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Heat-Shocked *Drosophila* Kc Cells Have Differential Sensitivity to Translation Inhibitors

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Abstract : The heat shock response is a universal stress response observed in all organisms and cultured cells. The response is regulated at both the transcriptional and translational level. Heat shocked *Drosophila melanogaster* Kc cells are used as the system for the study of translational regulation. In this system non-heat shock messages are associated with polysome but are not translated in a heat shocked condition. To figure out the change in the translation machinery, the effects of translation elongation inhibitors were tested on Kc cells. The result showed that the sensitivity of translation to these drugs changed in heat shocked cells. The significant changes were the decreased inhibition of heat shock protein synthesis by cycloheximide, emetine, and puromycin, and the increased inhibition of heat shock protein synthesis by verrucarin A, implying that the translation elongation mechanism in heat shocked cells changed.
Key words : antibiotics. *Drosophila melanogaster*. heat shock, protein synthesis.

Organisms or cultured cells respond to heat shock by synthesizing a special set of proteins. called heat shock proteins (hsps). This phenomenon is found in almost all organisms. including yeast. mammalian cells. Drosophila. and *E. coli*. The hsp synthesis is regulated at transcription and translation processes. Selective transcription of heat shock genes was reported as puffs on *Drosophila* polytene chromosomes (Tissier *et al*., 1974). The selective trascription has been found due to heat shock elements (HSE) and corresponding protein factors. heat shock factors (HSF). which are activated by heat shock (Wu *et al*., 1987). In many organisms. heat shock induces binding of HSF to HSE resulting in a rapid increase of hsp message synthesis and pre-existing non-heat shock message degradation. Thus, the transcription process has been considered as a main regulatory step of hsp synthesis. On the other hand, in *Drosophila* cells additional regulation mechanisms exist at a translation step under heat shock conditions. It was demonstrated that messenger RNAs encoding normal proteins were not broken down or irreversibly inactivated in heat shocked Drosophila cells. Rather the messages encoding normal proteins from heat shocked cells which were not used for protein synthesis during heat shock, could be translated into normal proteins in the recovery of heat shocked *Drosophila* cells (Storti *et al*., 1980). Therefore, there must be alterations in the translational component of heat shocked cells which are capable of causing selective translation of heat shock messages in *Drosophila* cells. In particular, translation elongation steps after initiation were altered in heat shocked *Drosophila* cells resulting in a 15 to 30-fold reduced elongation rate on normal messages relative to those of the efficiently translated heat shock mRNAs and implied a change of factors involved in elongation (Ballinger and Pardue, 1983). Antibiotics which have specific toxicity for protein synthesis have been used as a tool for investigating protein synthesis mechanisms. Here, I report the differential effects of antibiotics on protein synthesis machinery in heat shocked and control *Drosophila* Kc cells, including cycloheximide, puromycin, emetine, and verrucarin A, which all have inhibitory effects on translation elongation, to investigate the differences in protein synthesis in heat shocked and control cells.

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Materials and Methods

Materials

Puromycin, cycloheximide, emetine, and verrucarin A were obtained from Sigma Chemicals (St. Louis, USA). L-[^{35}S]-methionine (800~1,000 Ci/nmol) was purchased from Amersham (Arlington Heights, USA). Common laboratory reagents were from Fisher Scientific (Springfield, USA) or Sigma Chemicals.

Cell culturing and labeling conditions

Drosophila melanogaster Kc cells (Echalier and Ohanessian, 1970) were maintained at 25°C in D22 medium with heat inactivated fetal calf serum (FCS), as previously described (Sanders, 1981). Kc cells were grown to between 0.8×10^7 and 1.2×10^7 cells per ml and diluted 1:1 with D22+FCS the day before use. For the antibiotics treatment cells were aliquoted into test tubes after centrifugation followed by resuspending in D22 minus methionine medium and transferred to a 25°C or 37°C water bath at time zero. Antibiotics were added to give the designated final concentrations after 10 min. Twenty minutes after the drug addition (i.e., time 30 min), [^{35}S]-methionine (Amersham) was added to a final level of 25~35 μCi/ml. At the time of 70 min, cells were harvested in the benchtop centrifuge, the medium was discarded and the cell pellet was dissolved in Laemmli sample buffer. Constant numbers of cells per lane were loaded onto a SDS-polyacrylamide gel (PAGE) (10 or 12%) for electrophoresis (Laemmli, 1970). The sizes of marked proteins on autoradiograms were estimated by standard molecular markers (Bio-Rad).

Sucrose gradient for polysomes

Linear sucrose gradient was prepared from 0.5 M and 1.5 M sucrose solutions in 50 mM Tris-Cl pH 7.4, 100 mM KOAc, 5 mM $Mg(OAc)_2$, 5 mM dithiothreitol, and 0.1 mM EDTA (physiological buffer). For verrucarin A experiment cells were treated with antibiotics for 40 min at 25°C or 37°C in D22+FCS, and 5×10^7 cells were used for each sample. Metabolism was stopped by pouring the cell culture into 5 ml of crushed frozen PBS (150 mM NaCl, 10 mM Na phosphate, pH 6.9). After all the ice crystals were melted, cells were centrifuged at $2.500\times g$ at 4°C for 4 min and the pellets were lysed with 2 μl of RNasin (Promega, Madison, USA) and 200 μl lysis buffer (physiological buffer with 0.3 M sucrose, 0.35% Triton X-100, and 0.35% deoxycholate) by vortexing. Lysates were loaded onto the sucrose gradients and centrifuged for 1 h and 50 min at 37,000 rpm in an SW41 rotor in a Beckman ultracentrifuge. Polysome profiles were recorded with an ISCO fractionator by reading absorbance at 254 nm.

Results

Cycloheximide

To determine the sensitivity of *Drosophila* Kc cells in 25°C and 37°C conditions to the cycloheximide, a translation elongation inhibitor, a dose-response experiment was set up using three-fold increasing concentrations of the drug in a range between 0 and 1 μg/ml. Levels of inhibition were compared on autoradiograms of SDS-gels (Fig. 1). Protein synthesis in control cells at 25°C was dramatically inhibited by 0.03 μg/ml of cycloheximide (control lane 2) and was nearly undetectable at 0.1 μg/ml (control, lane 3). By contrast, hsp synthesis at 37°C did not show significant inhibition in this range of concentration of cycloheximide. In heat shocked cells, more than 70% of hsp70 and the smaller hsps as well as residual active synthesis continued at 0.03 μg/ml cycloheximide. Levels of inhibition seen at 0.03 μg/ml at 25°C were not observed until 0.3 μg/ml at 37°C (Fig. 1). It was concluded that hsp synthesis is up to thirty-fold less sensitive to cycloheximide than protein synthesis at 25°C by comparing the concentration of cycloheximide at which protein synthesis was 100% inhibited.

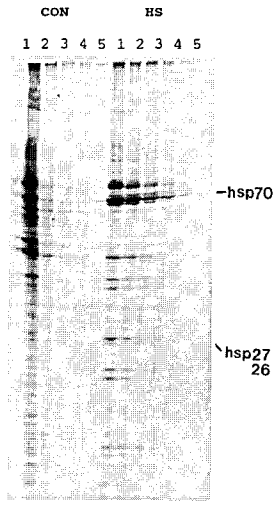

Fig. 1. Effect of cycloheximide on protein synthesis in Kc cells. Cells were labeled with 30 μCi of [^{35}S]-methionine per ml at 25°C and 37°C in the presence of cycloheximide. Following labeling, the cells were solubilized in sample buffer for 12.5% SDS gel. Autoradiogram shown is of cells incubated at 25°C (control) and 37°C (heat shock) with cycloheximide at final concentration of (lane 1) 0 μg, (lane 2) 0.03 μg, (lane 3) 0.1 μg, (lane 4) 0.3 μg and (lane 5) 1 μg per ml.

Emetine

The possibility that changed sensitivity to another translation elongation blocker, emetine, also occurred in heat shocked Kc cells was then tested. In the presence of emetine (Fig. 2), control protein synthesis was decreased to a very low level at 0.1 μg/ml (control, lane 3), while hsp synthesis continued at a high level at 0.3 μg/ml at 37°C (hs, lane 3). Furthermore, hsp synthesis, including histone H2b synthesis (Sanders, 1981), did not decrease to zero level at the highest concentration (3 μg/ml). While the difference in sensitivity to emetine is

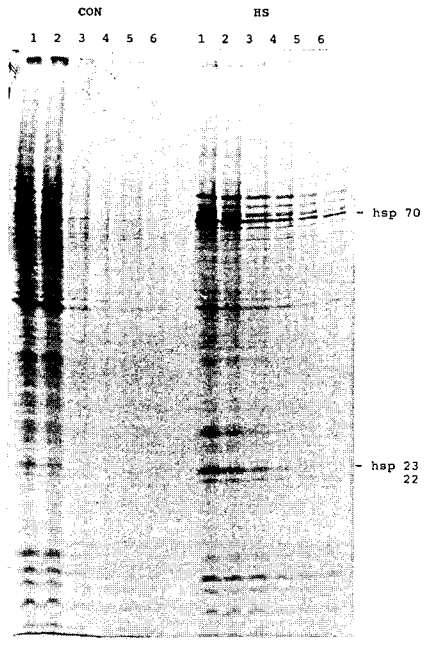

Fig. 2. Effect of emetine on protein synthesis. Kc cells were labeled with [^{35}S]-methionine for 40 min at 25°C (control) and 37°C (heat shock) in the presence of emetine as described in the text. The labeled cells were dissolved in sample buffer and electrophoresed on SDS gel. The gel was stained with Coomassie blue, dried and exposed to X-ray film. The samples shown on the autoradiogram are with (lane 1) 0 μg, (lane 2) 0.03 μg, (lane 3) 0.1 μg, (lane 4) 0.3 μg, (lane 5) 1 μg, and (lane 6) 3 μg per ml emetine.

not as large as that seen with cycloheximide, these experiments also show that the protein synthesis machinery is much less sensitive to inhibition of elongation in heat-shocked cells than in control cells.

Puromycin

Puromycin is known as a heat shock inducer in prokaryotes (Hausner *et al.*, 1988) and increases thermotolerance in Chinese hamster ovary cells (Lee and Dewey, 1987). Puromycin inhibits protein synthesis by getting into the A site on ribosomes and covalently binding the nascent peptide, thereby terminating translation elongation (Gale *et al.*, 1981). In *Drosophila* Kc cells, no hsp synthesis was induced by puromycin treatment; rather protein synthesis was inhibited by puromycin to different degrees in control and heat-shocked cells (Fig. 3). In both heat shock and normal conditions, higher molecular weight protein synthesis was constrained faster than low molecular weight protein synthesis. As with the inhibitors tested previously, control cells showed more sensitivity than heat-shocked cells; at 100 μM puromycin (control, lane 5), protein synthesis in control cells was shut down completely, while

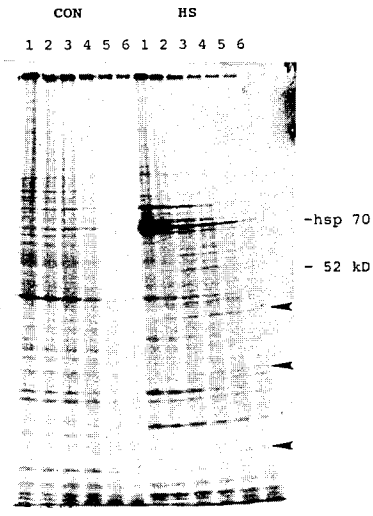

Fig. 3. Effect of puromycin on protein synthesis. Kc cells were labeled with [^{35}S]-methionine in the presence of puromycin for 40 min at 25°C (control) and at 37°C (heat shock). The lanes shown on the autoradiogram are with (lane 1) 0 μM, (lane 2) 3 μM, (lane 3) 10 μM, (lane 4) 30 μM, (lane 5) 100 μM and (lane 6) 300 μM puromycin. Arrowheads indicate small distinct fragments. The labeled cells were dissolved in sample buffer and electrophoresed on 12.5% SDS-PAGE and autoradiographed.

in heat-shocked cells hsp synthesis was still going on at 100 μM (hs, lane 5). One interesting finding in the puromycin treated heat-shocked cells was that new distinct smaller bands appeared on the autoradiogram in the presence of the drug. It was confirmed that these smaller bands were prematurely terminated hsps by a protease digestion pattern comparison (data not shown). These results imply that there are several preferred positions in the RNA sequence where puromycin binds to the A site of ribosomes and produces prematurely terminated peptides of a distinct size.

Verrucarin A

Verrucarin A is known as an inhibitor for the first peptide bond formed after initiation (Carter and Cannon, 1978). When Kc cells were treated with increasing concentrations of verrucarin A, inhibition of protein synthesis was observed in both control and heat shocked cells (Fig. 4A). But, in contrast to the other antibiotics tested above, protein synthesis in heat shocked cells was more sensitive to the drug than that in control cells. At 10 ng/ml (lane 5), almost no inhibition effect was detected on the autoradiogram in control cells compared with 80% protein synthesis inhibition estimated by measuring the amount of newly synthsized hsp70 in heat-shocked cells. Careful inspection of the heat shock lanes of the autoradiogram showed that inhibition of in-

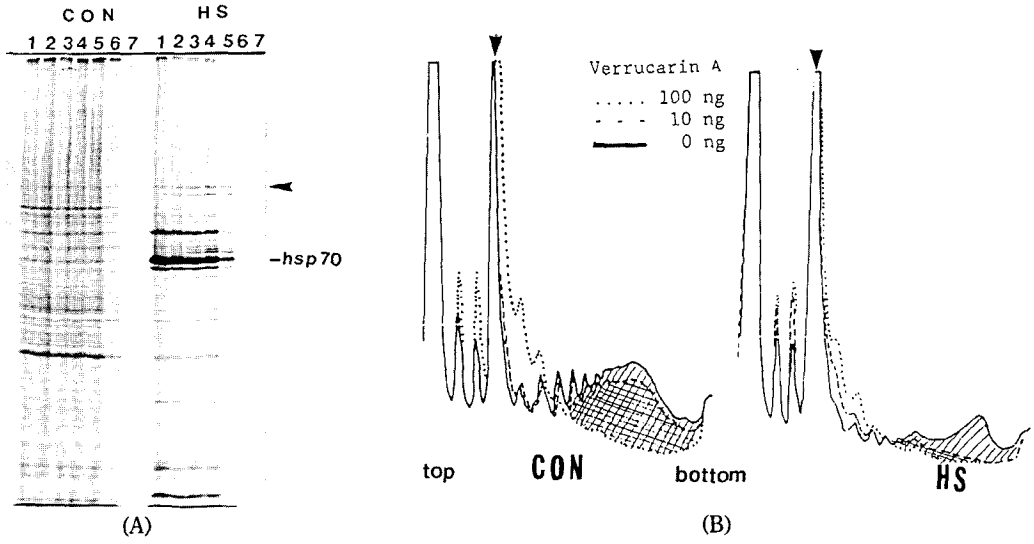

Fig. 4. Effect of verrucarin A on protein synthesis. (A) Kc cells were labeled with [^{35}S]-methionine for 45 min at 25°C (control) and 37°C (heat shock) in the presence of verrucarin A that was dissolve in DMSO. Samples were separated on 10% SDS-PAGE. The samples on autoradiogram are with (lane 1) no addition, (lane 2) 5% DMSO, (lane 3) 1 ng, (lane 4) 3 ng, (lane 5) 10 ng, (lane 6) 30 ng and (lane 7) 100 ng of verrucarin A per ml. Arrow indicates the protein synthesized both in control and heat shocked cells, but the synthesis is less inhibited by the drug than hsps in heat shock condition. (B) Polysome gradient profile with verrucarin A. Cells were incubated in D22 medium at 25°C or at 37°C for 10 min before adding verrucarin A to a final concentration of 10 ng or 100 ng per ml. The cells were then incubated an additional 30 min with drug. The cells were then lysed with lysis buffer as described in Materials and Methods, and loaded onto 0.5~1.5 M sucrose gradients. Samples were centrifuged for 1 h and 50 min in Beckman sw41 rotor. CON (25°C), HS (37°C) cells with or without verrucarin A as indicated. Arrowheads show the position of 80S monosomes and top and bottom of gradient are indicated.

corporation into hsps in heat shock conditions is greater than inhibition of incoporation into proteins also synthesized in a 25°C condition (indicated with an arrowhead). The differences in sensitivity to verrucarin A were clearly seen on polysome gradient profiles when the profiles were compared in both normal and heat shock conditions at two different concentrations of the drug, 10 and 100 ng per ml. In control cells at 25°C, the size of the polysome peak was reduced about 20% at 10 ng/ml, but in heat-shocked cells the polysomes were depressed about 83% in this condition (Fig. 4B). The results described above imply that there are some changes in the mechanism of translational elongation in heat-shocked cells.

Discussion

When *Drosophila* tissue culture cells are heat-shocked, pre-existing mRNAs remain in the cytoplasm, but their translation is repressed relative to that of the induced heat shock protein messages. The rates of both elongation and initiation on non-heat-shock mRNAs in heat-shocked cells are 15 to 30-fold reduced relative to those of the efficiently translated heat-shock mRNAs (Ballinger and Pardue, 1983). The lowered rates of translation elongation suggested there might be some changes in translation elongation machinery in heat shock. To investigate the possibility of change in translation machinery, the effects of a series of translation elongation inhibitors on hsp synthesis was carried out by comparing the [^{35}S]-methionine incorporation into protein in normal and heat-shock conditions on autoradiograms of gels. This approach provides more information on the dramatic and qualitative changes in protein synthesis typical of heat-shock cells in *Drosophila* than total count incorporated. The four antibiotics tested here can be classified into two groups which share common action steps in translation elongation. Cycloheximide, emetine (Gupta and Siminovitch, 1977) and puromycin all inhibit the translocation step after peptide bond formation (Gale *et al.*, 1981). A change in sensitivity to cycloheximide was observed in Kc cells in heat shock. Also, sensitivity to the drug during recovery from heat shock was tested (data not shown) and resulted in the same sensitivity to cycloheximide as control cells. The same sensitivity to the drug during recovery did not correlate with the known structure change in heat-shocked cells such as dephosphorylation of ribosomal small subunit protein S6 (Olsen *et al.*, 1983) in Kc cells. However, this finding does not rule out the possibility that other changes of the ribosome structure have occurred. With emetine, a

similar result was obtained: control protein synthesis has more sensitivity to emetine (Fig. 2). It has been clearly shown that emetine inhibits elongation factor 2 (EF-2) dependent translocation in contrast to cycloheximide's inhibition of non-enzyme dependent translocation (Carrasco *et al.*, 1976). But it is unlikely that EF-2 is modified in heat shocked Kc cells compared with that in control Kc cells (personal communication with Sanders). This suggests that the increased resistance of heat-shocked Kc cells to this drug is not due to post-translational modification of EF-2, such as phosphorylation. Hallberg *et al.* (1981) have reported that *Tetrahymena thermophilia* cells become more resistant to cycloheximide and emetine when the cells are starved, and they showed that phosphorylation of a ribosomal protein also occurred. They suggested a resulting conformational change in ribosome structure affected the interaction between the ribosome and antibiotics. The different sensitivity of Kc cells to those antibiotics in the heat-shocked condition reported here could be explained by conformational changes in the ribosomes. Puromycin also inhibited protein synthesis in control and heat shocked *Drosophila* Kc cells to different degrees unlike the induction of heat shock response in *E. coli* cells or the induction of thermotolerance in Chinese hamster ovary cells. But a possible explanation for the difference is not clear for the present time because no unusal codons not found in hsp messages by a computer analysis of the gene sequences, where the puromycin might stay longer at those points to give less sensitivity.

The second group of drugs tested is represented by verrucarin A which is known as an inhibitor of the formation of the first peptide bond after initiation, probably by affecting the peptidyl transferase reaction (Carter and Cannon, 1978) rather than the translocation reaction. In contrast with the decreased sensitivity to the antibiotics discussed above, heat shocked cells have more sensitivity to verrucarin A, showing an 4 fold reduction in heat shock polysomes compared with control cells. This result could be explained by assuming that ribosomal function changes. All these results could be explained by several other changes, e.g. by association with new proteins that appear and distribute the polysomes in heat shock, as suggested by McMullin and Hallberg (1986). Alternately, low molecular weight RNA molecules which are induced by heat-shock and which inhibit the translation of normal protein *Drosophila messages* in the reticulocyte lysate (Kawata *et al.*, 1988) could bind to ribosomes and hinder the binding of drugs to the ribosomes resulting in a different sensitivity to antibiotics. Such an induction of small RNAs under stress conditions has been reported in mammalian cells (Liu *et al.*, 1995) also. Therefore, the observed change might reflect a mechanism by which the state of association of the translation machinery with other factors is altered in heat-shock, resulting in the changed sensitivity to antibiotics, which could be investigated further.

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