc70 (small arrow) decay when compared to actin (A). The pH gradient of the isoelectric focusing gel is indicated across the top of the figures and the molecular weights, in kDa are indicated by the horizontal arrows.

Figure 4. Hsc70 made at 25°C and shifted to 35°C during the chase is stable while hsp70 made at 35°C and chased at 25°C is not. SDS-PAGE gel autoradiographs of 72 hour pupal wings labeled with ^{35}S-methionine for 40 minutes. The molecular weights, in kDa are indicated by the horizontal arrows. A. Wings labeled at 35°C and chased at 25°C for 0 or 3 hours. B. Wings labeled at 25°C and not chased at 25°C (labeled 0 under lane) or chased for 40 minutes at 35°C followed by 2 hours 20 minutes at 25°C (labeled 3 under lane).

Figure 5. The stability of hsp70 at various temperatures of synthesis. This bar graph shows that hsp70 made at 25°C, 28°C and 40°C is stable and hsp70 made at temperatures between 28°C and 40°C is not. The number inside the bars indicate the number of samples done at that temperature. The arrows at the top of the graph indicate that the actual value for that temperature is greater than maximum value on the y-axis. The error bars represent 1 standard deviation.

Figure 6. Hsp70 made at 33°C or 35°C has a half-life of approximately 3 hours while hsp70 made at 40°C is stable.
SDS-PAGE autoradiographs of 72 hour pupal wings heat-shocked in the presence of ^{35}S-methionine and chased with unlabeled methionine at 25°C show that hsp70 made at 40°C is stable but unstable when made at 33°C or 35°C. The molecular weights, in kDa are indicated by the arrows.

A. Wings labeled for thirty minutes at 33°C and chased at 25°C for 0 and 3 hours. B. Wings labeled for thirty minutes at 35°C and chased at 25°C for 0 and 4 hours. C. Wings labeled for thirty minutes at 40°C and chased at 25°C for 0 and 4 hours.

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Figure 1

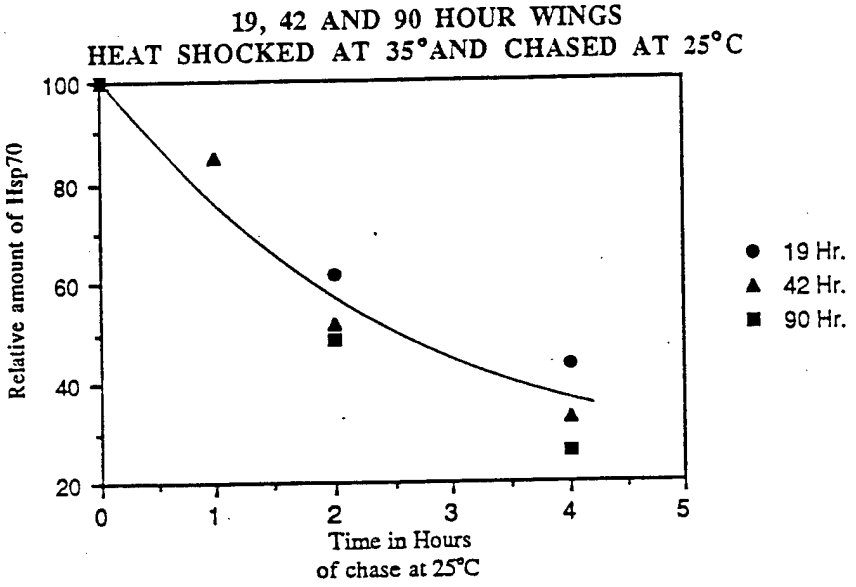

Figure 2

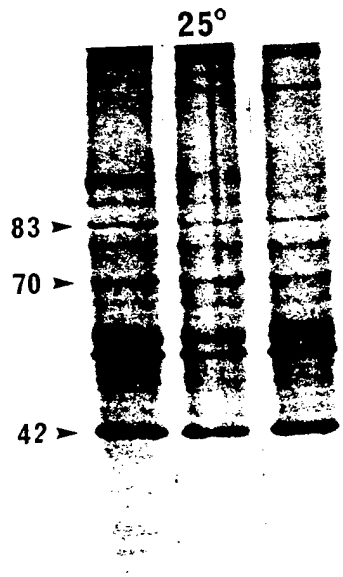

Figure 3

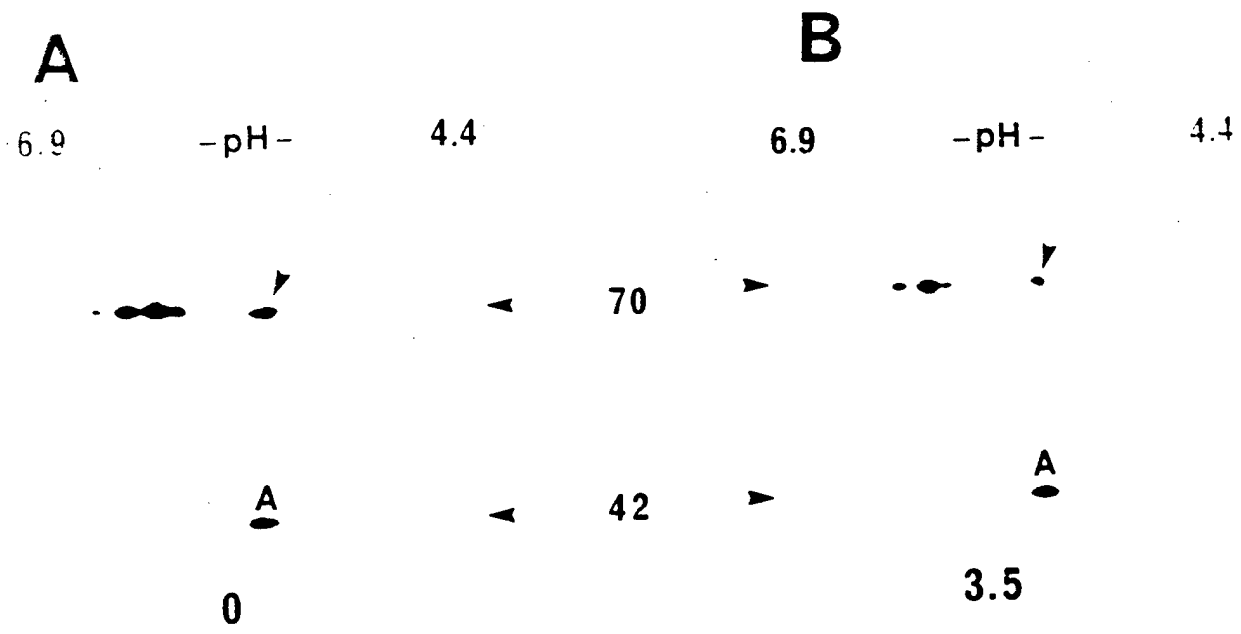

Figure 4

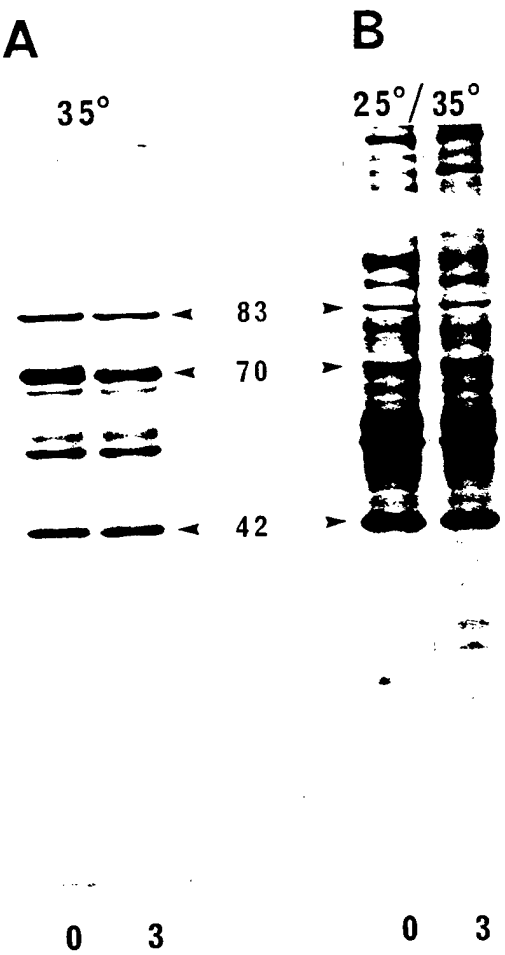

Figure 5

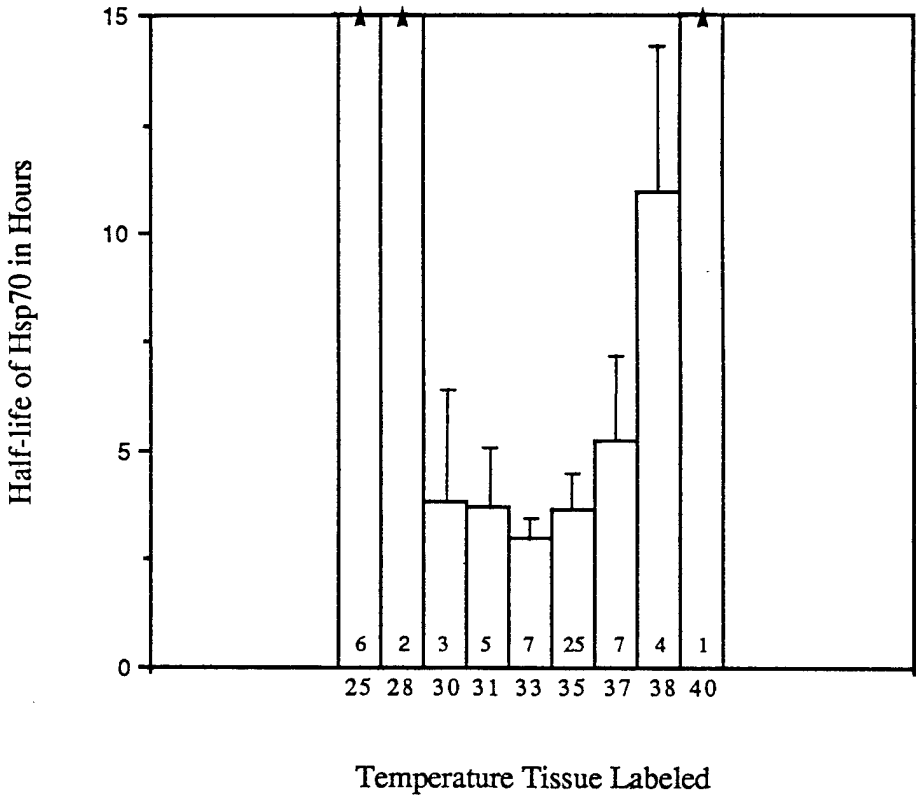
15
10
5
0
Half-life of Hsp70 in Hours
6
2
3
5
7
25
7
4
1
25 28 30 31 33 35 37 38 40
Temperature Tissue Labeled

Figure 6