Stress-Induced Proteins, pages 3–14

hsrω: A DIFFERENT SORT OF HEAT SHOCK LOCUS[1]

W.G.Bendena[2], M.E. Fini, J.C. Garbe, G.M. Kidder[3], S.C. Lakhotia[4], and M.L. Pardue

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

ABSTRACT The hsrω locus is very unusual. Each Drosophila species has one hsrω locus, identified by its puffing phenotype. This identification is confirmed by molecular analysis. The hsrω locus produces 3 major transcripts and appears to have both nuclear and cytoplasmic functions.

INTRODUCTION

Cytological studies of the major heat shock puffs of Drosophila led directly to the identification and characterization of the major heat shock proteins. However, one of the largest and most actively transcribed heat shock puffs has remained without an assigned protein product. This puff is in polytene region 93D in D. melanogaster, 48B in D. hydei, 20CD in D. virilis, and 58C in D. pseudoobscura. In spite of the different polytene band numbers in the different species, cytological studies have suggested that these puffs are equivalent. The puffs respond not only to agents that induce the heat shock response, but also to some agents that affect only this puff. The puffs contain 300 nM RNP granules not seen in other puffs (reviewed by 1). Recently the cytological

1. This work was supported by a grant from the National Institutes of Health.
2. Present address: Queen's University, Kingston, Ontario
3. Present address: University of Western Ontario, London, Ontario
4 Present address: Banaras Hindu University, Varanasi, India

evidence of the equivalence of these puffs has been confirmed by analyses of DNA sequences (2; 3).

Our studies on the 93D locus and its relatives in the other species suggest that the important products of this locus are RNAs, not proteins. For this reason, 93D and the equivalent loci in the other Drosophila species have been designated the hsrω (for heat shock RNA omega) locus. In each species this locus produces three major RNAs, named ω1, ω2, and ω3. These RNAs make up a set that is very different from the transcripts of any known locus.

THE MULTIPLE hsrω TRANSCRIPTS APPEAR TO HAVE SEPARATE ROLES IN THE NUCLEUS AND THE CYTOPLASM

Analyses of RNA from many different cell types show the same three major hsrω transcripts in each cell type. These transcripts are produced constitutively in most cell types but the level of the transcripts is increased severalfold by heat shock (Figure 1, A and B). In this respect the hsrω locus resembles the hsp83 locus. The hsrω locus also shows some developmental control of its activity (Figure 1, D-E), thus resembling the hsp22-27 group (reviewed by 4).

When different Drosophila species are compared there are minor differences in the sizes of the three hsrω transcripts: however, in general, the ω1 RNA is larger than 9 kb, the ω2 RNA is approximately 2 kb, and the ω3 RNA is approximately 1.2 kb. The ω1 and ω2 transcripts are found only in the nucleus; ω3 is found in the nucleus and cytoplasm (Figure 2). All three transcripts have the same transcriptional start site; however there are several reasons for thinking that ω1 is not a precursor to ω2 and ω3. First, there is a polyadenylation site near the end of the ω2 sequence, suggesting that ω2 transcripts are produced by alternate termination of transcription in this region. Second, we do not see any of the RNA fragments that would be expected if the terminal regions of ω1 were cleaved off to produce ω2. Third, ω1 is much more abundant in the nucleus than any known precursors. Finally, the pattern of evolution of the sequences that are found only in ω1 argues that these sequences have a specific nuclear function (see below). The ω2 RNA differs from the ω3 RNA only by the removal of a 700 nt intron. Thus ω2 does appear to be the nuclear precursor to ω3 (2).

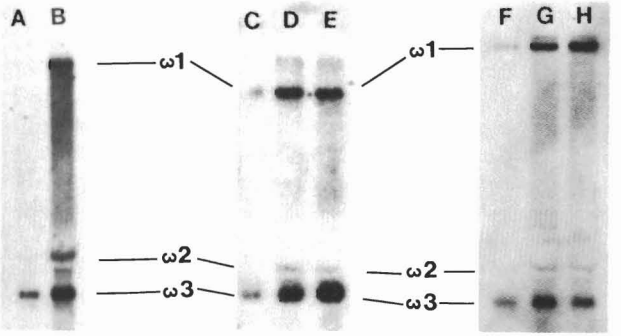

Figure 1. hsrω of D. melanogaster is heat shock inducible and developmentally regulated. Blot hybridization of control (A) and heat shock (B) RNA isolated from D. melanogaster embryos shows induction of the 3 major hsrω transcripts. Developmental increases in the level of hsrω transcripts are also detected. RNA from (non-heat shocked) early (C) middle (D) and late (E) stage embryos shows that hsrω transcripts increase during embryogenesis. Low levels of hsrω are detected during second instar (F) but increased levels are seen during third instar (G) and pupal (H) stages. Within each panel equal amounts of RNA were used for each lane. Blots were probed with a cDNA for hsrω3 to detect all three transcripts.

hsrω SEQUENCES ARE HIGHLY DIVERGENT BUT HAVE IMPORTANT CONSERVED FEATURES

The genes encoding heat shock proteins show strong DNA sequence conservation between animals, plants, and bacteria (4). In contrast, the hsrω loci have diverged even within the genus Drosophila. When small fragments of DNA from the D. melanogaster hsrω at 93D are hybridized to DNA from the D. hydei hsrω at 48B, the only significant cross-hybridization detected at any stringency is a fragment that includes the 3′ end of the intron and part of the second exon. The hybridization is due to 59 bp of perfect homology that begins 39 bp from the 3′ end of the intron. D. melanogaster and D. hydei are separated by 60 million years. When the sequence of D. melanogaster is compared with that of 58C from D. pseudoobscura, which is

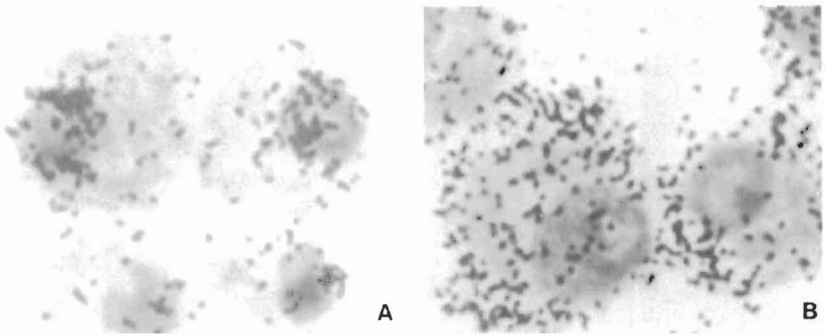

Figure 2. Autoradiograph of transcripts detected by in situ hybridization. Transcripts of hsrω show a different distribution from those of other heat shock genes. Cultured Schneider cells were heat shocked for one hour at 36°C, then spun onto slides, fixed, permeabilized, and hybridized to ^{3}H-RNA complementary to ω3 (A) or hsp70 (B). The ω3 probe detects all 3 hsrω transcripts. The hsrω transcripts are concentrated near the periphery of the nucleus with only a small fraction in the cytoplasm. hsp70 RNA is predominantly cytoplasmic with very little detected in the nucleus. Giemsa stain. Magnification x1850.

separated by 30 million years, the perfect homology has been extended by 2 bp in the intron. Cross-hybridization indicates that the conservation of this particular region extends throughout the genus (Garbe et al., in preparation).

The conservation of the intron/exon fragment argues that this sequence has an important function. One possible function would be to enable splicing under heat shock conditions. If the sequence is necessary for such splicing, one might expect the same conservation in the intron/exon region of the hsp83 transcript, the other heat shock RNA that is known to be spliced. That comparison gives no evidence that the hsrω conservation is necessary for heat shock splicing. The hsp83 sequence has been determined for D. melanogaster, D. pseudoobscura, and D. virilis (5). The hsp83 transcript shows very little sequence conservation in this region. There are only 9/39 matches among the three species at the 3'end of the hsp83

intron. There is a higher level of conservation in the exon (18/first 20 bp of the exon). However the exon is part of the protein coding sequence in hsp83 and the conservation may be explained by the demands of the protein sequence. The second exon of hsrω is not part of an ORF and thus seems likely to be conserved for some other reason (2; 6). One possibility that we are currently testing is that the hsrω intron-exon sequence might serve to regulate splicing and the entry of ω3 into the cytoplasm.

Although the intron-exon segment has the only sequence conservation that is easily detected by cross-hybridization, a comparison of the DNA sequences shows other, much shorter regions of homology between 93D and 2-48B. Hultmark et al., (7) have noted that the D. melanogaster hsp loci, with the exception of hsp 83, have identical nucleotides in the -1, +1, +7, +12, +15, and +20 positions. The three hsrω loci also have the same nucleotides in the first five of these sites, but have a T rather than an A in position +20. The hsrω loci all have 14 nucleotides conserved around the 5′ splice site and 14 nucleotides conserved at the polyadenylation site. All of the regions conserved between D. hydei and D. melanogaster are also conserved in the D. pseudoobscura transcript. These conserved regions suggest that it is important to the cell to be able to make and splice an RNA of this size and to be able to produce it under heat shock conditions.

The ωl transcripts contain all of the sequence in the ω2 transcript, followed by some "spacer" sequence and several kilobases of a short tandem repeat. Within each Drosophila species the repeats show less than 10% divergence from each other (8; 2). In spite of the homogeneity within a species, the repeat segments of the ωl transcript are highly divergent, both in the length of the repeat and in its sequence. The D. melanogaster repeat is about 280 bp. The D. hydei repeat is 115 bp. The only shared sequence is a nine nucleotide segment, ATAGGTAGG. Interestingly, the segment is found twice in the 280 bp repeat and once in the 115 bp repeat so that it is distributed about equally along the two RNAs. It seems very likely that this sequence is a binding site for something. If so, the two transcripts may be functionally equivalent in spite of the overall sequence divergence.

RELATIVE LEVELS OF THE hsrω TRANSCRIPTS VARY WITH THE PHYSIOLOGICAL STATE OF THE CELL

One phenotype of the hsrω puff is its induction by agents that do not induce other members of the heat shock puff set. For instance, both benzamide and colchicine induce puffing specifically at hsrω (1). In addition, during the developmental sequence of puffing, there is puffing at 93D. The evidence that hsrω responds to so many agents suggests that it is especially sensitive to environmental conditions. In addition, Lakhotia (1) found that treatments inducing a puff at 93D block subsequent induction by a second agent, if the two inducers are applied in a relatively short time. This evidence that the locus is refractory to a second closely-spaced induction suggests that it is autoregulated.

Analysis of RNA from different developmental stages shows clear evidence of developmental regulation of hsrω (Figure 1 D-E). No hsrω transcripts are detected in very early embryos but they appear to be among the first transcripts of the zygote genome. Levels of hsrω RNA are low in the early larval instars but rise in the third instar and remain high throughout pupal life. The developmental profile of hsrω transcript abundance is reminiscent of ecdysone-induced transcripts, suggesting that the hsrω locus is also sensitive to ecdysone. A D. melanogaster cell line, Schneider line S-3, that is sensitive to ecdysone was used to test ecdysone responsiveness. Ecdysone treatment (10^{-6} M) of these cells induces accumulation of the hsrω transcripts. This induction is not an indirect result of a heat shock since we can detect no induction of hsp 70 RNA. Ecdysone has been shown to induce hsp22 both in S-3 cells and in the intact animal (9). In ecdysone-treated S-3 cells, the rise in hsrω continues for at least 24 hrs, whereas hsp22 RNA increases for 4 hrs and then declines. This suggests that hsrω is involved in a later stage of the ecdysone-induced developmental program.

The slower induction of hsrω by ecdysone also suggests that hsrω transcription is a secondary response to the hormone. It is customary to test for secondary responses by giving ecdysone in the presence of cycloheximide to inhibit synthesis of any new proteins that might be induced by the hormone. We have found that cycloheximide alone leads to high levels of the ω3 transcript. The effect of cycloheximide on ω3 levels

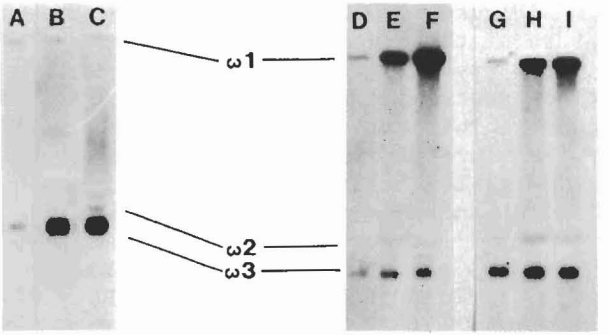

Figure 3. Inhibition of protein synthesis leads to increases in the levels of the hsrω3 transcript; treatment with benzamide or colchicine hyperinduces ω1.. RNA from Schneider S-3 cells was probed with the ω3 sequence. A, control; B and C, cells treated for 2 hours with cycloheximide (10^{-4} M) or pactamycin (10^{-7} M); D and G, control cells; E and F, benzamide (10 mM) for 12 and 24 hrs; H and J, colchicine (100 μg/ml) for 12 and 24 hr.

appears to be due to inhibition of protein synthesis since inhibitors of both initiation (pactamycin) and elongation (emetine, cycloheximide) produce the same result (Figure 3, A-C). The drugs appear to act largely, if not entirely, by inhibiting the turnover of the ω3 transcript. At least during recovery from heat shock, hsrω transcripts turn over much more rapidly than do other RNAs. Drugs affecting translation seem to specifically stabilize the ω3 transcript; we see no significant increase in either the ω2 or the ω1 RNAs. Cycloheximide-induced increases in ω3 levels are seen in salivary glands, as well as in diploid cells, yet we can see no puffing in the 93D region. This lack of puff induction supports the suggestion that cycloheximide is acting only to inhibit turnover of the cytoplasmic transcript.

We have also studied two of the drugs that induce puffing specifically at 93D, colchicine and benzamide. These drugs affect the levels of hsrω transcripts very differently than do the translational inhibitors. Both colchicine and benzamide treatments lead to high levels of the ω1 RNA but produce less dramatic changes in the levels

of ω2 and ω3 (Figure 3, D-I). We do not yet know what common feature of these two drugs leads them to affect hsrω in similar ways; however this increase in the ω1 transcript contrasts with the high levels of ω3 induced by translational inhibitors. The different patterns of hsrω transcripts that are induced by different drugs suggests that the relative levels of hsrω transcripts may reflect different disturbances in cellular metabolism.

THE SMALL OPEN READING FRAME IN THE ω3 TRANSCRIPT APPEARS TO BE TRANSLATED.

The ω3 transcript is both spliced and polyadenylated, two characteristics that are generally associated with mRNA, yet we have been unable to detect a polypeptide that might be encoded by this RNA (Figure 4, A-C). The potential open reading frames (ORFs) in the ω3 transcript are all very short in comparison to the size of the transcript. In addition, there is almost no conservation of the ORFs that are seen. The single exception is a small ORF (ORF-ω) beginning near position +120 from the transcription start site. This is the first ORF that is in what is thought to be an appropriate context for translation (10). The first four amino acids in ORF-ω are conserved in all three Drosophila species. The conservation is at the level of amino acids rather than nucleotide sequence. Surprisingly there is little obvious conservation of the rest of the ORF-ω polypeptides; however their lengths are almost the same. ORF-ω in D. melanogaster encodes 27 amino acids, 23 in D. hydei, and 24 amino acids in D. pseudoobscura.

Although we have not detected a polypeptide product of hsrω, we did find that the ω3 transcript sedimented with the monosome/disome peak on polysome gradients. This suggestion that the ω3 transcript was translated was supported by other types of analysis; buoyant density in metrizamide polysome gradients, EDTA release from polysomes, and studies with translational inhibitors. The several kinds of evidence that ω3 was associated with small polysomes, plus the evidence that any disruption of protein synthesis leads to stabilization of ω3 RNA, led us to suspect that ORF-ω was translated (Fini et al., in preparation).

If ORF-ω is translated, it would be expected to inhibit the translation of other ORFs on the same RNA

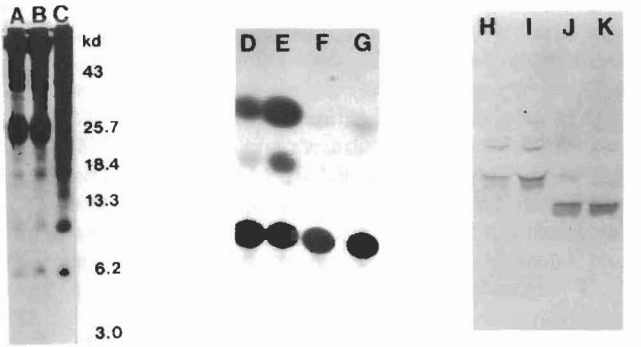

Figure 4. ORF-ω on the hsrω3 transcript appears to be translated although a heat shock induced polypeptide of the expected size is not detected. Proteins from Schneider S-3 cells labeled with ^{3}H-amino acids between 30 and 60 minutes of heat shock (A and B) are compared to control cells (C). Other heat shock proteins are clearly detected. The positions of molecular weight standards are indicated. The leader of D. melanogaster hsrω3 to +67 does not inhibit the translation of a downstream CAT gene at 25°C (D) or at 36°C (E). The D. melanogaster hsrω3 leader to +200 containing ORF-ω does inhibit the translation of a downstream CAT gene at both 25°C (F) and 36°C (G). The D. hydei ORF-ω fused in frame to CAT results in the synthesis of a fusion protein (H and I) larger than the original CAT protein (J and K). Both CAT and the ORF-ω/CAT fusion protein are detected with anti-CAT antibody.

molecule. To test this prediction, two recombinant DNA molecules were constructed utilizing the CAT/SV40 chimeric gene. Both of these constructs contained the upstream control regions of 93D, to ensure that the gene could be induced by heat shock, and part of the ω3 sequence. In the ω-leader-CAT construct, the ω3 sequence ends just in front of ORF-ω and is replaced by the bacterial chloramphenicol acetyl transferase (CAT) gene. In the ORF-ω-CAT construct, the CAT gene had been added 30 nucleotides behind ORF-ω. In both constructs the CAT gene has its own translation start. Both constructs were stably transformed into cultured Drosophila cells.

Although ORF-ω-CAT produced significantly more cytoplasmic RNA than ω-leader-CAT, only the ω-leader-CAT cells made significant amounts of the CAT enzyme (Figure 4, D-G). This result suggested that ORF-ω was being translated and inhibiting translation of the CAT gene immediately behind it. Direct evidence for the translation of ORF-ω came from a third construct in which the CAT coding region was linked in frame to ORF-ω of D. hydei. Cells stably transformed with this construct made a CAT protein that was larger than the wild type protein by the amount expected if translation had started in ORF-ω (Figure 4, H-K).

The ORF-ω-CAT fusion protein is easily detected in the transfected cells. Our hypothesis is that the normal translation product of ORF-ω is not detected because it is turned over very rapidly. Because only the first four amino acids are strongly conserved, it may be that it is the act of translation of ORF-ω, rather than its product that is important to the cell.

THE LOSS OF hsrω APPEARS TO BE DETRIMENTAL TO THE ORGANISM

An attempt to saturate the 93D region with lethal and visible mutations (11) produced no detectable point mutations in the sequences that are now identified as hsrω. This lack of mutations seems less surprising now that we know the hsrω sequence and the way in which it is evolving. It appears that there are only a few regions where nucleotide substitutions would disrupt the functions of the transcripts.

During the mutational analysis, several large deletions that removed hsrω were characterized. Two deletions were identified that seem to be particularly useful. One, $Df(3)e^{Gp4}$, removes hsrω and 11 complementation groups proximal to it. The other, Df(3)GC14, removes hsrω and 3 complementation groups distal to it. Animals heterozygous for these two deficiencies should be totally lacking only the hsrω locus. Such heterozygotes should make up one quarter of the progeny of a cross between two flies, each carrying one of the deficiencies over a balancer chromosome with an intact hsrω locus. Embryos lacking hsrω hatch as well as siblings of other genotypes; however, the egg was produced by a mother with an intact hsrω locus so we cannot draw

conclusions about the need for hsrω during embryogenesis. The lack of hsrω becomes apparent as soon as larvae hatch. Larvae heterozygous for the two deficiencies grow very slowly and most die before pupation. Less than 5% emerge as adults and these die very soon. We are in the process of using P-element-mediated transformation in an attempt to rescue the larvae with the hsrω sequence. If such rescue proves that the phenotype we see is actually due to the loss of hsrω, it will provide clear evidence that hsrω has an important effect on viability.

SUMMARY

In spite of sequence divergence, the hsrω loci have some conserved regions that give clues to their function. These clues suggest that the ω1 transcript could serve as a binding site for a nuclear protein while the ω3 transcript could give a measure of translational activity. It seems reasonable to suggest that hsrω may coordinate nuclear and cytoplasmic activities. Such a role is consistent with evidence that hsrω is very sensitive to environmental conditions and that its loss affects viability.

REFERENCES

1. Lakhotia SC (1987). The 93D heat shock locus in Drosophila: a review. J Genet 66:139.
2. Garbe JC, Bendena WG, Alfano MA, Pardue ML (1986). A Drosophila heat shock locus with a rapidly diverging sequence but a conserved structure. J Biol Chem 261: 16889.
3. Ryseck R-P, Walldorf U, Hoffman T, Hovemann B (1987). Heat shock loci 93D of Drosophila melanogaster and 48B of Drosophila hydei exhibit a common structural and transcriptional pattern. Nuc Acids Res 15:3317.
4. Lindquist S (1986). The heat-shock response. Annu Rev Biochem 55:1151.
5. Blackman RK, Meselson M (1986). Interspecific nucleotide comparisons used to identify regulatory and structural features of the Drosophila hsp82 gene. J Mol Biol 188:499.

6. Garbe JC (1988). Characterization of heat shock locus 93D in Drosophila melanogaster. PhD thesis Massachusetts Institute of Technology, Cambridge, MA
7. Hultmark D, Klemenz R, Gehring WJ (1986). Translational and transcriptional control elements in the untranslated leader of the heat shock gene hsp22. Cell 44:429.
8. Peters FPAMN, Lubsen NH, Walldorf U, Moormann RJM, Hovemann B (1984). The unusual structure of heat shock locus 2-48B in Drosophila hydei. Mol Gen Genet 197:392.
9. Ireland RC, Berger E, Sirotkin K, Yund MA, Osterbur D, Fristrom J (1982). Ecdysterone induces the transcription of four small heat shock genes in Drosophila S-3 cells and imaginal discs. Develop Biol 93:498.
10. Kozak M (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283.
11. Mohler J, Pardue ML (1984). Mutational analysis of the region surrounding the 93D heat shock locus of Drosophila melanogaster. Genetics 106:249.