Morphological and molecular modifications induced by heat shock in *Drosophila melanogaster* embryos

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**SUMMARY**

The early embryo of *Drosophila melanogaster* did not survive treatment at 37 °C (heat shock) for 25 min. The histological analysis of eggs treated in this way showed that the heat shock caused disintegration of nuclei and of cytoplasmic islands, displacement and swelling of nuclei and blocked mitoses. These effects were not observed in embryos treated after blastoderm formation. After this stage, we noticed that development was slowed down. The heat shock proteins (hsp 83, 70 and 68) were, under shock, synthesized at all developmental stages. There was little or no synthesis of hsp 70 and 68 in unfertilized eggs, but synthesis increased in proportion to the number of nuclei present. Most probably, hsp 70 synthesis was directed by zygotic mRNA. DNA synthesis was not blocked by the heat shock though the overall incorporation of \(^{3}P\)thymidine was substantially reduced, presumably because of the block of mitoses. We did not find a direct relation between survival pattern and hsp synthesis. We concluded that some, at least, of the heat shock genes can be activated at all developmental stages and that heat shock could be used for synchronizing mitoses.

**INTRODUCTION**

The usual temperature for raising *Drosophila melanogaster* is around 24 °C though this organism can also live at temperatures ranging from 15 to 34 °C (Hedman & Krogstad, 1963; Powsner, 1935). This fly can also survive relatively short exposure to more extreme temperatures such as 4 °C or 40 °C. During the exposure to high temperature, usually around 37 °C, *Drosophila* cells undergo considerable molecular and physiological changes (Ashburner & Bonner, 1979, review). Transcription and translation of the genes which were active before the heat shock are halted (Bonner & Pardue, 1976; Lindquist, 1980, 1981; McKenzie, Henikoff & Meselson, 1975; Spradling, Penman & Pardue, 1975), while a newly synthesized set of RNAs is translated into at least seven different proteins (Mirault *et al.* 1978; Tissières, Mitchell & Tracy, 1974; Henikoff & Meselson, 1977; Spradling, Pardue & Penman, 1977).

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Such a response has been described for tissue culture cells and for various organs at various developmental stages of *Drosophila* (Chomyn, Moller & Mitchell, 1979; McKenzie *et al.* 1975; Lewis, Helmsing & Ashburner, 1975; Peterson, Moller & Mitchell, 1979) and it has been found in all the eucariotic organisms so far tested (Barnett, Altschuler, McDaniel & Mascarenhas, 1980; Fink & Zuethen, 1970; Giudice, Roccheri & Di Bernardo, 1980; Kelley, Aliperti & Schlesinger, 1980; McAlister & Finkelstein, 1980). Early embryos are the only known possible exception to this response: the *Drosophila* embryo before blastoderm formation (Dura, 1981; Graziosi *et al.* 1980), and the sea urchin embryo before gastrulation (Giudice *et al.* 1980) synthesize little or no heat shock proteins. The fly embryo response to heat shock also differs from other *Drosophila* systems in other respects. Hsp 83 has been found in large amounts as a cold protein in eggs kept at normal temperature, although its synthesis can be elicited by a heat shock of 30 min (Graziosi *et al.* 1980; Savoini *et al.* 1981). Furthermore, early embryos are extremely sensitive to the exposure to high temperatures: 15 min of treatment cause a very high mortality rate as well as phenocopies (Mitchell & Lipps, 1978; Santamaria, 1979).

We undertook this study to investigate this high mortality rate in early embryos and, by quantitating the $^{35}$S methionine-labelled hsps, to correlate the survival pattern with hsp synthesis.

**MATERIALS AND METHODS**

**Egg harvesting and fly maintenance**

Eggs were collected from a population cage of roughly 5000 flies. The laying population was kept under a fixed regime of food supply and of light/dark cycle to ensure the deposition of a large amount of eggs between 10 am and 4 pm. The light was switched on at 5 am and switched off at 5 pm. Fresh baker's yeast deposited on Petri dishes of standard *Drosophila* food was offered every hour; starting at 9 am until 5 pm. The feeding dishes were also used for harvesting eggs, but they were left in the cage for 10 min only. An average age of 5 min was assumed when the eggs were withdrawn from the cage. The mass collections were used for the survival curve and they contained 5–10% of eggs in advanced developmental stages. The eggs used for two-dimensional electrophoresis and for the histological analysis were laid over a period of 5 min and were individually checked for age.

A small cage containing a few hundred flies was prepared to collect eggs from the cross of heterozygote flies *Df (3R) 229, Df (3R) kar*3J (Ish-Horowicz *et al.* 1979).

**Egg labelling and heat shock**

Upon removal of the chorion membrane with 20% sodium hypochlorite and several washes in *Drosophila* saline, the eggs were permeabilized according to
the method of Limbour & Zalokar (1973). Then the eggs were covered with 30 μl of incubation medium and transferred either to 24 °C (controls), or to 37 °C. [35S]methionine or [3H]thymidine (final concentrations of 1 mCi/ml and 0.5 mCi/ml respectively) were added 10 min after the transfer of the eggs to the desired temperature. Similarly the controls had a preincubation of 10 min at 24 °C before addition of the label. At the end of the incubation, the eggs were washed in cold medium and either fixed for the histological analysis or sonicated for protein extraction.

Electrophoresis and fluorography

Protein extracts underwent two-dimensional electrophoresis as reported by O’Farrell (1975). After sonication, the samples were freeze-dried, resuspended in 50 μl of lysis buffer, centrifuged for 30 min at 50 000 g and 3 × 1 μl aliquots of the supernatant were used to count the total radioactivity in the TCA precipitable material. All first dimensions were loaded with 100 000 c.p.m. All the other electrophoretic parameters were the same as reported by Savoini et al. (1981).

Since hsps 70 and 68 did not always appear as stainable spots, their foci were identified matching the stained gels with autoradiographs run for other purposes (Graziosi et al. 1980; Savoini et al. 1981). Hsp 83 and β-tubulin were always recognized as consistent stainable spots. The protein spots were cut out, placed in 1 ml of 30% H2O2 and evaporated at 60 °C until dry, resuspended in scintillation liquid and counted.

Single eggs were analysed on 9% polyacrylamide slab gels using the discontinuous sodium dodecyl sulphate (SDS) method of Laemmli (1970). These gels underwent fluorography as reported by Bonner & Laskey (1974) using Kodak film RP X-Omat.

Histology and autoradiography

Both chorion and vitelline membranes were removed from the eggs for histological analysis. After fixation using the method of Zalokar & Erk (1977), the eggs were embedded in paraffin and sectioned at 8 μm. The sections were stained with haematoxilin and eosin for 10 and 2 min respectively and permanently mounted in Canada balsam.

The autoradiographs were prepared by dipping the slides in the photographic emulsion Ilford K5 diluted 1:2 with water at 37 °C. The excess of emulsion was drained by leaving the slides vertical until completely dry. All slides were exposed for 7 days and developed by standard methods. The histological sections used for the autoradiography were stained after exposure and development.

RESULTS

Embryonic viability

A total of 5452 eggs, subdivided into 43 experimental groups, was heat
shocked for 25 min. Each egg collection was subdivided into four to eight batches of eggs and each batch was aged at 24 °C until the embryos had reached the desired developmental stage. Fig. 1 shows the percentage survival of heat-shocked embryos of different ages and the equation describing this survival. A third degree polynomial was the best fit which is statistically significant (F = 8.20; D.F. = 39). We calculated the second derivative to find the point of inflection which was around 130 min. Before this age the nuclei are engaged in a fast mitotic cycle and the viability is very low. Soon after 130 min, when mitoses stop and the embryos become cellularized, the survival rate was not dissimilar from that of the controls.

A few hundred embryos were dechorionated and inspected under a dissecting microscope 2–3 h after the treatment. The eggs treated before blastoderm formation showed no sign of development while the eggs treated at later stages showed a delayed development but normal morphology.

Heat-shock proteins

As reported previously (Graziosi et al. 1980) different amounts of hsp's were found at different developmental stages. To quantitate hsp's we ran two-dimensional gel electrophoresis of $^{35}$S methionine labelled extracts of embryos heat shocked for 30 min at seven different ages: from unfertilized eggs up to the beginning of gastrulation (3 h). After localization of the proteins on stained gels,
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the spots corresponding to hsp 83, 70 and 68 and β-tubulin were cut out and counted. Hsps 70 and 68 were counted together because they were not well separated in this gel system.

The counts of hsp 83 increased from 150 c.p.m. (out of 100,000 c.p.m. total counts) in unfertilized eggs to about 350 at gastrulation. Hsps 70 and 68 were virtually absent in very young eggs but, together, they showed 3000 c.p.m. at gastrulation (3% of the total counts). Because of the consistent trend of the curves, we considered the use of statistics unnecessary. The amount of synthesis of hsps and the number of nuclei at each stage are shown in Fig. 2.

In order to investigate whether hsp 70 was translated from maternal messages, we used the mutant Df (3R) 229, Df (3R) kar 31 deficient for the loci coding for this protein. We ran on SDS gels extracts of single eggs obtained from heterozygote flies 90 min and 3 h after deposition. Hsp 83 was always present, hsp 68 was found in all late embryos. Hsp 70 was recognized only in 27 late embryos, the remaining 8 hsp-deficient eggs were considered to be the hsp 70 null homozygotes (Fig. 3). This proportion is not dissimilar from the expected Mendelian ratio.

Fig. 2. Counts of [35S]methionine-labelled spots of hsp 83, hsp 70 + 68 and β-tubulin cut out from two-dimensional gel electrophoresis of embryos heat shocked at various developmental stages. We took β-tubulin as a possible reference protein because its synthesis was not suppressed by the heat shock. All first dimensions were loaded with 100,000 c.p.m. The embryonic ages refer to the mid-incorporation time. The theoretical number of nuclei was taken from Zalokar & Erk (1977) tables.
Fig. 3. Fluorography of SDS gel electrophoresis of 3 h-old eggs (lanes A to H) obtained from the cross between heterozygote flies deficient for the hsp 70 genes. The eggs were incubated 30 min at 37°C in medium containing 4 mCi/ml [35S]-methionine. We considered hsp 70 as missing in lanes B and F, we think that the faint band at the level of hsp 70 is due to a protein of similar molecular weight but of different isoelectric point as observed in two-dimensional gel electrophoresis. In lane I a 90-min-old egg was run. Exposure two months.

Morphological modifications of heat-shock embryos

We prepared histological sections of control and heat-shocked eggs to identify the possible cause of the early embryo mortality. Three experimental sets were considered: freshly laid eggs (subjected to heat shock 25 min after deposition, experiments A and B); advanced nuclear segmentation (50 min after deposition, experiments C and D); and beginning of gastrulation (210 min after deposition, experiments E and F). Each experimental set comprised three groups of about ten eggs; the first one underwent 30 min of heat shock (experiments A, C, E), the second one was heated for 60 min (experiments B, D, F) and the third one,
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DIAGRAM OF EXPERIMENTS

The control, was kept at 24°C until the end of the 60 min of heat shock and then fixed. These experimental regimes are summarized in Fig. 4.

Nuclear segmentation stages

After sectioning, the three-dimensional structure of the eggs treated during the nuclear segmentation stages was reconstructed to establish the exact number of nuclei in each egg. The control embryos fixed at 85 min were at stages 8 to 9
(Fig. 5A, Zalokar & Erk, 1977, staging) and showed cytoplasmic islands, interkinetic nuclei or mitotic figures.

In many of the heat-shocked eggs we could not recognize morphological structures resembling normal nuclei, while in the others we counted a limited number of nuclei (Table 1, Fig. 5B). We could not detect mitotic figures in the 40 heated eggs. From Table 1 it is also apparent that the longer the heat shock, the fewer the nuclei. In fact the average number of nuclei per egg in the experiments A and B was 1.2 and 0.7 respectively, while in the experiments C and D it was 9.9 and 5.7 respectively.
Table 1. Developmental stages reached by eggs after the heat shock

<table>
<thead>
<tr>
<th>Egg No</th>
<th>A</th>
<th>B</th>
<th>C</th>
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Controls at 85 minutes (experiments A and B): 128/256 nuclei
at 110 minutes (experiments C and D): 256/512 nuclei
at 270 minutes (experiments E and F): Stage 16.

The diameter of the control egg nuclei was 6–8 μm (Fig. 6A) while all the nuclei found in the treated eggs were considerably larger: 12–22 μm (Fig. 6B). These large nuclei were also located abnormally on the surface of the eggs. In one egg, treated for 1 h, we found a swollen nucleus showing damage of the nuclear membrane (Fig. 7A). In many cases we observed fibrillar structures which we suppose were disrupted nuclei (Fig. 7B) while similar structures were never observed in control eggs. We also noticed the absence or considerable reduction in the number of the cytoplasmic islands.

Gastrulæ

In gastrulating embryos the heat shock did not produce the dramatic changes reported above. The eggs were able to continue development while under shock. The two experimental groups E and F underwent heat shock at the same time, but the group of eggs treated for a longer period reached a more advanced stage of development. Nevertheless, this experimental group was less advanced than the control eggs (Table 1) even if they were fixed at the same time. Thus, in late embryos, the heat shock slowed development without blocking it.

During gastrulation we did not observe mitoses in either controls or heat-shocked eggs but, because of the stage-dependent loss of mitotic synchrony and the large number of cells, chromosomes could have escaped observation.

Thymidine incorporation

A preliminary pulse-chase experiment (Fig. 8) showed that, after 10 min of
heat shock, the total incorporation of thymidine was substantially repressed. Thus, in the following experiments, $[^3]$H]thymidine was added 10 min after the beginning of the heat shock to ensure that the label was incorporated under shock conditions.

**Segmentation stages**

In control eggs (experiment G) we found no unlabelled nuclei. The observed number of nuclei is indicated in Table 2. In many eggs there were some elongated radioactive traces indicating active mitosis. Judging from the number of nuclei, the control eggs (Fig. 9A) underwent two to four mitoses during the incubation period. The difference between the expected and the observed number of nuclei was due not only to the loss of some sections when coating the slides with the photographic emulsion, but also to a possible inhibition of mitosis by the incubation medium. Permeabilization itself does not interfere with normal development (Roberts & Graziosi, 1977).

In the absence of nuclear disintegration, the heat-shocked eggs should show a number of nuclei equal to, or higher than, that of controls. To test this, $[^3]$H]-thymidine was added to the heat-shock egg incubation medium at the time of fixation of the controls (see Fig. 4, experiments G and H). Labelled nuclei in the
Table 2. Number of nuclei labelled by $[^3]$H]thymidine

<table>
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<tr>
<th>Egg No</th>
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<tr>
<td></td>
<td>G</td>
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<td>15</td>
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<tr>
<td>Total</td>
<td>1510</td>
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<tr>
<td>Average</td>
<td>137.3/egg</td>
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</table>

Fig. 9. Autoradiographs of sections of eggs incubated in $[^3]$H]thymidine. (A) Young egg incubated at 24°C, experiment G. (B) Heat-shocked young egg, experiment H. Arrows point at radioactive nuclei. (C) 270-min-old control egg, experiment I. (D) 270-min-old heat-shocked egg. Arrows point at radioactive vitellophages, experiment L. Bar = 100 μm.
heat-shocked eggs (experiment H) were fewer than those counted in controls (Table 2). In heat-shocked eggs we never observed elongated nuclear radioactive traces. We found again large nuclei of 20 µm of diameter which were radioactive, and generally located at the surface of the egg (Fig. 9B). Only a few unlabelled nuclei were found.

**Gastrulae**

In the fifteen control eggs examined (experiment I), a large number of nuclei were radioactive. The majority of the radioactive nuclei was clustered in specific areas where the cells were invaginating: stomodeum, posterior midgut, germinal band and cephalic furrow (Fig. 9C). Few or no vitellophages were labelled.

Out of the 20 heat-shocked embryos (experiment L) only a few showed radioactive nuclei at the cephalic furrow and at the posterior midgut invagination. The majority of eggs had radioactive vitellophages (Fig. 9D).

**DISCUSSION**

In quantitating the amount of [35S]methionine incorporated into the hsps we did not intend to establish the absolute rate of synthesis of these proteins. Rather, our experimental procedure accounts only for the proportion of the label which ended up in the heat-shock proteins. The synthesis of hsp 70 and 68 rose steadily during development and did not show, on a logarithmic scale, any sharp increase at blastoderm formation. Consequently it appears that these proteins can be synthesized before blastoderm formation.

It has been reported that the bulk of the zygotic genome is activated at blastoderm formation (Anderson & Lengyel, 1979; Lamb & Laird, 1976; McKnight & Miller, 1976; Zalokar, 1976). If this is also true for the heat-shock genes then hsp synthesis during early stages should be supported by maternal mRNAs. At least as far as hsp 70 and, possibly, hsp 68 are concerned, we do not favour this hypothesis because: i) there was little or no synthesis in unfertilized or freshly laid eggs, ii) their synthesis rose during the exponential phase of nuclear multiplication and not at blastoderm formation and, iii) we did find mutant eggs (229 hat33) unable to synthesize hsp 70.

If hsps 70 and 68 are indeed translated from zygotic messages, their low synthesis or even absence in early stages is not surprising. If we compare the number of nuclei in the zygote with that of the egg at blastoderm formation we find a ratio 1:6000. Hence, assuming equal transcriptional and translational rates at all stages, the zygote is expected to produce far less hsps than the following stages. Furthermore many nuclei disintegrated and, presumably, disintegrated nuclei cannot be transcribed normally. This interpretation cannot be applied to hsp 83 whose synthesis was relatively high even in unfertilized eggs. Most probably its synthesis is sustained by maternal mRNAs, at least during the early stages.
The morphology of the heat-shocked eggs

In many eggs, heat shocked during the nuclear multiplication stages, we found no nuclei or just a few. It is obvious that eggs without nuclei or with damaged nuclei cannot undergo normal development and, on the basis of the mortality curve, we think that such damage was caused only in eggs which did not reach the stage of blastoderm formation. Our observations are very similar to those reported by Zalokar & Erk (1976), who obtained swollen nuclei upon treatment of eggs with dinitrophenol or anoxia. It is now known that both anoxia and dinitrophenol induce a ‘heat-shock-like’ response (Ashburner, 1970; Ellgaard, 1972; Rensing, 1973).

Although our results can explain why the embryos did not survive the heat shock, they cannot explain the degeneration of nuclei. The unique morphological and physiological characteristics of the early embryos, as the absence of plasmalemma and the fast mitotic cycles (Sonnenblick, 1950; Zalokar & Erk, 1976), could account for the nuclear sensitivity to heat. But such characteristics cannot fully explain why some nuclei survive the exposure to the high temperature and the subsequent reduction of nuclei number as reported in Table 1. The unequal sensitivity of nuclei could be more convincingly explained by the unequal distribution of some protective agents either the hsp5 or any other component of the egg. We know that the heat-shock proteins migrate into the nuclei (Arrigo, Fakan & Tissieres, 1980; Valazquez, Di Domenico & Lindquist, 1980) and it is possible that their concentration in the egg was too low to exert a possible protective action on all nuclei. Furthermore, the early embryo is not compartmentalized by plasmalemmas and substances could diffuse in nucleus-free areas.

Besides the disintegration of nuclei we found that the heat shock interfered with the mitotic cycle of early embryos. Frequently in control eggs, we observed mitotic figures which were never seen in sections of heat-shocked eggs. Thus the heated nuclei were unable to enter mitoses. Furthermore, taking into account the synchrony of mitoses and that almost all nuclei of all the labelled eggs were radioactive, it is apparent that the nuclei reached the S phase. Consequently the block should occur between S and M, presumably in G2. Alternatively it is possible that the nuclei did not reach M because they died just after the S phase. In any case DNA synthesis itself was not inhibited and the low incorporation of thymidine during the heat shock was due to a low number of nuclei entering the S phase.

As far as gastrulating heat-shocked embryos are concerned, we found only a delay in development. Similar results were obtained in embryo by Dura (1981) and in pupae by Lindsley & Poodry (1977). Delayed development could be caused by a variety of reasons including the block of mitoses.

Finally, we must take into account the possibility that nuclear disintegration as well as a slower development could be due to the perturbation of the normal
programme of embryonic gene expression and of coordinate maternal message translation, as reported for tissue culture cells (McKenzie et al. 1975; Moran et al. 1978; Tissières, Mitchell & Tracy, 1974). This hypothesis could be tested by an appropriate experimental design which could also show a way to use hyperthermia to discriminate between the contribution of the embryonic genome and that of the maternal genome to development. Further investigations could also indicate if the heat shock may be used as a physiological method for synchronizing mitoses, which might have practical applications in a variety of biological systems.

We wish to thank Dr D. Ish-Horowicz for the kind gift of the mutant stock 229 kar^y, and Dr D. B. Roberts and C. R. Bebbington for critical reading and improvements to the manuscript. This research has been supported by the CNR grant No. 81 00488, 85-115, 2143 and by the MPI grant 1981–82.

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(Accepted 27 April 1983)