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Heat shock but not benzamide and colchicine response elements are present within the — 844 bp upstream region of the *hrsω* gene of *Drosophila melanogaster*

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Abstract. The selective inducibility of *hsrω* gene by heat shock and several chemical agents and its selective non-inducibility by heat shock under certain conditions led to suggestion that this locus is subject to multiple controls at the level of transcription. With a view to delimit these different control elements, transgenic lines horbouring *hsrω* 5' promoter deletion variants tagged to the *lacZ* reporter gene were used. Three different assays, viz., staining for *β*-galactosidase activity in different larval tissues using chromogenic X-gal substrate, [^{3}H] uridine labelling of polytene nuclei and *in situ* DNA-DNA hybridization with a non-radioactive probe to polytene chrmosome spreads for checking the puffing status of the resident and the transgene in larval salivary glands, were applied to monitor the activiy of the reporter gene following different treatments. Our results showed that the – 844 bp to +107 bp sequence was sufficient for heat shock induction of the transgene in all tissues. An analysis of the base sequence of the *hsrω* promoter revealed the presence of three consensus heat shock elements at – 466, – 250 and at – 57 bp and of two GAGA factor binding sites at – 496 and at – 68bp within the – 844 bp region. Germline transformants carrying the – 346 bp to – 844 bp region of the *hsrω* promoter showed only a very weak heat shock inducibility of the reporter gene in agreement with the presence of only one of the three putative heat shock elements and one of the two GAGA factor binding sites in this region. Interestingly, neither of the transformed lines (carrying the – 844 bp to + 107 bp or the – 844 bp to –346 bp of the *hsrω* promoter region) showed any response of the transgene to benzamide or colchicine treatments. These results showed that while the heat shock response elements of the *hsrω* are included within the – 844 bp region the response elements for benzamide and colchicine treatments are outside this region.

Keywords. 93D locus; HSE; polytene chromosomes; GAGA factor; non protein coding gene.

1. Introduction

The *hsrω* gene at the 93D locus in *Drosophila melanogaster* is an unusual heat shock gene which, although highly induced by heat shock, does not code for any of the heat shock proteins and is also singularly inducible by a variety of other agents like benzamide, colchicine etc., (reviewed by Lakhotia and Sharma 1995a). It is significant that the kinetics of induction of this locus by heat shock are different from all other heat shock loci (Mukherjee and Lakhotia 1979; Vazquez *et al* 1993) and under certain conditions of heat shock, the *hsrω* locus remains refractory to heat shock induction (see Lakhotia 1987, 1989; Lakhotia and Sharma 1996). Furthermore, the kinetics of

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binding of the heat shock transcription factor (HSF) to the 93D locus during heat shock is also different from that at the other heat shock loci (Westwood *et al* 1991; Wu *et al* 1994). Some of the earlier studies also suggested that the upstream region of the *hsrω* gene did not carry a typical heat shock element (HSE) and this was believed to be responsible for its unusual inducible properties (Hovemann *et al* 1986; Garbe *et al* 1989; Lakhotia 1989). However, a direct analysis of the promoter region of the *hsrω* locus for the regulation of its various inducible properties has not yet been made. We have recently studied the promoter region of the *hsrω* gene for its developmental expression through promoter-deletion variants driving a reporter gene (*lacZ*) in germline transformed lines (Mutsuddi and Lakhotia 1995). In the present study, we used these transgenic lines to characterize the upstream region controlling its heat shock and other inducibility. Our results showed that a region up to – 844 bp of this gene is sufficient for heat shock inducibility of *hsrω* gene but the regulatory sequences involved in its colchicine and benzamide inducibility are not included in this region.

2. Materials and methods

All flies and larvae were reared under uncrowded condition at 22° ± 1°C on the standard *Drosophila* food supplemented with additional yeast.

2.1 *Transgenic lines*

The transgenic lines carrying different lengths of the 93D promoter region used in this study were the same as described earlier (Mutsuddi and Lakhotia 1995). One set of lines carried a 951 bp region (from – 844 bp to + 107 bp) upstream of the *lacZ* reporter gene in pCaSpeR-AUG-*β*-gal vector; the other set of lines carried the region from – 844 bp to – 346 bp of the *hsrω* gene upstream of the *lacZ* reporter gene in pCaSpeR-hs43-*β*-gal vector (for details see Mutsuddi and Lakhotia 1995). The lines carrying 951 bp of *hsrω* were designated as *951 lacZ* and those carrying 498 bp of *hsrω* promoter as *498lacZ* lines. Two different transgenic lines were used for the *951lacZ* construct transgene in the first line (*951lacZ1*) was inserted at 30EF on 2L and was a semi-lethal insertion due to which it was maintained against the CyO balancer (Lindsley and Zimin 1992) while in the other line (*951lacZ2*), the insertion was at 30B on 2L and was homozygous viable. The *498lacZ1* line used in this study had the transgene inserted at 44B on 2R and was homozygous viable. Earlier studies using these transgenic lines (Mutsuddi and Lakhotia 1995) had established that the insertion in each case was free of position effect etc.

2.2 *Heat shock, benzamide and colchicine treatments*

Different tissues were dissected out from actively migrating late third instar larvae of the different transgenic lines in Poels' salt solution (PSS, Lakhotia and Mukherjee 1980) and were immediately subjected to one of the following treatments.

Control: incubated in PSS at 24°C for 30 min;
Heat shock: incubated in PSS at 37°C for 40 min;
Benzamide incubated at 24°C for 10 min in PSS containing benzamide (BDH, Poole, UK) at a concerntration of 1·0 mg/ml;

Colchicine: incubated at 24°C for 40 min in PSS containing colchicine (Russel Doglas, France) at a concentration of 0·1 mg/ml.

In addition to the above *in vitro* treatment, *in vivo* heat shock treatment was also applied to late third instar larvae from the desired transgenic lines by placing them in a moist Petri dish at 37°C for 30 min; following the heat shock, they were allowed to recover for 30 to 60 min at room temperature before an assessment of the induced *lacZ* reporter gene activity.

The *lacZ* reporter gene activity in the different larval tissues following the various treatments was assessed by X-gal staining as described earlier (Mutsuddi and Lakhotia 1995). In the case of larval salivary glands, the following two additional methods were employed.

2.3 [^3H] *uridine labelling of salivary glands*

Control, heat shocked, benzamide and colchicine treated salivary glands from *951lacZ1* (either with the CyO balancer or the few surviving homozygotes with transgene insertion at 30EF) and *951lacZ2* (with transgene insertion at 30B) larvae were labelled with [^{3}H] uridine (Activity 250 μCi/ml, Sp. Act. 18Ci/mM, BRIT, Bombay) for 10 min at 24°C or at 37°C (in the case of HS treatment) following which glands were squashed and processed for autoradiography (Lakhotia *et al* 1990).

2.4 *In situ hybridization to polytene chromosomes* (*DNA-DNA*)

DNA-DNA non-radioactive *in situ* hybridization was employed to determine the puffing status of the resident (93 D) and the transgene insertion site following the various treatments to salivary glands of *951lacZ2* and *4981acZl* lines. The squash preparations of variously treated salivary glands from the transgenic lines were processed for *in situ* hybridization with the Digoxigenin (DIG) labelled 951 bp *Eco*RI-*Bam*HI fragment of the *p951lacZ* clone to chromosomal DNA as described (see Mutsuddi and Lakhotia 1995 for details). Hybridization signal was detected colorimetrically following the manufacturer's (Boehringer and Mannheim) instructions. After examining and photographing the hybridization signal under phase optics, the chromosomes were stained with Giemsa and re-examined. At leat 20 nuclei were examined in each case.

3. Results

3.1 *Activity of hsrω promoter deletion variants following different treatments as revealed by X-gal staining for the reporter gene activity*

The level of induction of the transgene by heat shock, benzamide and colchicine in different larval tissues was checked by X-gal staining for β-galactosidase acivity. Heat shock (applied either *in vitro* or *in vivo,* see §2) led to an enormous increase in the intensity of staining in all the third instar larval tissues (various imaginal discs, brain-ganglia, gut, Malpighian tubules and salivary glands etc) from either of the *951lacZ* lines. However, heat shock treatment to tissues from the *498lacZ1* line caused only a small increase in the intensity of X-gal staining. Representative examples of the

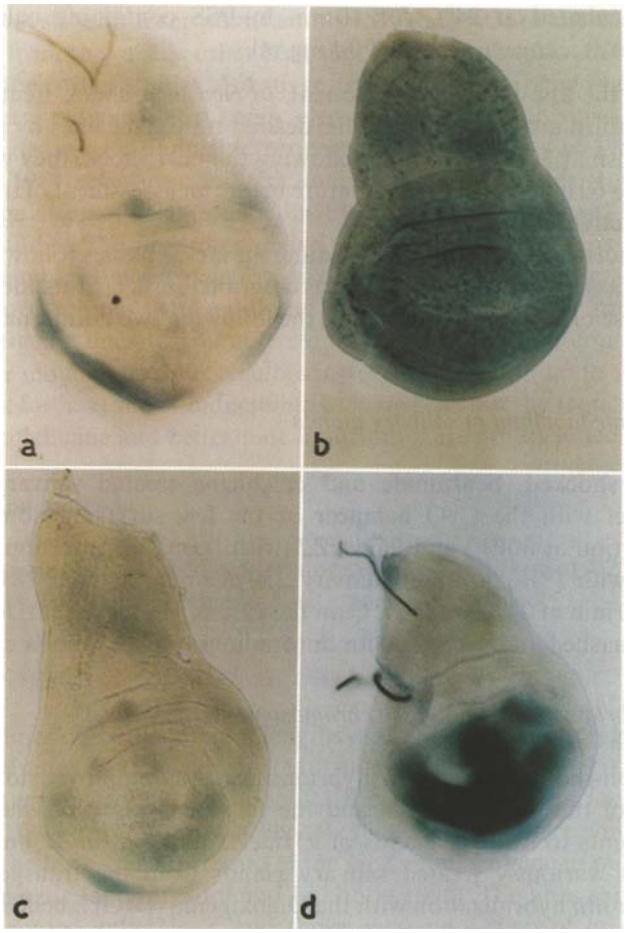

Figure 1. *lacZ* expression in wing imaginal discs of *951lacZ2* (**a,b**) and *498lacZ1* (**c,d**)**:** compared to the control (**a),** the heat shocked disc from *951lacZ2* larvae shows an enormous increase in X-gal staining (**b**)**;** however, compared to control (**c**) only a marginal increase in staining is seen in heat shocked disc (**d**) from the *498lacZ1* line.

X-gal staining in control and heat shocked larval wing imaginal discs from the *951lacZ2* and *498lacZ1* lines are shown in figure 1.

Benzamide or colchicine treatment did not lead to any increase in X-gal staining, beyond the control level, in any of the larval tissues neither in *951lacZ* nor in *4981acZ* lines (not shown).

3.2 [^3H] *uridine incorporation at the transgene and at the resident hsrω gene after different treatments in 951lacZ2 line*

Salivary glands from late third instar larvae of *951lacZ2* line were lablled with [^{3}H] uridine under control conditions, or after heat shock, benzamide or colchicine treatment and the number of silver grains at the resident 93D locus and at the site of transgene insertion (30B on 2L) were recorded. Heat shock treatment led to a well

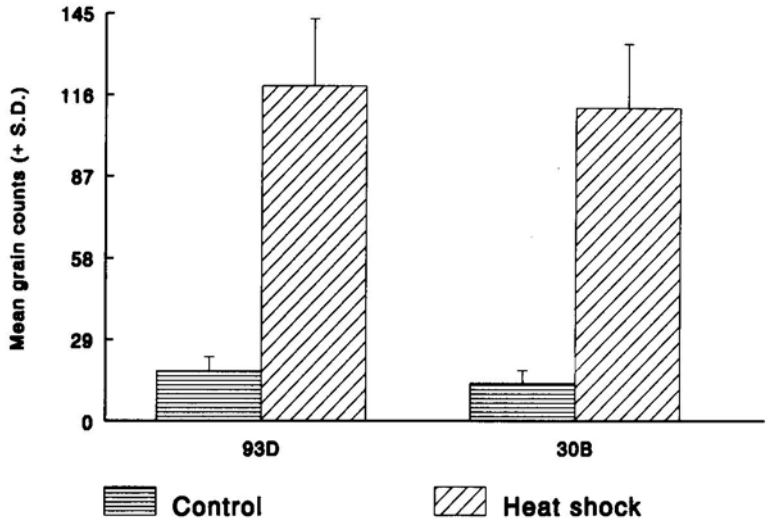

Figure 2. Histogram showing the mean numbers of silver grains al 93D and 30B sites in control and heat shocked *951lacZ2* salivary glands labelled with [^{3}H] uridine (the numbers of nuclei examined in each case is same as in table 1). Note the massive increase in ^{3}H-uridine incorporation at both the sites following heat shock.

Table 1. Comparison of the ratio of the numbers of silver grains at 93D and at the 30B site in control and heat shocked salivary glands of *951lacZ2* line.

Treatment	Mean($\pm$ SD)93D/30B ratio
Control	$1{\cdot}347 \pm 0{\cdot}086 (n = 16)$
Heat shock	$1{\cdot}279 \pm 0{\cdot}062 (n = 28)$

develped puff at the resident 93D site as well as at the 30B region where the transgene was inserted and parallel to the induction of puffs at these sites, the [^{3}H] uridine labelling at both these sites recorded a large increase in the numbers of silver grains after heat shock (see figure 2). The data in table 1 show that heat shock induced increase in [^{3}H] uridine incorporation (also see figure 3a) at the 30B site was of the same order as at the resident 93D site since the 93D/30B grain ratios in control and heat shocked nuclei did not differ significantly (Student's "*t*" = 1·883, $P > 0{\cdot}05$ at 42 degrees of freedom).

Interestingly, the benzamide or colchicine treatments to salivary glands of *951lacZ2* larvae caused neither a visible puff nor any increase in the number of silver grains at the site of transgene insertion (30B), although the resident *hsrω* gene at the 93D site showed, as expected, a good puff and significantly increased levels of [^{3}H] uridine incorporation after either of these treatments (see figure 3b, c).

The *951lacZ1* line, which is a semi-lethal line with insertion at 30EF, and in which a few larvae homozygous for the transgene insertion survive to late third instar stage, was also examined for [^{3}H] uridine incorporation at the transgene following heat shock, benzamide or colchicine treatment. In this line too, heat shock led to a good induction of the transgene at 30EF (data not presented) whether in single copy as in the *951lacZ1*/*CyO* heterozygous larvae or in two copies as in the rare *951lacZ1/951lacZ1*

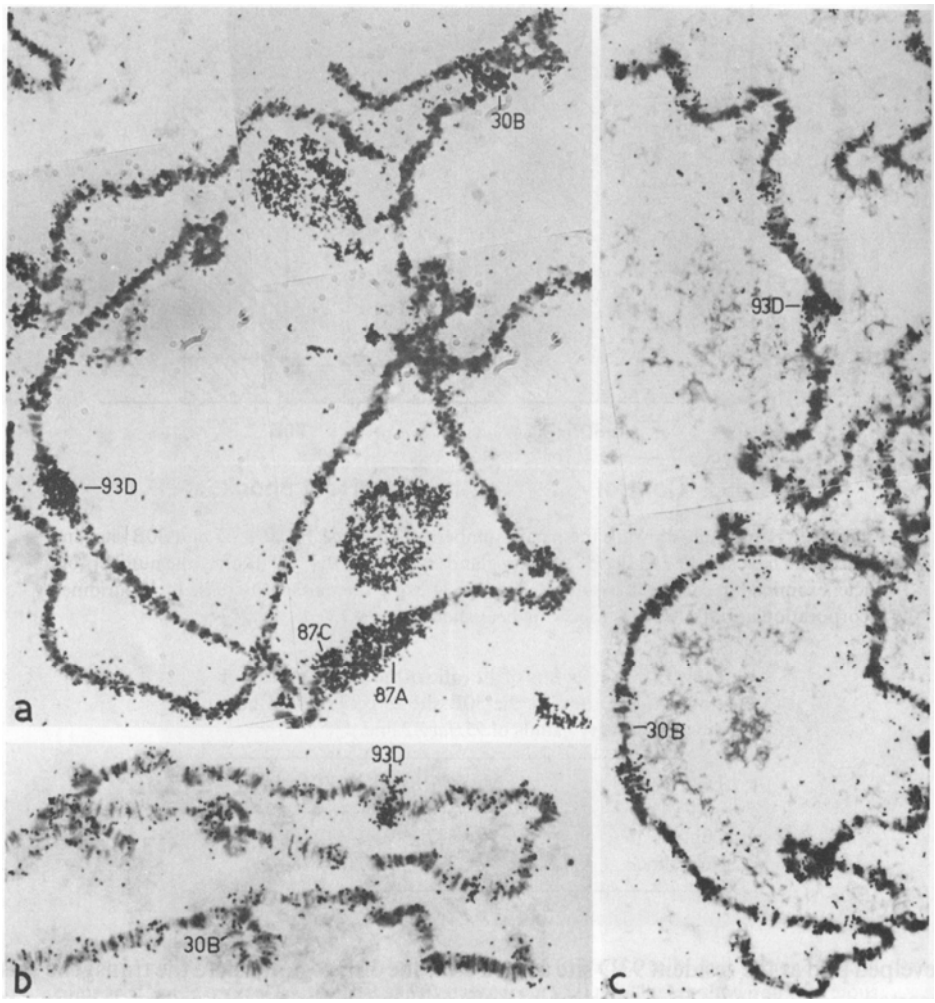

Figure 3. ^{3}H-uridine labelling of polytene chromosomes in salivary glands of *951lacZ2* larvae following heat shock (**a**)**,** colchicine (**b**) or benzamide (**c**) treatment. Note the good induction of the resident 93D as well as the transgeneat 30B following heat shock (**a**); in (**b**) and (**c**)**,** on the other hand, only the resident 93D is strongly labelled with [^{3}H] uridine following colchicine or benzamide treatment while the transgene at 30B remains inhibited. The 87A and 87C puffs, the duplicate loci for HSP70, are also highly induced by heat shock in (**a**)**.**

homozygous surviving larvae. Further, as in the *951lacZ2* line, benzamide or colchicine treatment failed to induce the transgene at 30EF, although the resident 93D puff was, as expected, well induced (not shown).

3.3 *Puffing activity of the transgene and the resident hsrω gene after different treatments as revealed by in situ hybridization*

A DIG-labelled probe for the 93D promoter region was hybridized *in situ* to chromosomal DNA in polytene spreads from the variously treated salivary glands of *951lacZ2*

and *498lacZ1* lines to allow an unambiguous identification of the resident as well as the transgene sites. Since puffing is a good indicator of transcriptional activation (Beermann 1967), the band-like or the spread out appearance of the signal distinctly revealed the activity status of these sites. The resident 93D gene in each case provided a very good internal control. In control salivary gland nuclei of the *951lacZ2* as well as the *498lacZ1* lines, the resident 93D as well as the trangenes were not puffed and the hybridization signal was seen as a more or less well-defined band (figure 4a, c). As shown in the example in figure 4, large puffs were always induced at the resident *hsrω* and at the transgene sites in heat shocked polytene nuclei from the *951lacZ2* line (figure 4b). However, in the *498lacZ* line (figure 4d) while the resident 93D locus was always puffed after heat shock as expected, the site of the insertion (44EF) of the chimeric gene was only marginally puffed.

Benzamide or colchicine treatment induced the resident 93D gene in polytene chromosomes from both *951lacZ* and *498lacZ* lines; however, the transgene in all cases remained as unpuffed as in the corresponding control nuclei (figure 4e-h).

4. Discussion

The selective inducibility of *hsrω* by several chemical agents and its selective non-inducibility by heat shock under certain conditions showed that this locus is subject to mutiple controls at the level of transcription (Lakhotia 1987,1989; Lakhotia and Sharma 1996). We have used 5' promoter mutants tagged to *lacZ* reporter gene to delimit the rgulatory elements that control the inducibility of this locus under heat shock and non-heat shock conditions. The three transgenic lines used in this study were earlier used by us (Mutsuddi and Lakhotia 1995) to characterize the promoter region of the *hsrω* locus that regulated its developmental expression. Since the reporter gene expression in all these lines was found to be unaffected by the site of insertion, we are certain that the reporter gene activity in response to the various inducers used in this study also reflected the promoter activity of the upstream region present in each of the two transgene constructs.

Our results from these different assay systems, viz., X-gal staining for *β*-galactosidase activity in different tissues, [^{3}H] uridine incorporation and DNA-DNA *in situ* hybridization to monitor the puffing status in polytene chromosomes, clearly showed that – 844 bp to + 107 bp of *hsrω* sequence was sufficient for heat inducibility of the reporter gene. The region of *hsrω* unpstream sequence (from – 844 bp to – 346 bp) present in the *498lacZ* line was, however, not sufficient for a typical heat shock inducibility of this transgene since heat shock resulted in a very small puff at the transgene insertion site and only a marginal increase in X-gal staining. It appears, therefore, that the upstream region up to – 346 bp is crucial for heat shock inducibility of *hsrω*.

The response to heat shock depends primarily on binding of the activated H S F with multiple HSEs present in the upstream region of heat shock genes (Perisic *et al* 1989; Xiao *et al* 1991; also see Srinivas and Swamynathan 1996). Earlier imunocytochemical localization studies showed that the HSF binds poorly at the 93D heat shock puff (Westwood *et al* 1991). Maximum binding of HSF at this locus was observed up to 30s after the heat shock but at later time points the intensity of staining for HSF was considerably weak (Wu *et al* 1994). The HSE was initially defined as a 14 bp consensus sequence, 5'CTNGAANNTTCNAG3' (Pelham 1982). Hovemann *et al* (1986) and Garbe *et al* (1989) analysed the promoter region of the *hsrω* locus and looked for HSEs based on this 14 bp consensus and suggested that the *hsrω* promoter region showed

only imperfect matches to the HSE. However, subsequent studies have led to a redefinition of HSE as a repeating array of the 5 bp sequence 5'-nGAAn-3', where each repeat is inverted relative to the immediately adjacent repeat (Amin *et al* 1988; Xiao and Lis 1988; Xiao *et al* 1991). The G at position 2 is highly conserved and base substitution at this region abolishes heat inducibility (Xiao and Lis 1988), whereas the conservation at positions 3 and 4 is less marked. Recent studies have also shown that though the base at position 1 is variable, an A is preferred at this site (Fernandes *et al* 1994). Mutsuddi and Lakhotia (1995) have recently extended the sequence information for the *hsrω* promoter up to the – 844 bp (see figure 5, EMBL accession no. X85039). We have reanalysed the

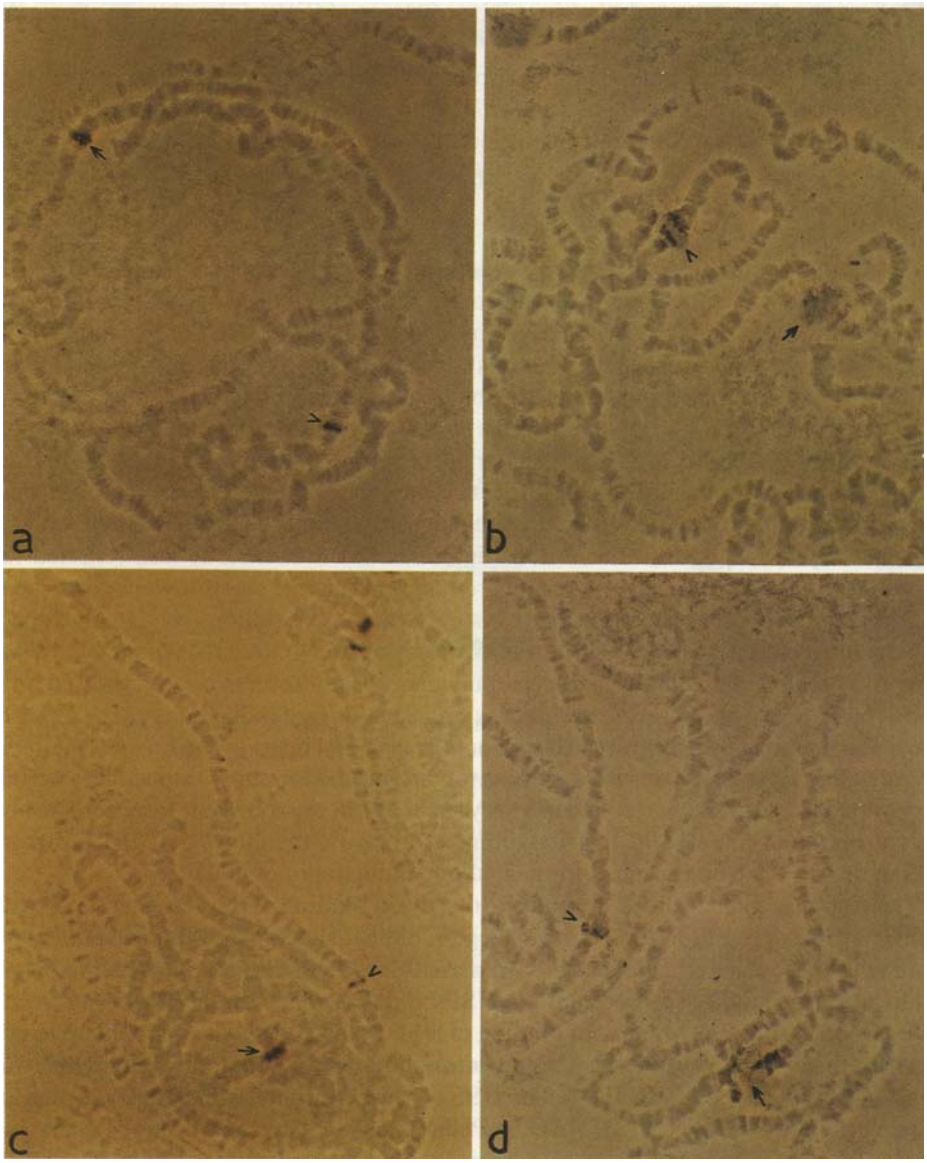

Figure 4. a-d.

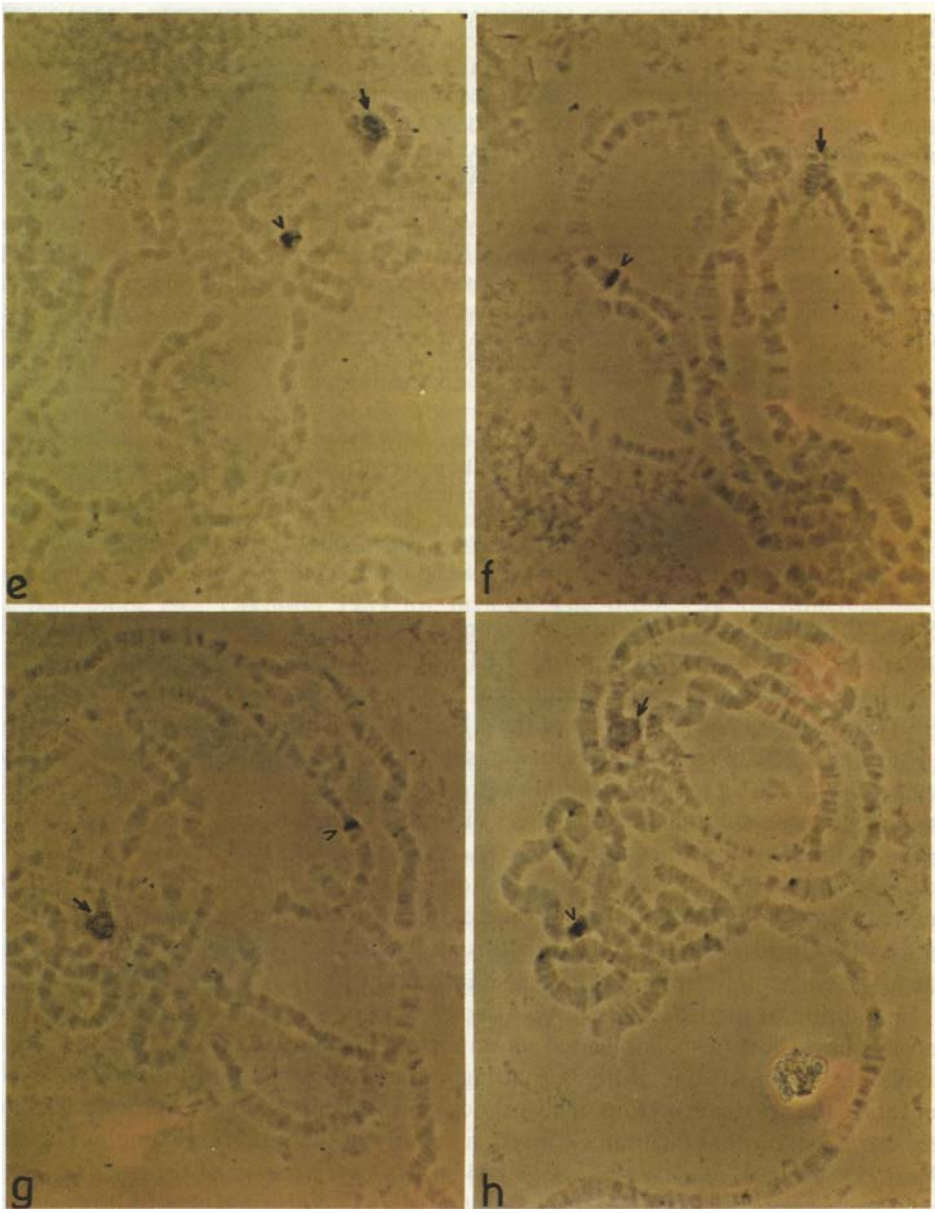

Figure 4. *In situ* hybridization of Dig-labelled 93D probe to polytene spreads from control (**a, c**): heat shock (**b,d**), benzamide (**e, g**) or colchicine treated (**f, h**) salivary glands from *951lacZ2* (**a, b, e** and **f)** and *498lazZ1* (**c, d, g** and **h)** lines. The chromosomes are viewed under phase contrast optics; the resident *hsrω* gene is marked by arrow and the transgene by arrow-head. Note that the hybridization signal is either a sharp band or is diffused depending upon the unpuffed or puffed state of the site.

– 844 bp region of the 93D promoter for putative HSE consensus sequence using the redefined HSE motif; as may be seen in figure 5, this search revealed three HSE consensus motifs in the – 844 bp of the 93D promoter at (i) –466 bp to –452 bp

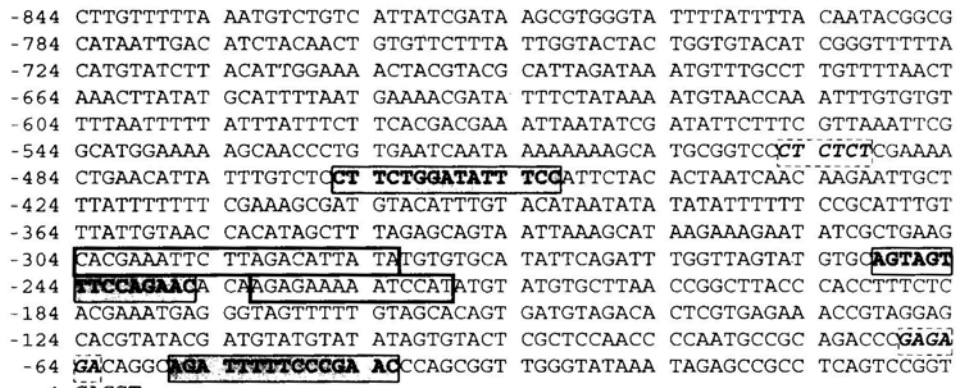

Figure 5. Base sequence of the upstream region up to – 844 bp of the *hsrω* locus as described by Mutsuddi and Lakhotia (1995) showing the proposed locations of HSEs (bold letters in shaded boxes) and GAGA-factor binding sites (italic latters in broken line boxes). The bold T at position + 1 is taken as the transcription start point following Hovemann *et al* (1986). The other regions suggested as possible HSEs (see text) in the *hsrω* sequence in the EMBL database (Accession no. U18307) are boxed without shading.

(5'C*TTC*TG*GAT*AT*TTC*C3'), (ii) – 250 bp to – 236 bp (5'A*GTA*GT*TTC*CA*GAA*C3') and (iii) at – 57 bp to – 43 bp (5'A*GAT*TT*TTC*CC*GAA*C3'), respectively. The second HSE has A at position 1 of the pentamer at two places and thus should be a very strong HSE (Fernandes *et al* 1994). Two of the 4 HSE consensus regions (from – 57 to – 43 and from – 250 to –236 bp regions, respectively) identified by Garbe *et al* (1989) within the – 360bp region of the *hsrω* promoter overlap with two of the three HSE sequence motifs identified in this study. The other regions suggested to be possible HSEs in the EMBL sequence database (Accession no. U18307) from – 232 to – 218 and from – 304 to – 283 bp (see figure 5), do not seem to show a good match with the revised (Xiao *et al* 1991) HSE motifs. Therefore, we do not think those to be functional HSEs. The third HSE recognized in this study (from – 466 to – 452 bp) was not identified earlier (Hovemann *et al* 1986; Garbe *et al* 1989).

In addition to the HSEs, the GAGA-factor binding sites [$(GA)_n$ or $(CT)_n$ sequence motifs] have been also implicated as important regulators of heat shock induced activation of genes (Lu *et al* 1993). Binding of the GAGA factor at the $(GA)_n$ or $(CT)_n$ repeats in the upstream regions of heat shock genes is believed to provide a nucleosome free environment that facilitates efficient assembly of the initiation complex (Lis and Wu 1993; Granok *et al* 1995). The – 844 bp promoter region of the *hsrω* (figure 5) includes two putative GAGA factor binding sites, at – 496 bp and at – 68 bp positions, respectively (Mutsuddi and Lakhotia 1995).

In agreement with the fact that the – 844bp promoter region in the *951lacZ* lines included all the three putative HSEs and the two GAGA factor binding sites, it was found to be sufficient for heat shock inducibility of the *hsrω* promoter in the *951lacZ* transgene. The transgene in the *498lacZ* lines included only the distal HSE (at – 466 to – 452 bp) and this transgene showed only a very weak heat inducibility: this makes it likely that the distal HSE is also functional but not sufficient for a complete induction of the *hsrω* promoter by heat shock. Another factor responsible for the weak heat shock activity of the *498lacZ* transgene appears to be the presence of only one of the GAGA factor binding site at – 496 bp (figure 5).

Although the present study suggests the presence of typical HSEs and putative GAGA-factor binding sites on the 93D promoter as expected of a heat shock locus, response of the *hsrω* gene to heat shock is indeed different from all other heat shock loci: its level of induction after heat shock is not correlated with the other heat shock sites which respond in a more coordinated fashion (Mukherjee and Lakhotia 1979) and the response of this locus to heat shock can be idependently varied by a number of other conditions (see reviews in Lakhotia 1987, 1989). Further more, as already noted, unlike the other heat shock loci, the binding of HSF at the 93D heat shock puff is transient (Westwood *et al* 1991; Wu *et al* 1994). The observation that the 93D promoter is induced by heat shock in larval Malpighian tubules is also significant in this context since earlier studies from our laboratory (Lakhotia and Singh 1989; Singh and Lakhotia 1995) showed that none of the common heat shock protein genes is inducible in Malpighian tubules of *Drosophila* larvae. All thsese observations suggest that the heat shock response of this locus is not simply due to binding of the HSF to HSEs. Obviously, factors other than typical HSEs and the $(CT)_n$ repeats are also involved in heat shock response of this unusual gene. These additional factors/elements remain to be identified.

Earlier studies from our laboratory showed that although the *hsrω* locus was not inducible by heat shock under certain conditions, it remained responsive to colchicine or benzamide (see Lakhotia 1989 for review). The profiles of the different *hsrω* transcripts and kinetics of their turnover etc., in response to its induction by heat shock or by the different chemical inducers too were different (Lakhotia and Sharma 1996). Our present results confirmed the earlier suggestions that the cis-acting regulatory elements for its activation by different agents were distinct since while the – 844 bp of the *hsrω* promoter was sufficient for most of its developmental expression (Mutsuddi and Lakhotia 1995) and for its heat shock inducibility (present results), this region did not include the cis-acting response elements for benzamide or colchicine. Additional support for distinct regulatory elements has been obtained in other studies in our laboratory using small chromosomal deletions that map upstream of the *hsrω* locus: it has been found that in a deletion with breakpoint about 20 kb upstream of the *hsrω* transcription start point, the benzamide and colchicine inducibility is lost although its heat shock indicibility is not affected (Tapadia and Lakhotia 1993 and in preparation). Further studies are necessary for identification of the upstream benzamide and colchicine response elements.

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