

Multiple Inducers of the *Drosophila* Heat Shock Locus 93D (*hsr ω*): Inducer-specific Patterns of the Three Transcripts

William G. Bendena, James C. Garbe, Karen Lahey Traverse, Subhash C. Lakhotia, and Mary Lou Pardue

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. The *Drosophila hsr ω* locus produces one of the largest and most active heat shock puffs, yet it does not encode a heat shock protein. Instead, this locus produces a distinctive set of three transcripts, all from the same start site. The largest transcript, ω 1, is limited to the nucleus and appears to have a role there. A second nuclear transcript, ω 2, is produced by alternative termination and contains the sequence found in the 5' 20–25% of ω 1 (depending on the *Drosophila* species). The cytoplasmic transcript, ω 3, is produced by removal of a 700-bp intron from ω 2. All three *hsr ω* RNAs are produced constitutively and production is enhanced by heat shock. In addition to being a member of the set of heat shock puffs, the *hsr ω* puff is induced by agents that do not affect other heat shock loci, suggesting that *hsr ω* is more sensitive to environ-

mental changes than other loci.

We report here that agents that induce puffing of *hsr ω* loci in polytene nuclei also lead to an increase in *hsr ω* transcripts in diploid cells. We also show that the relative levels of ω 1 and ω 3 can be modulated independently by several agents. All drugs that inhibit translation, either initiation or elongation, stabilize the ω 3 transcript, which normally turns over within minutes in control cells. Drugs (such as benzamide and colchicine) that induce puffing of *hsr ω* , but not other heat shock loci, lead to large increases in ω 1. Although the constitutive level of ω 1 is relatively stable, the drug-induced excess is lost rapidly when the drug is withdrawn. The relative levels of *hsr ω* transcripts may reflect different states in cellular metabolism.

STUDIES of the heat shock response have shown that cells from animals, plants, and bacteria are poised to respond rapidly to increases in temperature and to a number of other environmental stresses (reviewed by Craig, 1985; Lindquist, 1986). The response to such stresses (the "heat shock response") involves the synthesis of a set of at least three families of polypeptides, which are termed the heat shock proteins (hsp).¹ If the stress is sufficiently severe, the response also includes other changes in cell structure and metabolism; the intermediate filament network collapses around the nucleus while transcription and translation of mRNAs for the normal set of polypeptides is suspended. There is evidence that the heat shock response can protect the cell during periods of transient stress; mild heat shocks enable the cell to withstand a subsequent exposure to temperatures above the temperature which would ordinarily be lethal. Although the hsp would be expected to be involved in this thermoprotection, it is not known what the exact role of

any hsp is in heat shock. However, each of the major families of hsp can also be found in nonstressed cells (at least in some cell types), encoded by transcripts of either the gene used in heat shock or by a closely related gene. The roles of these proteins in heat shock may not be fundamentally different from their roles in nonstressed cells.

The mRNAs for hsp have received a great deal of attention but much less is known about other types of heat shock-induced transcripts. In *Drosophila*, where polytene puffs give a unique view of gene transcription, it can be seen that there is one very large puff that does not encode any of the known hsp (Garbe and Pardue, 1986). Although this puff is clearly a member of the heat shock puff set, it can also be induced independently of the other heat shock puffs by several agents. All *Drosophila* species studied have one member of the heat shock puff set that is induced by these other agents. In *Drosophila melanogaster* this puff is in polytene region 93D, but in different species it has different locations (Lakhotia and Mukherjee, 1980; Lakhotia and Singh, 1982; Burma and Lakhotia, 1984; Lakhotia, 1987). The locus has been named the *hsr ω* locus (because it was originally detected as a gene producing heat shock RNA ω , although we now know that it is active in almost all cells). The *hsr ω* locus has been cloned from *D. melanogaster* (Walldorf et al.,

William G. Bendena's present address is Department of Biology, Queen's University, Kingston, Ontario, Canada. Subhash C. Lakhotia's present address is Department of Zoology, Banaras Hindu University, Varanasi, India.

1. Abbreviations used in this paper: DPBS, *Drosophila* phosphate-buffered saline; hsp, heat shock proteins; nt, nucleotides.

1984; Garbe and Pardue, 1986), *D. hydei* (Peters et al., 1984; Garbe et al., 1986), and *D. pseudoobscura* (Garbe et al., 1989). In each case the sequence analysis confirms the homology suggested by the phenotype of the puff.

The *hsr ω* locus is expressed in nonstressed cells but the level of the transcripts is significantly increased by heat shock. In both stressed and control cells, three transcripts (Fig. 1) are detected, all with the same transcription start site (Ryseck et al., 1987; Garbe, J. C., unpublished observations). The exact size of each transcript varies slightly among the *Drosophila* species, but the general pattern of transcripts is highly conserved. A 10–20-kb transcript, which spans the transcription unit, is composed of \sim 3 kb of unique region followed by 7–17 kb of short tandem repeats. This transcript, ω 1, is limited to the nucleus. A second nuclear transcript, ω 2, contains only the first 2–3 kb of the unique region. A cytoplasmic transcript, ω 3, has the sequence of ω 2 minus an intron of \sim 0.7 kb (Garbe et al., 1986; Pardue et al., 1987).

Cytological studies have shown that several agents, including benzamide, colchicine, and colcemid can induce puffing at 93D (and the equivalent loci in other species) without inducing other heat shock puffs (Lakhotia and Singh, 1982). Generally, a polytene puff extends over several chromosomal bands and must contain enough DNA for a number of transcription units. Thus, agents that produce a puff at this locus might be inducing transcription of sequences other than the *hsr ω* sequences that are transcribed when the cell is heat shocked. If, however, the agents are actually inducing the *hsr ω* sequences, the puffing studies suggest that the *hsr ω* sequences are sensitive to more environmental agents than are the other heat shock genes. In addition, treatments inducing a puff at 93D can block subsequent induction by a second agent, if the two inducers are applied in a relatively short time (Lakhotia, 1987). If all inducers are affecting the same transcription unit, the evidence that the locus is refractory to a second closely spaced induction suggests that the locus is autoregulated.

To determine whether the *hsr ω* heat shock locus can in fact be induced by the chemicals that induce puffing of 93D in salivary gland cells, we tested the effect of the agents on cultured cell lines (nonpolyploid) of *D. melanogaster*. We find that all of the agents that induce puffing of 93D in polytene cells also lead to increased accumulation of RNA from the heat shock origin of transcription of *hsr ω* in cultured cells. Surprisingly, the relative levels of the three *hsr ω* transcripts vary in ways that may reflect the physiological state of the cell. The relative levels change with time during a heat shock. In addition the nonheat shock puff inducers cause higher levels of the ω 1 transcript. In contrast, all tested inhibitors of protein synthesis lead to very high levels of the ω 3 transcript, although none of these inhibitors induce polytene puffing at 93D.

Materials and Methods

Cell Lines

Two cultured cell lines, both derived from *D. melanogaster* embryos, have been used in these experiments. Schneider lines 2 and 3 were made by Schneider (1972). Schneider line 2-L was later adapted to grow in suspension in DME (Lengyel et al., 1975). Schneider line 3 is grown on plates in Schneider's medium (Schneider, 1972). In spite of differences in growth conditions, when we have tested the two lines in the same experiment, they have given equivalent results, giving no evidence that the results are an idiosyncrasy of that particular cell line.

Cell Growth

Schneider line 3 cells were grown in plates at 25°C in Schneider's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 μ g/ml streptomycin (complete media). Cells were routinely maintained at 6×10^6 cells/ml. Schneider line 2-L cells were grown in spinner culture at 25°C in DME (Gibco Laboratories) supplemented with 0.5% lactalbumin hydrolysate, MEM nonessential amino acids, serum, and antibiotics, as above. Benzamide (BDH Chemicals, Ltd., Poole, England) was prepared at concentrations of \leq 20 mM in complete medium before addition to cells. Colchicine, colcemid, and cycloheximide (Sigma Chemical Co., St. Louis, MO) were prepared as concentrated stock solutions in water before addition to cells. Actinomycin D and nocodazole were prepared as concentrated stock solutions in DMSO. Although several concentrations of each drug were used in initial experiments, for the experiments discussed here final concentrations were: 1 μ g/ml actinomycin D, 10 mM benzamide, 5 μ M colcemid, 100 μ g/ml colchicine, 100 μ M cycloheximide, 100 μ M emetine, 3 μ g/ml nocodazole, and 1 μ M pactamycin.

Salivary Gland Incubations

Salivary glands were isolated from third instar larvae in Poels' solution (Mukherjee and Lakhotia, 1979). They were incubated for 2 h at room temperature in Poels' solution, Poels' solution plus 10 mM benzamide, or Poels' solution plus 100 μ M cycloheximide and then frozen. RNA was prepared from the glands using the guanidine-HCl procedure (Garbe et al., 1986). In each experiment the pooled glands were split into two aliquots. One aliquot was incubated in buffer as a control; the other aliquot was incubated with the drug.

RNA Analysis

Total RNA was extracted from 1.2×10^7 cells using the guanidine-HCl procedure (Garbe et al., 1986). Extracted RNA was denatured by glyoxal treatment, separated on 1% agarose gels, and transferred to Hybond-N membranes (Amersham Corp., Arlington Heights, IL) as described by Thomas (1980). Hybridization probes were 32 P-labeled by random priming (Feinberg and Vogelstein, 1983), using gel-purified DNA fragments as template.

Hybridization Probes

All hybridization experiments shown (except Fig. 9, lanes E-H) were done with a cDNA clone padml29F5 (Garbe and Pardue, 1986). This contains all but the first 91 nucleotides (nt) of the ω 3 transcript and therefore is present in all *hsr ω* transcripts in equal amounts. The intron probe used in Fig. 9 was a subcloned genomic fragment that contained the entire sequence of the intron plus 64 nt of the first exon and 19 nt of the second exon. This probe detects ω 1 and ω 2 with equal efficiency.

Antibody Staining of Schneider 2-L Cells

Cells were spun onto glass slides with a cytocentrifuge and immediately fixed for 30 min with 3.7% formaldehyde in *Drosophila* phosphate-buffered saline (DPBS) plus 2 mM MgCl₂ (DPBS = 10 mM Na-phosphate, pH 7.4, 130 mM NaCl). Cells were washed in DPBS, permeabilized in DPBS plus 0.1% Triton X-100 for 2 min, and washed again in DPBS. Cells were blocked with DPBS plus 5% fetal calf serum and 0.1% Tween 20 for 20 min and then a mouse monoclonal antibody against *Drosophila* α -tubulin, monoclonal 2A6 from M. Fuller (University of Colorado, Boulder, CO), made in the same series as 3A5 (Piperno and Fuller, 1985), was applied in the blocking solution. Cells were held at 25°C for 1 h, washed with DPBS, and incubated at 25°C for 30 min with fluorescein-labeled goat anti-mouse IgG. Cells were washed and mounted in 90% glycerol plus *p*-phenylenediamine. Photography was on Tri-X film developed with Dfaiine.

Results

The Relative Ratios of *hsr ω* Transcripts Change with Time During Heat Shock

Cultured *Drosophila* cells were heat shocked at 36°C, a temperature that induces maximal expression of the heat shock response in these cells. Samples were taken periodically for 4 h and analyzed for the presence of several RNAs (Fig. 1).

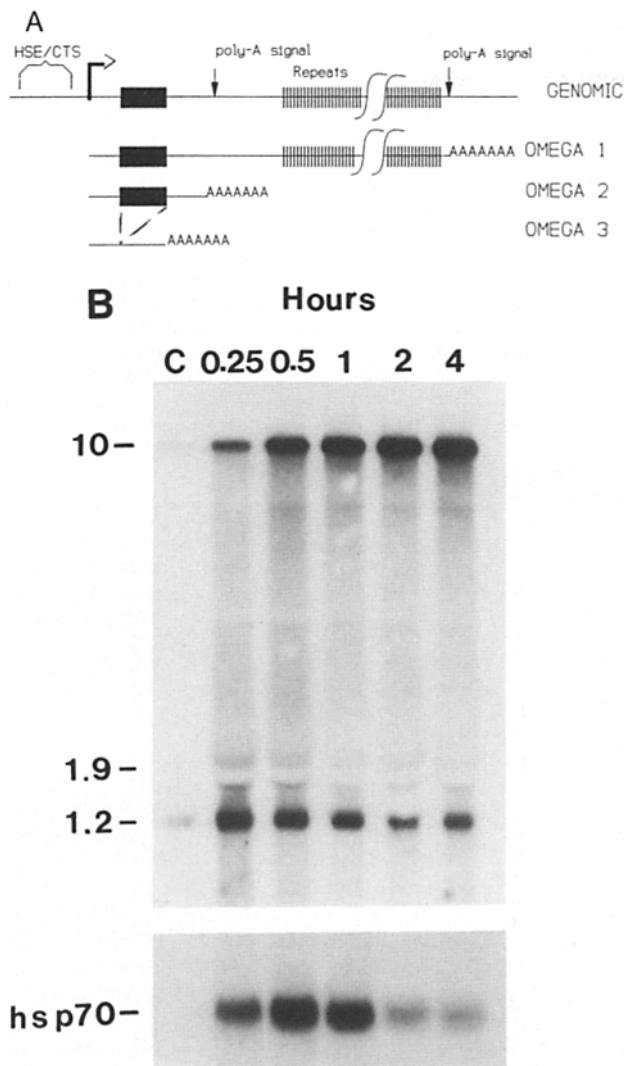


Figure 1. The pattern of *hsr ω* transcripts changes with time during heat shock. (A) Schematic diagram showing the genomic organization of the *hsr ω* locus (*GENOMIC*) and the structure of its transcripts (*OMEGA 1*, *2*, and *3*) in the *Drosophila* species studied. *HSE/CTS* indicates a region containing heat shock elements and at least some of the constitutive transcription signals. The bent arrow marks the start of transcription for all three transcripts. The black box represents the sequences that are spliced out to make $\omega 3$. The first poly-A signal appears to be used for termination of $\omega 2$. The second poly-A signal appears to be used for termination of $\omega 1$. The region marked repeats is the 8–15-kb segment of tandem direct repeats; this region has been greatly truncated in the diagram. The unique region between the transcription start and the repeats is ~ 3 kb. The $\omega 1$ transcript is colinear with the entire transcription unit and is limited to the nucleus. The $\omega 2$ transcript is also nuclear and is processed to yield the cytoplasmic transcript, $\omega 3$, by removal of a 700-nt intron (sequences indicated by black box). *AAAAAAA* indicates polyadenylation of transcripts. The $\omega 3$ transcript is 1.2 kb and $\omega 2$ is 1.9 kb. The size of $\omega 1$ varies somewhat between alleles. (B) Autoradiograph showing the *hsr ω* transcripts in Schneider 3 cells after different periods of heat shock. Similar results are obtained for Schneider 2-L cells. C, control cells held at 25°C. Other lanes contain equal amounts of total RNA from cells held at 36°C for the number of hours indicated above the lane and then processed immediately. RNA was separated on an agarose gel, transferred to a filter, and hybridized with 32 P-labeled restriction endonuclease fragments representing the sequence of $\omega 3$. The insert below shows the appropriate region of this filter after reprobing with sequences for the *D. melanogaster* hsp 70. Like $\omega 3$, the hsp 70 transcript level

Throughout the heat shock, all three *hsr ω* transcripts were detected at levels considerably above those seen at 25°C; however, the levels of $\omega 1$ and $\omega 3$ changed markedly during the heat shock, and they changed in opposite directions with respect to each other. The levels of $\omega 1$ increased throughout the 4 h. Levels of $\omega 3$ decreased somewhat after the first hour. Transcripts encoding hsp 70 showed some decrease paralleling (with a slight lag) the time course of the $\omega 3$ decrease.

The Three *hsr ω* Transcripts Differ in Their Turnover Rates

When heat shocked cells were allowed to recover at 25°C, all *hsr ω* transcripts returned to their control level within the first hour. In contrast, mRNA for hsp 83, the other heat shock RNA that is also expressed constitutively, remained at an elevated level for several hours (Fig. 2). To study turnover of *hsr ω* transcripts in nonstressed cells, Schneider 3 cells were treated with 1 μ g/ml of actinomycin D at 25°C for ≤ 4 h and sampled at intervals (Fig. 3). Under these conditions, the $\omega 2$ and $\omega 3$ transcripts turned over extremely rapidly. The earliest cell sample was taken after 15 min of treatment; no $\omega 2$ or $\omega 3$ could be detected even after this short interval. In contrast, $\omega 1$ appeared to be very stable; the level of $\omega 1$ showed no change over the 4 h of the experiment. Histone mRNA turns over rapidly in most cells (Stein et al., 1984). In this experiment, the level of histone mRNA began to drop after the first 30 min of drug treatment.

Benzamide, Colchicine, and Colcemid Preferentially Increase Levels of $\omega 1$

When *D. melanogaster* salivary glands are incubated with benzamide at 22°C, specific puffing in region 93D is seen within 10 min (Fig. 4; Lakhotia and Mukherjee, 1980). The same specific induction of the 93D region is seen when the glands are incubated with colchicine or colcemid for >30–45 min (Lakhotia and Mukherjee, 1984). Similar responses are observed at the equivalent *hsr ω* sites in other species. To monitor RNA levels from the *hsr ω* locus in diploid cultured cells, Schneider cells were treated with the chemicals and sampled for RNA periodically during the drug treatment. All three drugs led to elevated levels of transcripts from the heat shock locus, as predicted by the polytene puff. Surprisingly, however, all of the chemicals preferentially increased the level of the $\omega 1$ RNA.

When cells were treated with 10 mM benzamide at 25°C, the level of $\omega 1$ rose as a function of time, achieving an ~ 20 -fold induction after 6 h (Fig. 5 A). The transcripts remained at this elevated level for the rest of the 24-h period tested. Maintenance of the high level of $\omega 1$ depended on the presence of benzamide; when benzamide was removed after a period of induction, transcript $\omega 1$ rapidly returned to its constitutive level. There was no detectable increase in the level of $\omega 3$ until after 12 h of incubation in benzamide; induction of $\omega 3$ was only twofold when it occurred.

decreases in the later stages of heat shock but the decrease of the hsp 70 RNA lags slightly behind that of $\omega 3$. The level of $\omega 1$ continues to rise throughout the experiment. Hybridization was at 60°C in 0.6 M NaCl, 0.12 M Tris-HCl, and 8 mM EDTA, pH 7.0, 0.5% SDS. Filters were washed in 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0, 0.5% SDS at 60°C.

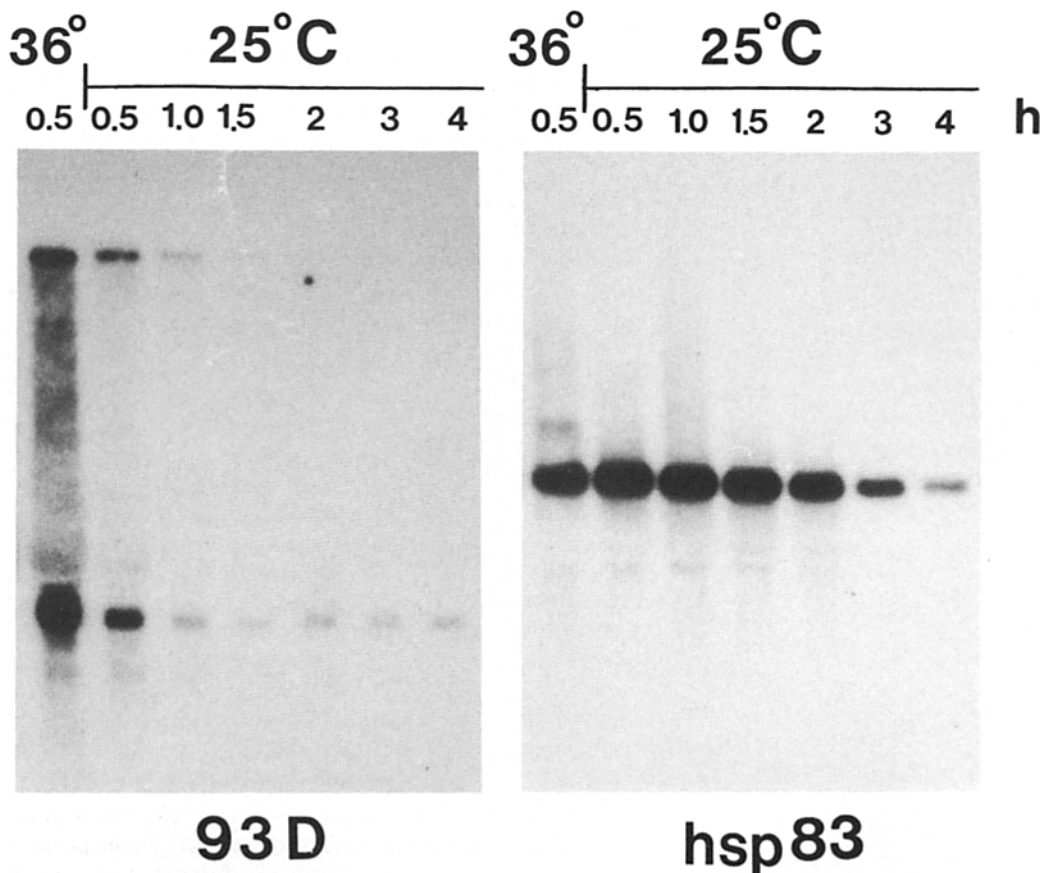


Figure 2. *hsr* ω transcripts return to control levels more rapidly than hsp 83 transcripts upon recovery from heat shock. Schneider 3 cells were heat shocked for 30 min at 36°C and then returned to 25°C. Aliquots were taken at the times indicated above each lane and the RNA was analyzed as in Fig. 1. The panel marked 93D shows the *hsr* ω transcripts as detected by the probe for the ω 3 sequence. The panel marked hsp 83 shows the same samples probed for the sequences encoding the *D. melanogaster* hsp 83. The *hsr* ω transcripts return to control levels at 1–1.5 h while the hsp 83 transcripts do not return to control levels until 4 h after return to control temperatures. (The autoradiogram shows only samples taken up to the return of hsp 83 RNA to control levels. The control lane is not shown.)

When cells were incubated with 100 μ g/ml colchicine at 25°C, ω 1 was maximally induced after 6 h, achieving a 15–20-fold induction over the constitutive level (Fig. 5 B). This level remained stable over a 48-h test period. As with benzamide, the ω 3 transcript showed little or no change. There was an increase in the ω 2, but the increase was relatively small. Treatment with colcemid or nocodazole gave similar patterns of induction, but, at the concentrations tested, colcemid and nocodazole gave weaker inductions than benzamide and colchicine, resulting in some eightfold induction after a 24-h period. Nocodazole also took a longer time to induce the transcripts; no increase was seen in the first 12 h of incubation with the drug (data not shown).

None of the chemicals tested induce puffing of heat shock loci other than *hsr* ω . To test that cultured cells were not being stressed by long incubations in the chemicals, the RNA filters were probed with sequences encoding hsps 70 and 22. No induction of hsp 70 RNA was noted in these experiments, although it is the most abundant RNA in heat shocked cells and is a sensitive indicator of even low levels of heat shock. In spite of the lack of hsp 70 induction, there was a slight induction of hsp 22 transcripts. The hsp 22 level appeared maximal after 6 h of treatment with benzamide and colchi-

cine. No hsp 22 induction was detected after treatment with colcemid or nocodazole (data not shown).

Studies of actinomycin D treatment of cells in benzamide suggest that the additional ω 1 induced by benzamide is also stabilized by the drug or by actinomycin D (Fig. 6). Cells were incubated with benzamide and actinomycin D was added to the culture 1 or 2 h later. All cultures were analyzed 4 h from the initial addition of benzamide. Although the level of ω 1 increased with time during the 4 h in benzamide, the cultures with actinomycin D added showed, at 4 h, approximately the level of ω 1 found in parallel cultures, treated with benzamide, and sampled at the same time that actinomycin D was added to the other cultures. This implies that actinomycin D has stopped the transcription of ω 1 but does not lead to its turnover. On the other hand, when the benzamide was washed out by replacing the culture medium, the level of ω 1 rapidly returned to control levels. Unexpectedly, this return to control levels was not complete if the benzamide was washed out and replaced by medium containing actinomycin D, raising the possibility that new RNA synthesis is necessary for the turnover of the ω 1 transcript, an explanation that may explain the stability of the ω 1 transcript in the earlier turnover experiment.

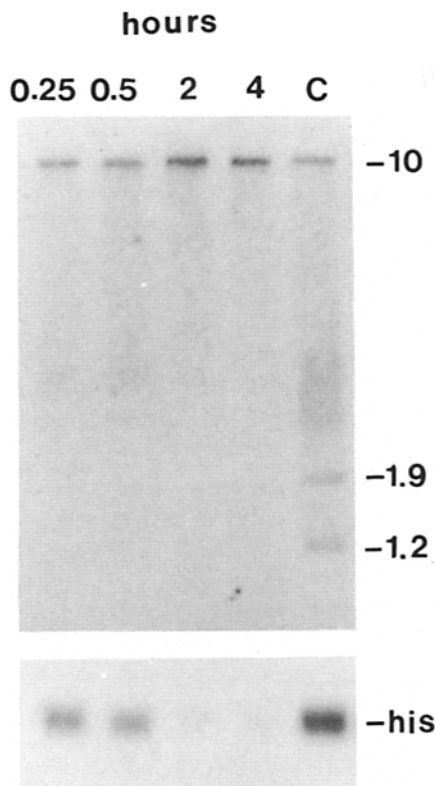


Figure 3. The constitutive ω_2 and ω_3 transcripts turn over rapidly while ω_1 transcripts are more stable. Aliquots of Schneider 3 cells were incubated with actinomycin D at 25°C for the times indicated above each lane. RNA was extracted and analyzed as in Fig. 1. The *hsr ω* transcripts were detected by sequences from ω_3 . Inset below is the appropriate region from the filter after it had been reprobbed with sequences complementary to the *D. melanogaster* histone genes. The histone transcripts are short lived but they do not turn over as rapidly as do ω_2 and ω_3 . (The control was cells in medium plus the DMSO used to dissolve actinomycin.)

Inhibitors of Protein Synthesis Lead to an Increase in the Level of the ω_3 Transcript

Cycloheximide does not induce puffs on polytene chromosomes (Ashburner, 1974; Fig. 4) so we were surprised when the control for another experiment revealed that cycloheximide-treated Schneider 3 cells had very high levels of the ω_3 transcript, although there was no detectable increase in the ω_1 or ω_2 transcripts (Fig. 7). The cycloheximide concentration used had been sufficient to inhibit protein synthesis by 98% (data not shown). The protein synthesis inhibitors emetine and pactamycin were equally effective in inducing the rapid, preferential appearance of the ω_3 transcript (Fig. 7). The three drugs inhibit protein synthesis by different mechanisms, suggesting that it is their shared effect, inhibition of protein synthesis, that is affecting the level of ω_3 .

Regardless of which protein synthesis inhibitor was used, within 2 h after addition of the inhibitor the steady state level of ω_3 surpassed that achieved by a 1-h heat shock at 36°C. The protein synthesis inhibitors appear to produce effects primarily by stabilizing the turnover of ω_3 (which, as discussed above, is normally extremely rapid). We have tested the effects of actinomycin D on cycloheximide-treated cells

in several conditions and find that, if cycloheximide is present, the ω_3 level is stabilized at the level present in the cell when the actinomycin D was added. In one experiment, cells were heat shocked at 36°C for 30 min to enhance the level of *hsr ω* transcripts. Cells were then returned to 25°C either (a) without further treatment, (b) in the presence of cycloheximide alone, or (c) in the presence of cycloheximide and actinomycin D. Total RNA was extracted from cells at each time point and analyzed (Fig. 8). Cells shifted down to 25°C in the presence of cycloheximide and actinomycin did not show the decrease in the heat shock level of ω_3 that was seen when untreated cells were shifted down. Although actinomycin prevents new transcription in control cells (Fig. 7), in these shift down conditions there appeared to be some new transcription in addition to the cycloheximide stabilization of ω_3 . We have noted other conditions under which actinomycin is not completely effective in these cells (Scott et al., 1980). Cells shifted down in the presence of cycloheximide alone showed even higher levels of ω_3 , suggesting that the drug was stabilizing not only the ω_3 that had been made during heat shock but the new transcripts that were made at 25°C.

Further evidence that the apparent induction of the ω_3 transcript is actually due to RNA stabilization comes from studies of the intron that is excised in the processing of ω_3 (Fig. 9). The excised intron, which differentiates ω_2 from ω_3 is stable during heat shock (data not shown) and thus provides another measure of the amount of transcription and processing that occurs during the heat shock. Schneider 3 cells were incubated with cycloheximide for 15 min and then either held at 25°C or transferred to 37°C for 40 min. Parallel cultures received the heat treatment in the absence of the drug. RNA was extracted from cells in each culture and the level of ω_3 and the excised intron fragment compared. As expected, the level of ω_3 is increased by the heat shock. It is also increased by exposure to the drug. The drug treatment and the heat shock are additive. In contrast, the level of the intron fragment, which is increased by the heat shock, is not further increased by the cycloheximide. If enhanced transcription and processing were playing a role in the increase

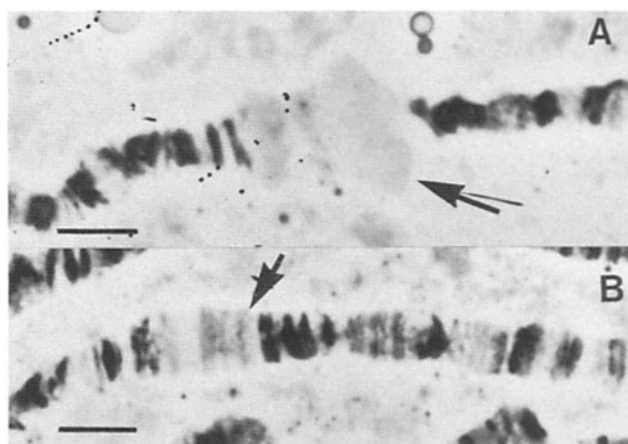


Figure 4. The *hsr ω* locus (93D) is puffed after treatment with benzamide but not cycloheximide. (A) Segment of chromosome 3R from a salivary gland incubated in benzamide. (B) Segment of chromosome 3R after incubation in cycloheximide. The 93D site is indicated by arrows. Orcein stain.

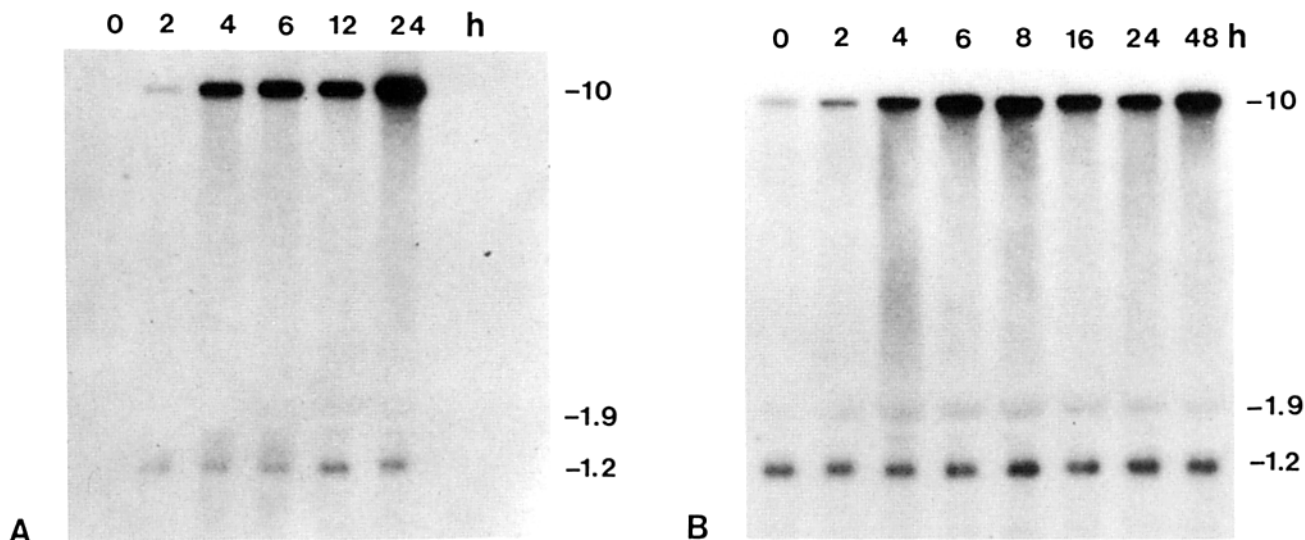


Figure 5. Chemical inducers which specifically induce puffing at the *hsrw* locus lead to enhanced expression of transcript $\omega 1$. (A) Cells treated with benzamide. (B) Cells treated with colchicine. Schneider 3 cells were treated with the drugs for the times indicated. Total RNA was extracted from equal numbers of cells and analyzed as in Fig. 1. The probe was sequences from $\omega 3$.

of $\omega 3$, we would expect cycloheximide to cause a similar increase in the intron fragment in heat shocked cells where it is stabilized.

The Drug Response of Polytene Cells Resembles that of Diploid Cells

The conclusions of our studies on transcript accumulation in

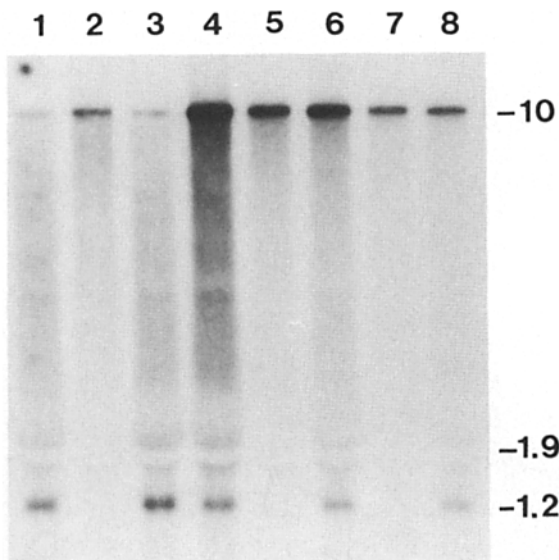


Figure 6. Drug-induced increases in $\omega 1$ are lost rapidly when drugs are removed. Aliquots of cells were incubated, at 25°C, with the following solutions for the times indicated: (lane 1) control in drug-free medium; (lane 2) benzamide for 2 h, followed by fresh medium plus actinomycin D for 2 h; (lane 3) benzamide for 2 h, followed by drug-free medium 2 h; (lane 4) benzamide for 4 h; (lane 5) benzamide for 4 h, with actinomycin D added for last 2 h; (lane 6) benzamide for 2 h; (lane 7) benzamide for 4 h, with actinomycin D added for last 3 h; (lane 8) benzamide for 1 h. Transcripts were analyzed as in Fig. 1. The probe was sequences from $\omega 3$. The $\omega 1$ level returns to the control level after the removal of benzamide unless actinomycin D is present.

the diploid cells are consistent with the studies of drug-induced puffing of chromosomes in polytene cells. To make a more direct comparison between the cell types we have isolated RNA from the polytene salivary glands of third instar larvae (Fig. 10). The glands had been incubated for 2 h at 25°C in Poels' solution containing either 100 $\mu\text{g/ml}$ cycloheximide or 10 mM benzamide. The 2-h period was chosen as a time long enough to allow RNA accumulation but short enough to ensure that the excised glands were still healthy. RNA from these glands, plus control glands incubated without any drug, was analyzed as with the cultured cells. The results show that both of the drugs affect the salivary gland cells in very much the way that they affect the cultured cell line. Benzamide led to a large increase in the $\omega 1$ level. Cycloheximide led to an increase in $\omega 3$. We note that, in addition to the expected increase in $\omega 1$, benzamide leads to some elevation in the level of $\omega 3$, possibly indicating a decline in protein synthesis in the cultured glands. This transcript analysis suggests that, for this locus, the polytene puffing studies are predictive of results in other cell types.

An Increase in $\omega 3$ Is Not Sufficient for Thermoprotection of the Cell

There have been reports that cells from several organisms could be transiently protected from temperature increases (thermoprotected) by treatments that include incubation in cycloheximide. To test the possibility that the thermoprotection was simply due to cycloheximide-induced increase in $\omega 3$, we have studied thermoprotection under conditions in which we had such an increase (Fig. 11). Cells growing at 25°C were treated with cycloheximide for 10 min or 2 h before shifting equal aliquots, plus aliquots of untreated cells, to the heat shock temperatures of 36, 37, 38, or 39°C for 40 min. The effect of the heat on cell activity was monitored by examining the accumulation of the *hsrw* transcripts; the ability to accumulate these transcripts is one measure of the ability of the cell to survive a heat stress. Cells that received no drug treatment rapidly accumulate all three *hsrw* transcripts at 36 and 37°C. The 38°C treatment is more drastic; accumu-

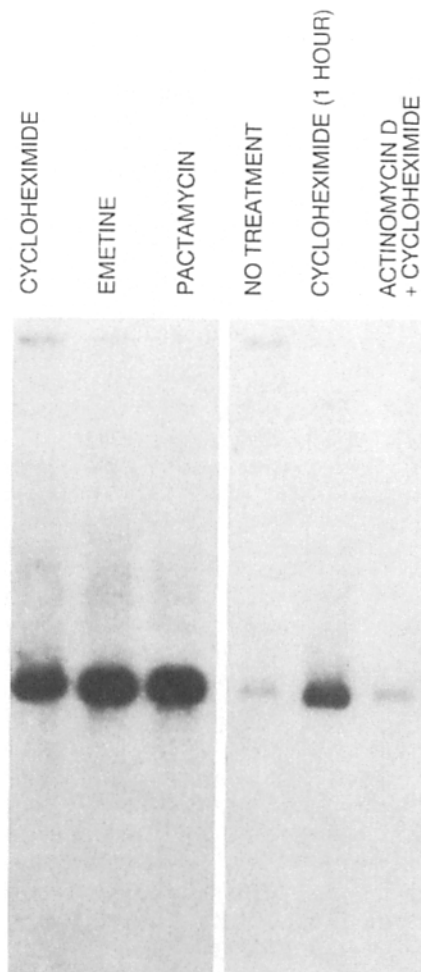


Figure 7. Inhibition of protein synthesis leads to large increases in levels of ω_3 . Schneider 3 cells were incubated with the protein synthesis inhibitors for 2 h (first 3 lanes), or with cycloheximide or cycloheximide plus actinomycin D for 1 h (last 2 lanes). At the end of the incubation, RNA was processed as for Fig. 1 and probed with ^{32}P -labeled sequences of ω_3 . All protein synthesis inhibitors lead to preferential accumulation of ω_3 . Although ω_3 normally decays rapidly in the presence of actinomycin D (Fig. 3), the control level of ω_3 is maintained if cycloheximide is also present, suggesting that the inhibition of protein synthesis stabilizes the ω_3 transcript.

lation of ω_3 is no longer apparent. This change in the ω_3 level is probably due, at least in part, to an inhibition of splicing at 38°C since accumulation of splicing precursors increases abruptly at this temperature (data not shown). When the heat stress is increased to 39°C, accumulation of all *hsw* transcripts is severely repressed. Transcripts for hsp 70 and 83 show similar decreases at 39°C, with inhibition of hsp 83 splicing becoming marked at 38°C.

If the cycloheximide-induced increase in ω_3 had a thermoprotective effect, we would have expected drug-treated cells to be able to carry out splicing, transcription, and other activities at higher temperatures than nontreated cells. In addition, cells that had longer drug treatment, and thus more ω_3 accumulation, might be expected to show more protection. Such a shift in temperature profile has been seen in cells thermoprotected by induction of low levels of heat shock synthesis (Yost and Lindquist, 1986). This is not the result for

our cycloheximide-treated cells. Both sets of drug-treated cells showed the same temperature profiles as the control cells. Thus, it appears that the increase in ω_3 is not sufficient for thermoprotection, although we have not eliminated the possibility that such an increase is one component in thermoprotection.

Discussion

These results demonstrate that the chemical agents that induce a puff specifically at 93D (and equivalent loci in other *Drosophila* species, the *hsw* loci) also affect the levels of *hsw* transcripts in both diploid and polytene cells. Studies of polytene puffing had suggested that the *hsw* locus was more sensitive to environmental agents than were the other heat shock loci. The analyses of transcript accumulation reported here support that conclusion.

The most striking feature of these results is the evidence that ω_1 and ω_3 can be regulated independently of each other. (In the case of colchicine there was also a differential effect on ω_2 but this was small and will not be considered further here.) Although all three *hsw* transcripts originate at the same transcription start site (Ryseck et al., 1987; Garbe, J. C., unpublished results), we have several reasons to believe that ω_1 is not a precursor to ω_2 and ω_3 . For example, we can detect no processing intermediates between ω_1 and

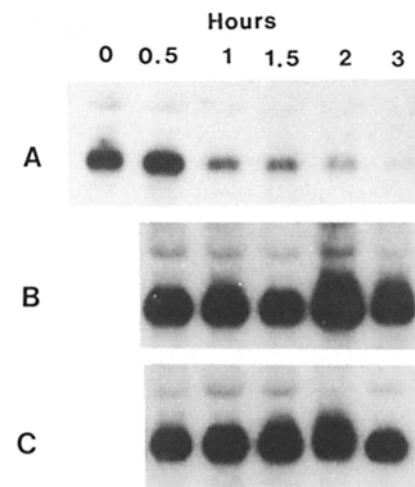


Figure 8. Inhibition of protein synthesis enhances the stability of transcript ω_3 . Aliquots of Schneider 3 cells were heat shocked at 36°C for 30 min in drug-free medium and then split into three aliquots and returned to 25°C for the number of hours indicated over each lane. *A* shows cells recovering from heat shock in drug-free medium. *B* shows cells to which cycloheximide was added when they were returned to 25°C. *C* shows cells to which cycloheximide plus actinomycin D was added when they were returned to 25°C. RNA was extracted and analyzed as in Fig. 1, using sequences of ω_3 as the probe. Only the region of the gel with the ω_2 and ω_3 transcripts is shown. The high levels of the transcripts seen in *B* suggest that the drug is stabilizing both the heat shock-induced and the newly synthesized transcripts (and possibly increasing transcription). Transcript levels in *C* are slightly lower than those in *B* but are still higher than the heat shocked control (lane 0), suggesting that in this situation actinomycin D may not have completely stopped transcription.

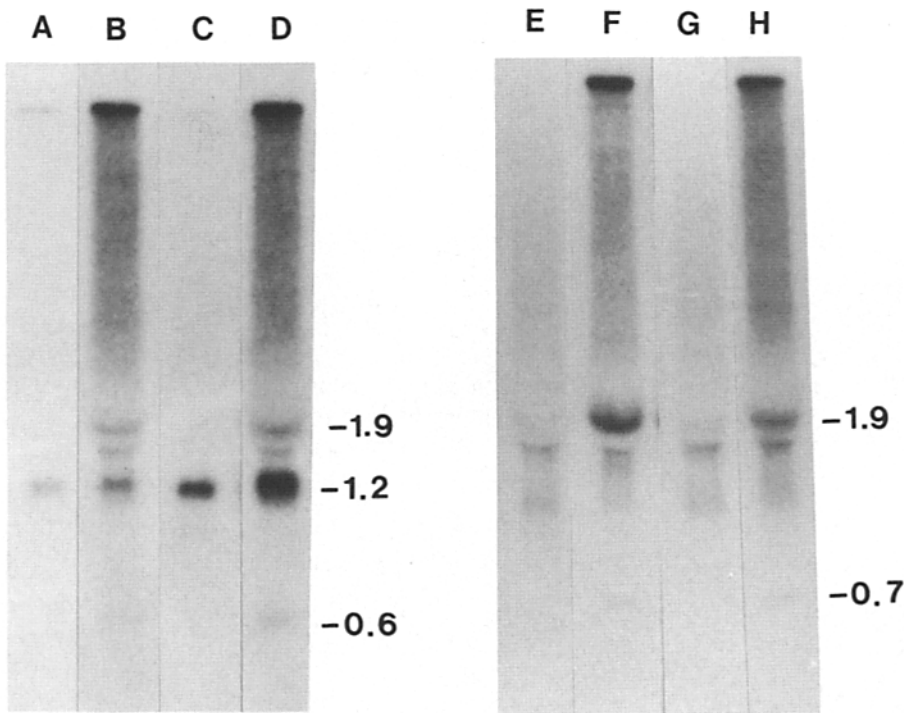


Figure 9. Cycloheximide does not enhance the level of the excised 0.7-kb *hsr ω* intron. Parallel cultures of Schneider 3 cells were incubated in the presence or absence of cycloheximide for 15 min and then either held at 25°C or transferred to 37°C for 40 min. RNA was processed as in Fig. 1. Lanes A–D were probed with sequences of ω 3. Lanes E–H were probed with sequence from the *hsr ω* intron (see Materials and Methods). Lanes A and E, 25°C, no treatment; lanes C and G, 25°C, cycloheximide; lanes B and F, 37°C, no treatment; lanes D and H, 37°C, cycloheximide. Although the ω 3 transcript (1.2 kb) is much enhanced by cycloheximide (compare lanes A and C with lanes B and D), the excised intron (0.7 kb) is not increased (compare lanes F and H). The 0.6-kb fragment that is detected by ω 3 sequence is a putative processing product now under study. Neither it nor the ω 2 precursor (1.9 kb) show significant elevation by cycloheximide.

ω 2 or 3. Instead, we believe that the two shorter transcripts are the result of alternative termination near the polyadenylation signal that is found \sim 2–3 kb into the unique region in each of the species studied (Bendena et al., 1989). The dif-

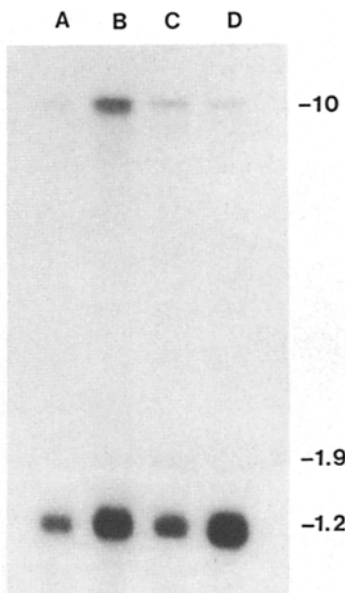


Figure 10. *hsr ω* transcript patterns induced by benzamide and cycloheximide in polytene cells resemble those of diploid cells. Salivary glands were dissected from third instar larvae in Poels' medium. Equal numbers of glands were incubated at 25°C in Poels' medium alone (control), in Poels' medium plus 10 mM benzamide, or in Poels' medium plus 100 μ g/ml cycloheximide. RNA was extracted and analyzed as in Fig. 1. The probe was ω 3 sequence. Lanes A and C are each the control for the lane to their right. Lane B, benzamide, 2 h; lane D, cycloheximide, 2 h.

ferent responses of the transcripts reported here are completely consistent with this hypothesis (although alternative explanations are also possible). For instance, inhibition of transcription with actinomycin D leads to rapid turnover of ω 3 and its presumed precursor, ω 2, but has no effect on the levels of ω 1, suggesting that ω 1 cannot be chased into ω 3. In addition, the specific enhancement of the level of ω 1 by benzamide, colchicine, etc. does not lead to increased amounts of ω 3, either during drug treatment or when the ω 1 levels return to control values on recovery. The higher levels of ω 1 in these drug experiments do not result from a general inhibition of RNA processing by these drugs; we find that other transcripts are processed normally in the presence of these drugs (data not shown).

Polytene puffing experiments give strong evidence that puffing indicates induction at the level of transcription (Bonner and Pardue, 1977). Results of our drug studies are consistent with transcriptional induction by benzamide but they also show that the excess ω 1 induced by benzamide is maintained only in the presence of that drug. The amount of ω 1 returns to its constitutive level soon after benzamide is washed out of the cells. Similarly, 93D puffing is maintained only in the presence of benzamide and regresses as soon as the drug is withdrawn (Lakhotia and Mukherjee, 1980). On the other hand, the high levels of ω 1 that are induced by benzamide are maintained when transcription is blocked by actinomycin D if benzamide is also present. The rapid decrease in the ω 1 levels after removal of inducing drugs may require synthesis of some new RNA since the decrease is much slower when benzamide is washed out in the presence of actinomycin D.

The stabilization of the ω 3 transcript by cycloheximide appears to be due to the drug's ability to inhibit protein synthesis. All of the drugs that we have used successfully to inhibit protein synthesis in these cells also lead to increases in ω 3,

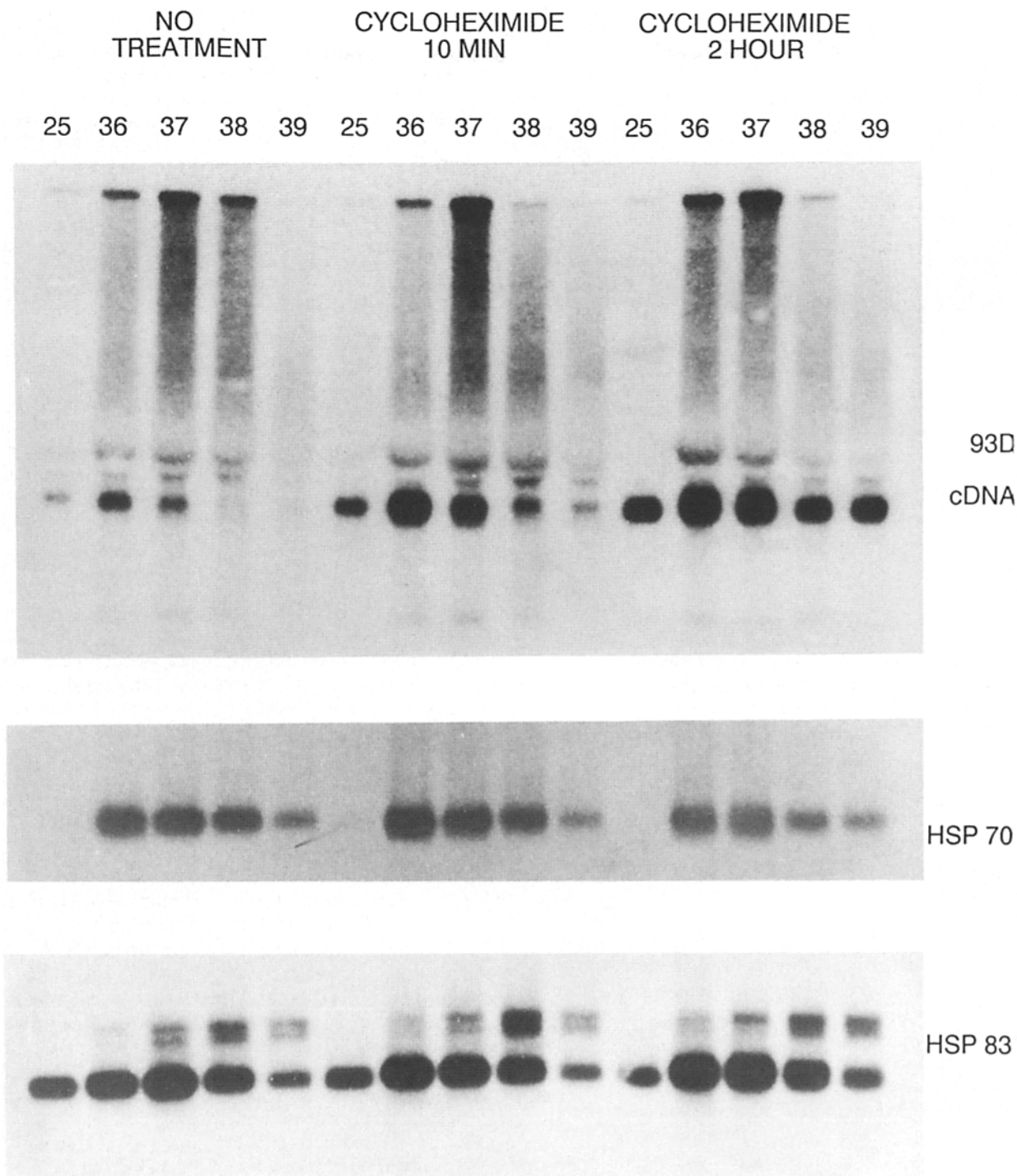


Figure 11. An increase in $\omega 3$ is not sufficient for thermoprotection. Schneider 3 cells were grown at 25°C and then shifted to the indicated temperature for 40 min. Parallel RNA extractions were made from cells with no drug and cells incubated for 10 min or 2 h (as indicated) in the presence of 100 $\mu\text{g}/\text{ml}$ cycloheximide before shifting to the indicated temperature. RNA was analyzed as in Fig. 1. The probe was the 93D cDNA (sequences of $\omega 3$). The two panels below show the same filter probed with sequences coding for *D. melanogaster* hsp 70 and hsp 83. The unspliced precursors of hsp 83 RNA become apparent at the higher temperatures. Neither drug treatment protects the cells well enough to permit synthesis and processing of new transcripts at temperatures higher than those tolerated by control cells.

without affecting levels of $\omega 1$. The result is the same whether the drugs act at the level of initiation (pactamycin) or elongation (cycloheximide, emetine). This result also suggests that the enhanced stability of the $\omega 3$ transcript is not due to poly-some shielding from nucleases since pactamycin is as effec-

tive as cycloheximide in increasing $\omega 3$. Instead it appears that continued protein synthesis is necessary to ensure the decay of $\omega 3$ RNA. The stability of *c-myc* (Ledal et al., 1985) is controlled in this manner as is the turnover of histone mRNA when DNA synthesis is arrested (Sive et al., 1984). The $\omega 3$

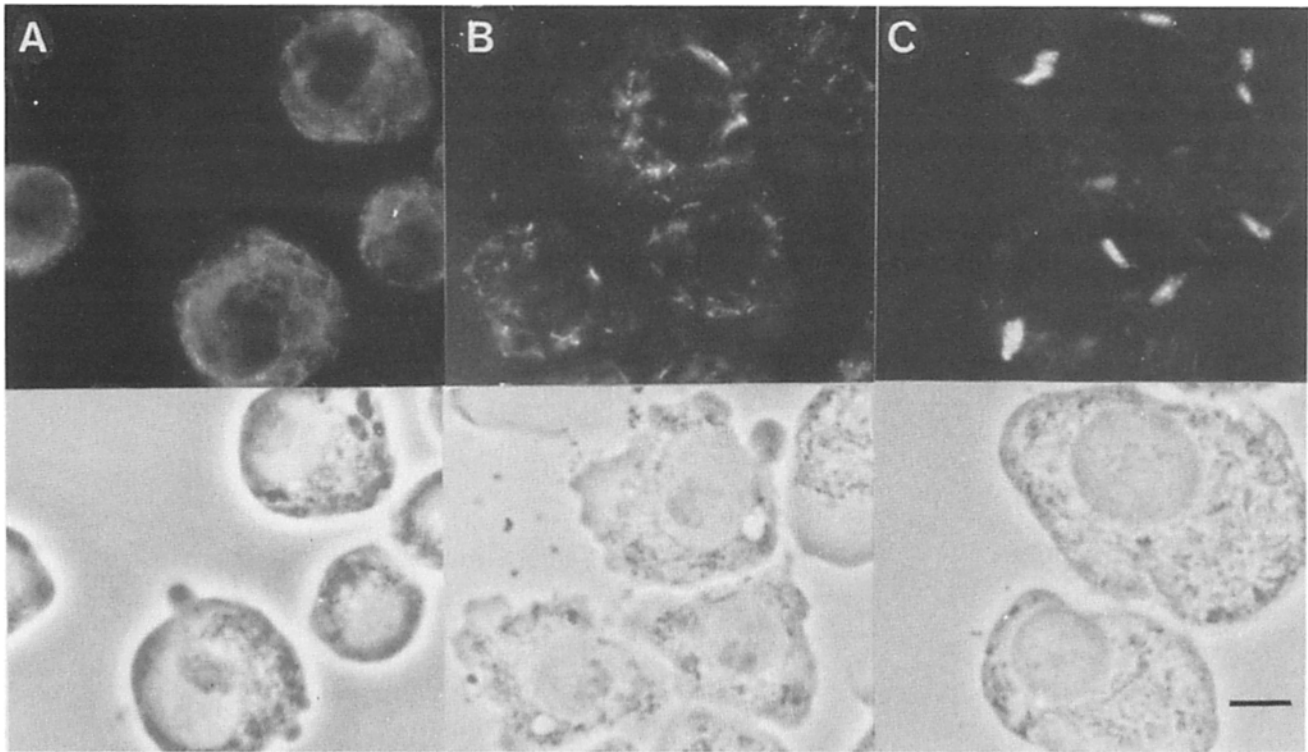


Figure 12. The effect of drugs on microtubules in *Drosophila* cells. The top row of photomicrographs shows cells stained with a monoclonal antibody against *Drosophila* α -tubulin, detected with fluorescein-labeled anti-mouse IgG. The bottom row shows the same cells in phase contrast. (A) Control cells, showing a fine network of cytoplasmic microtubules. (B) Cells after an 8-h treatment with colchicine. Only a spiked ring of microtubules around the nucleus is detected. Nocodazole-treated cells showed a similar distribution of microtubules only when treated with the drug for 24 h. (C) Cells treated for 2 h with vinblastin. Paracrystalline arrays of microtubular components are scattered throughout the cytoplasm. The perinuclear rings detected with colchicine and nocodazole are not seen. Bar, 5 μ m.

transcript resembles the *c-myc* and histone mRNAs in that it has a very short half-life. There is evidence that a very short open reading frame in $\omega 3$ is translated (Bendena et al., 1989; Fini et al., 1989). The translation may be related to the turnover of $\omega 3$.

Although it is possible to group all the drugs that lead to specific increases in $\omega 3$ as inhibitors of protein synthesis, it is not obvious that the drugs leading to increases in $\omega 1$ act by a common mechanism. One effect of benzamide is the inhibition of ADP-ribosyl transferase, while colchicine and colcemid are usually used to inhibit microtubule polymerization. It seems unlikely that benzamide is acting through effects on ADP-ribosyl transferase since the other inhibitors of this enzyme that we have tested, 3-aminobenzamide, niacinamide, and theophylline, have no effect on the transcripts of *hsw* or any other heat shock locus over a 48-h exposure (data not shown).

Singh and Lakhota (1984) have reported that several drugs affecting microtubules, including vinblastin and nocodazole, did not induce puffing of the 93D region, suggesting that colchicine and colcemid might not be acting through the microtubules to induce $\omega 1$. The RNA analyses reported here also show that vinblastin does not lead to an increase in $\omega 1$. However, we find that nocodazole treatment does lead to increases in the level of $\omega 1$, although these increases come much later than those produced by colchicine (and later than the times studied in the puffing experiments). Interestingly, we find that breakdown of the cytoplasmic microtubules in

Schneider cells is much slower when cells are treated with nocodazole than with colchicine. Induction of $\omega 1$ was not seen in the first 12 h of nocodazole treatment but was detected after 24 h. The complete breakdown of the microtubule network by nocodazole follows approximately this time schedule, while the $\omega 1$ increase and the complete breakdown of the microtubule network occur over a much shorter time scale in colchicine-treated cells. Thus, in these experiments most of the microtubule poisons do affect $\omega 1$ on a time scale that approximates that of their breakdown of the microtubule network. Vinblastin is the only exception and vinblastin also leads to a unique redistribution of microtubular components; instead of depolymerizing the microtubules, vinblastin causes the formation of large paracrystalline aggregates of tubulin (Fig. 12; Gundersen et al., 1987). The correlations suggest that the $\omega 1$ levels may be reflecting drug-induced changes in the cytoskeleton. It is possible that benzamide also produces its effects through the cytoskeleton. The drug has been reported to depolymerize contractile fibrils in *Physarum* (Korohoda and Wohlfarth-Bettermann, 1976) and to affect the mitotic spindle and centromeric functions of mammalian cells (Babu et al., 1980). Perhaps it plays a similar role in *Drosophila* cells even though we detect no changes in the microtubule network.

Since the presumed targets of the drugs that increase the $\omega 1$ transcript are predominantly cytoplasmic, it is interesting that the level of the nuclear transcript responds so reproducibly and quickly. In the absence of environmental perturba-

tions the constitutive level of $\omega 1$ is very constant but it rises rapidly in response to the drugs and returns quickly to its lower level when the drug is withdrawn. The striking feature of the $\omega 1$ transcript is the 7–13 kb of short tandem repeats. The repeats are conserved within each *Drosophila* species studied but between species the repeats have diverged sharply in both length and sequence. What is conserved is a nine nt sequence that is distributed rather uniformly along the length of the repeated region in each $\omega 1$ transcript (Garbe et al., 1986). This structure suggests that $\omega 1$ acts by binding one or more nuclear components, possibly forming the 300-nm RNP particles that are found in *hsr* ω puffs (Dangli et al., 1983). Perhaps $\omega 1$ is regulating the level of some nuclear component in response to a cellular state, detected through the cytoskeleton.

Thermotolerance is a transient state of ability to tolerate temperature increases. The acquisition of a thermotolerant state has been noted after cells are treated at a mild heat shock temperature before being shifted to a temperature which would have been lethal, had the cells not been pre-treated. In certain cell types, the acquisition of thermotolerance appears to proceed in the absence of protein synthesis (Widelitz et al., 1986). Other investigations (Landry et al., 1982; Li and Werb, 1982; Subjeck and Sciandra, 1982) have suggested that hsp's have a functional role in the development of thermotolerance. Since $\omega 3$ levels are increased both by mild heat shocks and by lack of protein synthesis, we tested the ability of preinduced $\omega 3$ transcripts to protect cells at higher temperatures. We detected no thermoprotection. This result is, perhaps, not surprising. There are several cases in which increases in hsp's are not sufficient to confer thermotolerance (Lindquist, 1986). Possibly *hsr* ω transcripts act in conjunction with hsp's or some as yet uncharacterized factor to enable the cell to be thermotolerant.

The experiments on thermotolerance also suggest that hsp's are not necessary for splicing transcripts (*hsr* ω and hsp 83) at the usual *Drosophila* heat shock temperatures of 36 and 37°C. Splicing at these temperatures was about equivalent whether the cells were incubated in cycloheximide or not. Since the drug was present before the beginning of the heat shock, the cycloheximide-treated cells could not synthesize hsp's yet splicing was normal. When cells were incubated at 38 or 39°C splicing precursors increased significantly. Thus hsp's may be required for protection of splicing at higher temperatures, as suggested by Yost and Lindquist (1986).

We suggested earlier (Garbe et al., 1986; Bendena et al., 1989), on the basis of sequence conservation in several *Drosophila* species, that the $\omega 1$ and $\omega 3$ transcripts had different functions, one acting in the nuclear compartment and the other in the cytoplasmic compartment of the cell. We speculated that these transcripts might coordinate some aspect of nucleo-cytoplasmic interaction. The results presented here show that the two transcripts are capable of being modulated independently. Furthermore, different classes of environmental agents produce different patterns of modulation. These findings are consistent with the earlier hypothesis and offer clues toward deciphering the mechanism of action of this unusual locus.

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