

The non-induction of HSP70 in heat shocked Malpighian tubules of *Drosophila* larvae is not due to constitutive presence of HSP70 or HSC70

Bhupendra N. Singh and S. C. Lakhotia

Department of Zoology, Banaras Hindu University, Varanasi 221 005, India

To understand reasons for the earlier reported non-induction of any of the common heat shock polypeptides in Malpighian tubules of *Drosophila* larvae, we examined the levels of the 70 kDa heat shock polypeptide (HSP70) and of its cognates (HSC70) in control and heat shocked larval tissues, including the Malpighian tubules, by ³⁵S-methionine labelling in conjunction with Western blotting or by immunocytochemical localization using appropriate antibodies against the heat inducible and constitutively expressed members of the HSP70 family. The HSP70 was totally undetectable in western blots of control as well as heat shocked Malpighian tubules of *Drosophila* larvae; the levels of different HSC70 in untreated Malpighian tubules were comparable to those in other larval tissues and also did not increase following heat shock. Immunocytochemical localization of HSP70 showed that none of the highly polytenized cells of Malpighian tubules showed induction of HSP70 after heat shock. The non-induction of the common heat shock proteins in Malpighian tubules of *Drosophila* larvae, therefore, is not due to au-

toeregulation by constitutively high levels of HSP70 or HSC70.

CELLS of virtually every organism respond to a sudden exposure to elevated temperature (heat shock) by transiently increased synthesis of a specific set of polypeptides, commonly referred to as heat shock proteins (HSPs) or stress proteins, which help cells survive the thermal and other damages¹⁻³. This response, first discovered in *Drosophila*^{4,5}, is a highly conserved biological phenomenon. However, contrary to the general dogma that all cells of an organism respond more or less uniformly to heat shock¹⁻³, Lakhotia and Singh⁶, using ³⁵S-methionine to label the newly synthesized proteins, found that none of the well-characterized HSPs, including the most abundant 70 kDa heat shock protein (HSP70), was induced in heat shocked Malpighian tubules of *Drosophila* larvae. However, the basal levels of HSPs in the Malpighian tubules were not examined in that study⁶. Since synthesis of HSPs is autoregulated, particularly by HSP70 levels^{2,3,7}, the lack of induction of HSPs in heat-shocked Malpighian tubules⁶ could be due to a constitutive presence of these proteins in threshold quantities so that further synthesis was inhibited. We have examined this possibility in the present study. Using ³⁵S-methionine labelling in conjunction with HSP70-specific antibodies, we reconfirm the earlier observation⁶ on non-inducibility of the common HSPs in this tissue of *Drosophila* larvae and further show that the HSP70 is totally absent, before as well as after heat shock in this tissue; the levels of the various forms of 70 kDa

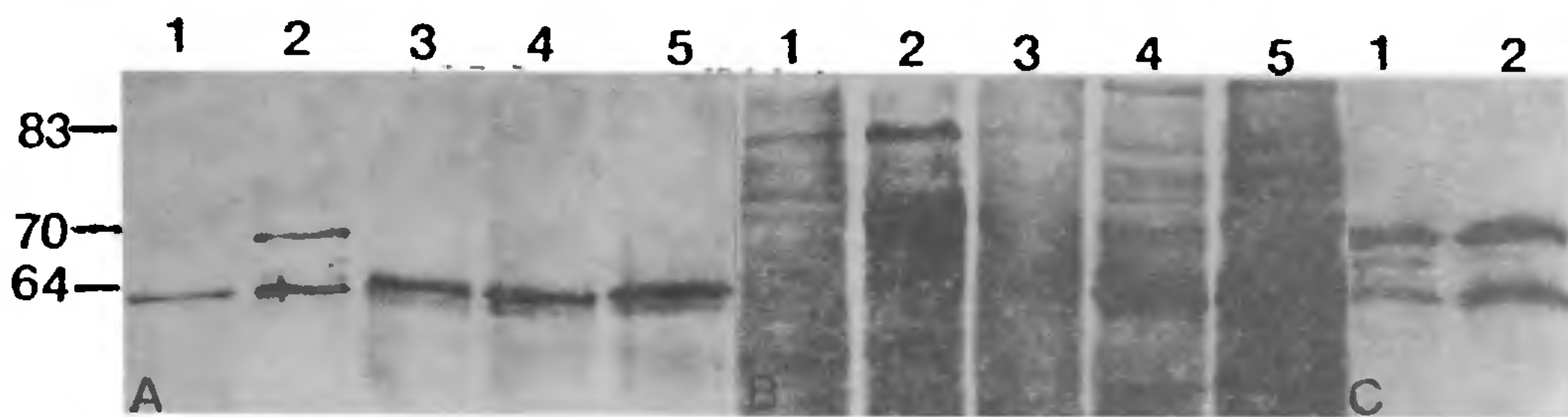


Figure 1. Showing absence of HSP70 in Malpighian tubules of *Drosophila* larvae. *a*, Western blot of control (lanes 1 and 3) and heat-shocked (lanes 2, 4 and 5) samples of salivary glands (lanes 1 and 2) and Malpighian tubules (lanes 3–5) challenged with the HSP60 and HSP70 (7Fb) antibodies; lane 5 has 1.5 times more protein than lanes 3 and 4. *b*, Fluorogram of the Western blot in (*a*). *c*, Western blot of a sample of heat-shocked salivary glands alone (lane 1) or of a mixed sample of heat shocked salivary glands and Malpighian tubules challenged with HSP60 and HSP70 antibodies. For details, see text. The molecular sizes (in kDa) of polypeptides are indicated on sides.

heat shock cognate proteins (HSC70)^{8,9} in the larval Malpighian tubules are not higher than in other tissues. Therefore, the non-induction of HSP70 and other common HSPs in Malpighian tubules following heat shock is not due to inhibition by the constitutively present members of the HSP70 family.

A wild-type strain (Oregon R⁺) of *Drosophila melanogaster* was used for these studies. The flies and larvae were reared on standard cornmeal–agar–yeast food at 22 ± 1°C.

Different organs (salivary glands, Malpighian tubules, brain ganglia) from late third-instar larvae were dissected in Poels' salt solution (PSS)¹⁰, transferred to fresh PSS in microfuge tubes and were immediately either heat shocked at 37°C for 30 min or were kept at 22°C for 30 min as control. The heat shocked and control tissues were labelled with ³⁵S-methionine (BRIT, Bombay, 200 µCi/ml; sp.act. 1000 Ci/mM) for 30 min at 37°C and 22°C, respectively. Protein samples were prepared in the Laemmli sample buffer¹¹ and electrophoresed by SDS-PAGE as described⁶. Equal numbers of organs of a given type were taken for control and heat shock samples.

The electrophoresed polypeptides were transferred to nitrocellulose membrane (Schleicher & Schuell, Germany) by semi-dry blotting following the method of Lakhota and Singh¹². Two rat monoclonal antibodies, viz. 7Fb (HSP70-specific) and 7.10 (recognizing the cognates (HSC70) as well as the heat inducible form of HSP70 of *Drosophila*)¹³, obtained from S. Lindquist (USA), were used to detect the HSP70 and HSC70, respectively, in western blots at 1 : 1000 dilution. In some cases (Figure 1), the blots were first challenged with an anti-HSP60 antibody (SPA-805, Stressgen, Canada) and then with the HSP70-specific 7Fb antibody. The primary antibodies were detected by using the corresponding HRP-conjugated secondary antibodies (Sigma, USA, or

Bangalore Genei, Bangalore) at recommended dilutions. Following the immunodetection, the blots with ³⁵S-methionine labelled samples were fluorographed by dipping in toluene with 22% PPO for 3 min and drying for 1 h at room temperature before exposing to X-ray film to detect radiolabelled newly synthesized polypeptides. The Western blots and the fluorograms were then compared.

For immunocytochemical localization of HSP70, the salivary glands and Malpighian tubules with associated parts of the gut were dissected out from late third instar larvae and incubated in PSS at 22°C (control) or at 37°C (heat shock) for 30 min. Tissues were fixed in 4% paraformaldehyde in PBT (130 mM NaCl, 7 mM Na₂PO₄ · 2H₂O, 3 mM NaH₂PO₄ · 2H₂O, 0.2% Triton-X-100, pH 7.0) and processed for immunocytochemical localization of HSP70 using the 7Fb antibody (1 : 200 dilution) and goat anti-rat secondary antibody (HRP conjugate, Sigma) at recommended dilution following the standard procedure¹⁴.

Figure 1 *a* shows a Western blot of control and heat shocked salivary glands and Malpighian tubules from late third-instar larvae challenged sequentially with HSP60-specific (SPA-805) and HSP70-specific (7Fb) antibodies; fluorogram of the same blot is shown in Figure 1 *b*. Synthesis of all the HSPs was typically induced in salivary glands by heat shock (only HSP83, HSP70 and HSP68 are seen since only the upper half of the gels was always blotted). However, in agreement with the earlier finding of Lakhota and Singh⁶, heat shock to Malpighian tubules resulted in enhanced synthesis of some other polypeptides with a major band at 64 kDa (lanes 3–5 in Figure 1 *b*), but not of any of the usual HSPs (it may be mentioned here that this 64 kDa polypeptide was erroneously estimated as 58 kDa band by Lakhota and Singh⁶). The 7Fb antibody, which exclusively recognizes the heat shock inducible form of HSP70 of *Drosophila*¹³, revealed the presence of HSP70 only in heat

shocked and not in control salivary glands. Neither the control nor the heat shocked Malpighian tubules showed any trace of HSP70 (lanes 3–5 in Figure 1). That the failure to get a signal with HSP70 antibody in Malpighian tubules was not due to the generally lower amount of proteins in Malpighian tubule samples was evident from the similar extent of labelling seen in the lanes for salivary glands and Malpighian tubules in the fluorogram in Figure 1 *b*. This was further confirmed by deliberately loading a greater amount of labelled sample from heat shocked Malpighian tubules in lane 5 (Figure 1 *a, b*); this also failed to reveal any trace of HSP70 in the Malpighian tubules. In addition, the same blot when challenged with an antibody for HSP60, which is constitutively present in all tissues of *Drosophila*^{12, 15}, showed more or less equal signal in all lanes, except in lane 5, which showed a more intense signal in proportion to the greater amount of protein loaded.

The complete absence of HSP70 in the Malpighian tubules can also not be due to a general failure of heat shock in these samples since the fluorogram clearly showed a significantly increased synthesis of the 64 kDa and other heat inducible polypeptides which are known to be heat shock induced in this tissue⁶.

The HSP70 protein of *Drosophila* can decay *in vivo* as well as *in vitro* at a much faster rate: degradation, which is mediated by a proteolytic action of HSP70 protein upon itself, may sometimes occur rapidly, even during the course of electrophoresis¹⁶. To check that the absence of HSP70 in Malpighian tubules was not due to a tissue-specific degradation of this polypeptide, the Malpighian tubules and salivary glands excised from late third instar larvae were pooled, heat shocked together, dissolved in the sample buffer and run in one lane: Western blot revealed a distinct presence of the HSP70 in this mix (lane 2 in Figure 1 *c*), similar to that in the heat shocked sample of salivary glands alone (lane 1 in Figure 1 *c*). This demonstrated that the Malpighian tubule samples did not specifically lose their HSP70 due to degradation.

The HSP70 protein family in *Drosophila* includes both the heat-inducible (HSP70) and constitutively expressed homologues, the heat shock cognates (HSC70), which exist in several isoforms and show spatial and temporal tissue specificity^{8, 17}. *Drosophila* embryos till the blastoderm stage do not synthesize HSP70 after heat shock, but are known to have an HSC70 in abundance⁸. Since it is possible that the total level of HSP70-related proteins in a cell is highly regulated¹⁸, we examined whether the Malpighian tubules of *D. melanogaster* larvae contained higher levels of HSC70, which may prevent induction of HSP70. For this purpose, we used the 7.10 antibody, which recognizes^{8, 13} both cognates and heat inducible forms of HSP70. Western blots of samples from different tissues (brain ganglia, salivary glands and Malpighian tubules of larvae), challenged with the 7.10 HSC70 monoclonal antibody, showed (Figure 2) a nearly similar

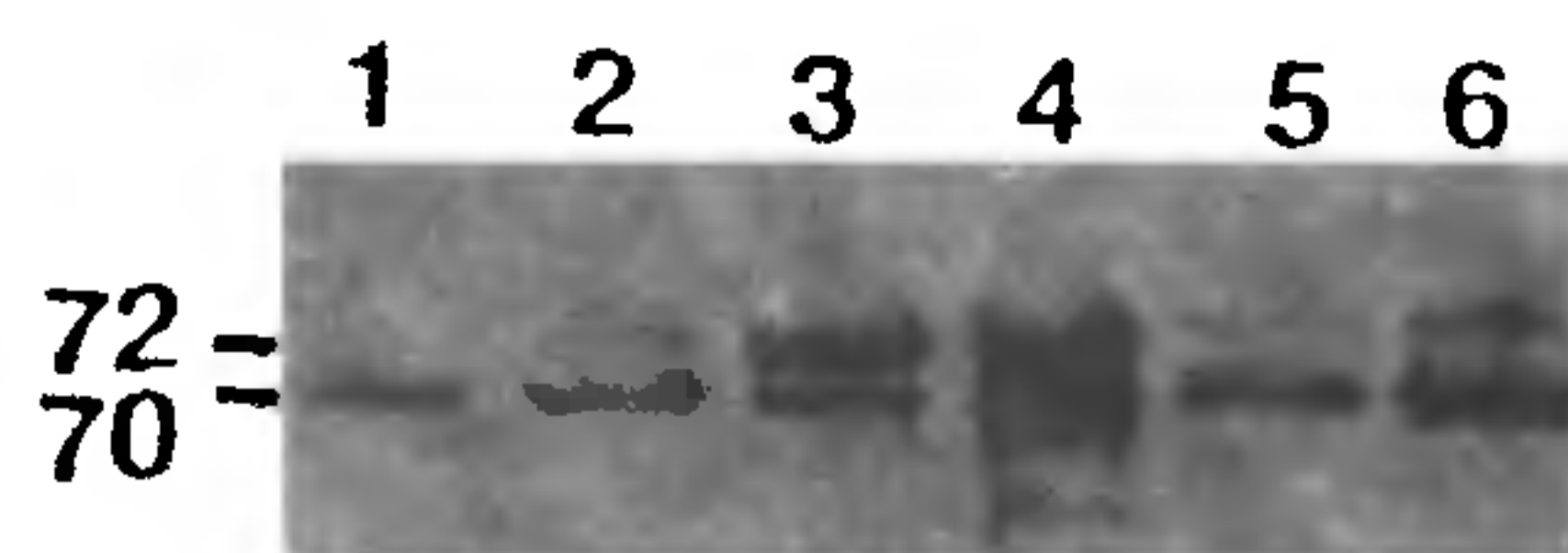


Figure 2. Levels of HSC70 in different larval tissues are similar. Western blot of control (lanes 1, 3 and 5) and heat shocked (lanes 2, 4 and 6) larval brain ganglia (lanes 1 and 2), salivary glands (lanes 3 and 4) and Malpighian tubules (lanes 5 and 6) challenged with the HSC70 antibody (7.10). The molecular sizes (in kDa) are indicated on the side.

pattern of reacting bands (68–72 kDa) in control and heat shocked samples of all the tissues. The more intense signal for salivary glands (lanes 3 and 4 in Figure 2) in this blot is due to more protein in these lanes. There was no noticeable increase in the levels of any of the HSC70 polypeptides in control or heat shocked Malpighian tubules (lanes 5 and 6 in Figure 2). This leads us to believe that the non-inducibility of HSP70 in Malpighian tubules is not due to a constitutive presence of high levels of HSC70.

Immunocytochemical staining with the HSP70-specific antibody (7Fb) confirmed the absence of HSP70 in Malpighian tubules since while the heat shocked salivary glands (Figure 3 *b*) and midgut (Figure 3 *d*) showed a distinct positive staining with this antibody, cells in control salivary glands (Figure 3 *a*), midgut (Figure 3 *c*) and in control as well as heat shocked Malpighian tubules (Figure 3 *c, d*) were negative for the HSP70 antibody. It is notable, however, that while the highly polytenized large cells of Malpighian tubules did not show any staining for HSP70, the fewer, smaller and flatter stellate cells¹⁹ showed heat shock induced presence of HSP70 (see Figure 3 *e*). Apparently, the induced HSP70 in these cells was not enough to be detectable in the Western blots of whole Malpighian tubules. The strong nuclear signal in heat shocked salivary gland cells (Figure 3 *b*) is in agreement with the earlier report²⁰ that during heat shock the HSP70 concentrates strongly in nuclei.

It may be noted that while preparing the Malpighian tubule samples for electrophoresis, we took extreme care to exclude any part of the midgut to which these organs remain attached *in situ* (see Figure 3 *c, d*); we believe that the induction of all the common HSPs in Malpighian tubules of *Drosophila* larvae as reported in an earlier publication⁵ was due to contaminating pieces of midgut, which like other tissues shows strong induction of HSP70 (Figure 3 *d*) and other HSPs.

The present results thus reconfirm the earlier report from our laboratory⁶ that the Malpighian tubules of *Drosophila* larvae fail to synthesize any of the common

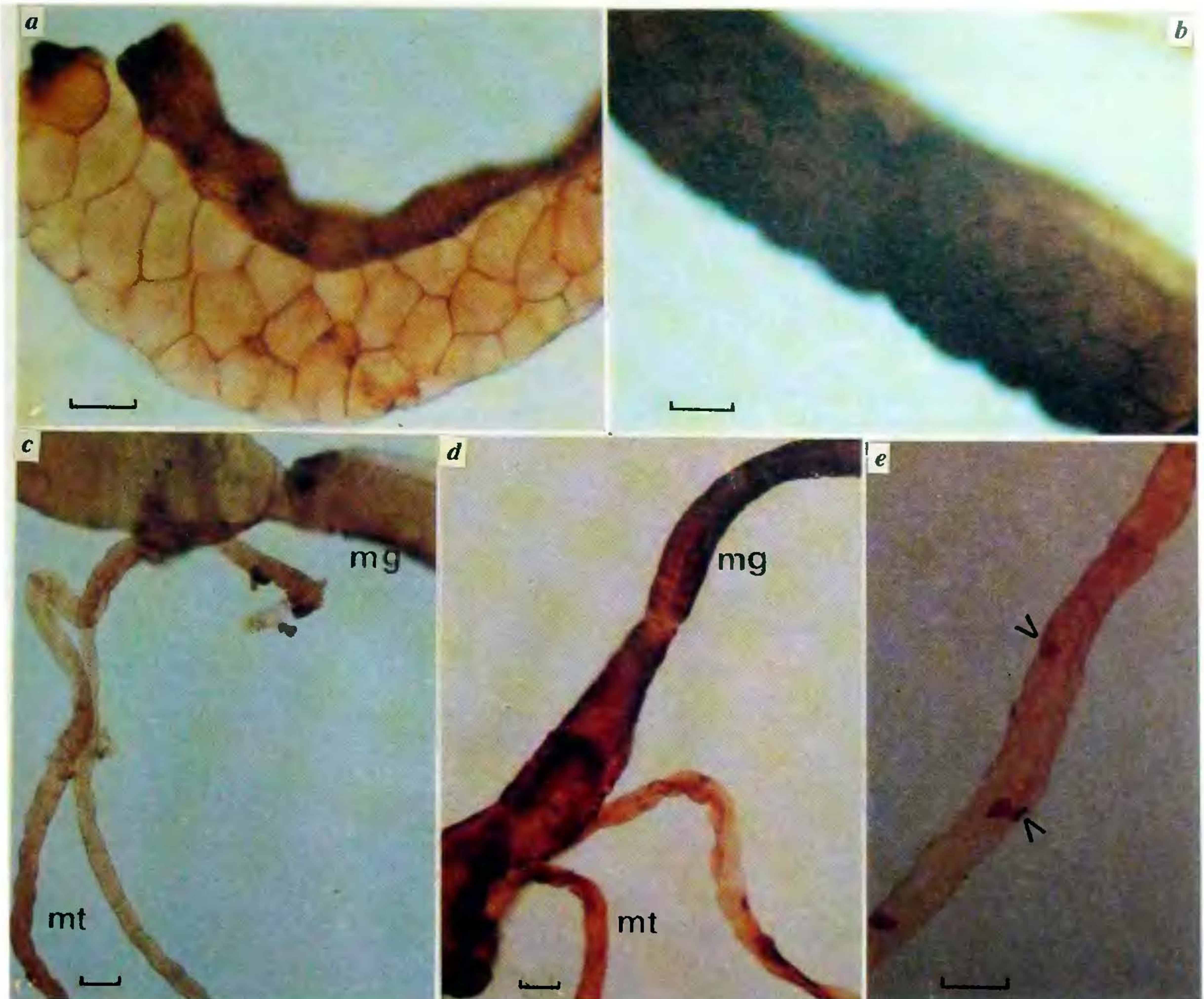


Figure 3. Immunocytochemical localization of HSP70 in control (*a* and *c*) and heat shocked (*b*, *d* and *e*) larval salivary glands (*a* and *b*), midgut (mg in *c* and *d*) and Malpighian tubules (mt in *c* and *d*). Part of a Malpighian tubule is shown at a higher magnification in (*e*) to reveal HSP70 induction in the peripherally located smaller stellate cells (arrowheads). Also note the stronger antibody binding in the nuclei of heat shocked salivary glands (*b*). The scale bars indicate 50 μm

HSPs in response to heat shock. This study further shows that the non-induction of HSP70 in this tissue is not due to autoregulation^{7, 18} by constitutively present HSP70 or HSC70. The same perhaps also applies to non-induction of the other HSPs in this tissue. The only other cell types of *Drosophila* which fail to mount the typical heat shock response are the nurse cells in late stages of oogenesis, oocyte and the pre-blastoderm embryos²¹. However, these cells, unlike the Malpighian tubules, do not show induction of a different set of polypeptides in response to heat shock, and also in these cases the non-induction of the HSPs may be correlated with a constitutively high level⁸ of HSC70. Certain types

of cells of *Sarcophaga*²² also do not show synthesis of the typical HSP70 when heat shocked; however, in these cases a different form of HSP70, the HSP65, is induced instead. Unlike in *Sarcophaga*, the 64 kDa HSP induced in Malpighian tubules of *Drosophila* larvae is not a homologue of HSP70 but is a member of the HSP60 family^{12, 15}.

Several cell types in mammals also differ in inducibility of the HSP70 when heat shocked²³; however, in most of these cases, unlike the situation in Malpighian tubules of *Drosophila* larvae, only the HSP70 is perhaps affected. Non-induction of the HSP70 in these mammalian cell types has been ascribed to transcriptional regulation by factors other than the heat shock transcription

factor²³. Thus, compared to the above cases, the heat shock response of Malpighian tubules of *Drosophila* larvae is unique. The regulatory pathways responsible for non-induction of all the common HSPs in Malpighian tubules of *Drosophila* larvae may involve the heat shock transcription factor²⁴ and/or other auxiliary transcription factors²⁵ necessary for transcriptional activation of the heat shock genes under conditions of stress. Further studies will help understand the mechanism of this regulation as also the biological significance of this unique situation of non-inducibility of all the common HSPs and induction of a different set in the larger polytenized cells of this particular tissue.

- 1 Schlesinger, M J., Ashburner, M and Tissieres, A. (eds), *Heat Shock Proteins From Bacteria to Man*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
- 2 Lindquist, S, *Annu Rev. Biochem*, 1986, **55**, 1151-1191.
- 3 Morimoto, R., Tissieres, A. and Georgopoulos, C. (eds), *Stress Proteins in Biology and Medicine*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990.
- 4 Ritossa, F. M., *Experientia*, 1962, **18**, 571-573
- 5 Tissieres, A., Mitchell, H. K. and Tracy, U., *J Mol. Biol*, 1974, **84**, 389-398
- 6 Lakhota, S C. and Singh, A K., *J Genet*, 1989, **68**, 129-137
- 7 DiDomenico, B., Bugaisky, G E. and Lindquist, S., *Cell*, 1982, **31**, 593-603.
- 8 Palter, B. K., Watanabe, M., Stinson, L., Mahowald, A. P. and Craig, E A., *Mol Cell Biol.*, 1986, **6**, 1187-1203.
- 9 Lindquist, S. and Craig, E. A., *Annu Rev. Genet.*, 1988, **163**, 631-677
- 10 Lakhota, S. C. and Mukherjee, T., *Chromosoma*, 1980, **81**, 125-136.
- 11 Laemmli, U. K., *Nature*, 1970, **227**, 680-685
12. Lakhota, S. C. and Singh, B N., *Indian J Exp Biol*, 1993, **31**, 301-302
- 13 Kurtz, S., Rossi, J., Petko, L. and Lindquist, S., *Science*, 1986, **231**, 1154-1157
- 14 Patel, N H., *Methods Cell Biol*, 1994, **44**, 445-488
- 15 Singh, B N. and Lakhota, S C. (in preparation)
- 16 Mitchell, H K., Petersen, N S. and Buzin, C H., *Proc Natl Acad Sci*, 1985, **82**, 4969-4973
- 17 Rubin, D M., Mehta, A D., Zhu, J., Shoham, S., Chen, X., Wells, Q R. and Palter, K. B., *Gene*, 1993, **128**, 155-163
- 18 Feder, J H., Rossi, J. M., Solomon, J., Solomon, N. and Lindquist, S., *Genes Develop*, 1992, **6**, 1402-1413
- 19 Berridge, M J. and Oschman, J L., *Tissue Cell*, 1969, **1**, 247-272
20. Velazquez, J M. and Lindquist, S., *Cell*, 1984, **36**, 655-662
- 21 Zimmermann, J L., Petri, W. and Meselson, M., *Cell*, 1983, **32**, 1161-1170
- 22 Joplin, K H. and Denlinger, D L., *J Insect Physiol*, 1990, **36**, 239-249
- 23 Morimoto, R I., Jurivich, D A., Kroeger, P. E., Mathur, S. K., Murphy, S P., Nakai, A., Sarge, K., Abravaya, K. and Sistonem, L. T., in *The Biology of Heat Shock Proteins and Molecular Chaperons* (eds Morimoto, R I., Tissieres, A. and Georgopoulos, C.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 417-455
24. Parker, C S. and Topol, J., *Cell*, 1984, **37**, 273-283
25. Lu, Q., Wallrath, L. L., Allan, B D., Glaser, R L., Lis, J T. and Elgin, S C R., *J Mol Biol*, 1992, **222**, 985-998

ACKNOWLEDGEMENTS This work was supported by a research grant from the Council of Scientific & Industrial Research, New Delhi, to SCL and by a research fellowship from Banaras Hindu University to BNS. We gratefully acknowledge the kind gift of 710 and 7Fb monoclonal antibodies by Prof S Lindquist

Received 21 April 1995, accepted 23 June 1995