Chromosome Research 1995, **3**, 151–161

RNA metabolism *in situ* at the 93D heat shock locus in polytene nuclei of *Drosophila melanogaster* after various treatments

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Received 8 July 1994; received in revised form 27 September 1994
Accepted for publication by M. Schmid 27 September 1994

Quantitative *in situ* hybridization to RNA on polytene chromosome spreads, using the 93D exon-, intron- and repeat-specific ^{35}S-labeled antisense RNA probes, revealed treatment- (heat shock, benzamide, colchicine, heat shock followed by benzamide and heat shock in the presence of colchicine) specific differences in the metabolism (synthesis and/or accumulation at the puff site) of the various hsr-omega transcripts, namely hsr-omega-nuclear (omega-n), omega-pre-cytoplasmic (omega-pre-c) and omega-cytoplasmic (omega-c). While heat shock increased the levels of all the three transcripts at the 93D puff site in a coordinated manner, benzamide led to a significant increase in the levels of hsr-omega-n and pre-c; on the other hand, colchicine caused increased levels of the omega-n and omega-c RNA species at 93D. The results also suggested splicing of hsr-omega-pre-c RNA at the site of synthesis with the spliced-out 'free' intron (hsr-omega-fi) accumulating at the puff site. The rate of splicing and/or turnover of the hsr-omega-fi varied in a treatment-specific manner. Although a combined treatment to salivary glands with heat shock and benzamide or colchicine is known to inhibit puffing and [^{3}H]uridine incorporation at 93D, the two treatments resulted in a treatment-specific increase in the *in situ* levels of different hsr-omega transcripts at the 93D site, suggesting a reduced turnover of specific transcripts from the site under these conditions. We suggest that the different 93D transcripts have roles in turnover and/or transport of RNA in nucleus as well as some role in cytoplasmic translation.

Key words: benzamide, colchicine, hsp70, hsr-omega, intron

Introduction

The 93D locus of *Drosophila melanogaster*, one of the major heat shock-induced genes, does not code for any known heat shock or other protein and holds a very interesting position owing to several unusual properties. This locus (and its homolog in other species of *Drosophila*) is singularly and specifically induced, independent of heat shock, by benzamide, colchicine or colcemid, thiamphenicol, vitamin B_6, etc. (see reviews by Lakhotia 1987, 1989, Pardue *et al.* 1990). Although this locus is functionally conserved in all species of *Drosophila*, its DNA base sequence has diverged in different species (Lakhotia & Singh 1982, Pardue *et al.* 1990). The 93D and 93D-like loci have been termed *'hsr-omega'* (Bendena *et al.* 1989). The transcription unit of the *hsr-omega* locus consists of a 5′ unique region followed on the 3′ end by a 10–12 kb stretch comprising tandem repeats of 115 – 280 bp unit length in different species. The 5′ unique region contains two exons interrupted by an intron. Normal as well as stressed cells produce three hsr-omega transcripts, namely hsr-omega-n, hsr-omega-pre-c and hsr-omega-c (Hogan *et al.* 1994), all originating from the same start point (see Lakhotia 1987, Pardue *et al.* 1990). Omega-n is the full-length (~10–12 kb) transcript, encompasses the entire transcription unit of *hsr-omega* and is restricted to the nucleus (hence termed omega-n). Omega-pre-c is ~1.9 kb in length and results from alternative termination at a polyadenylation site upstream of the tandem repeats and is the precursor of the cytoplasmic omega-c (1.2 kb), produced by splicing of the single intron (~700 bp).

Earlier studies in our laboratory revealed an intriguing relation between activities of the 93D, 87A and 87C heat shock loci. The 87A and 87C loci contain two and three copies, respectively, of *hsp70* genes; the 87C locus, in addition, also harbors multiple copies of non-coding but heat-inducible *alpha-beta* sequences (see Hellmund & Serfling 1984). It was shown that when heat shock was applied along with another inducer of *hsr-omega*, the 93D puff was not induced and at the same time the 87A and 87C loci were unequally puffed.

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The failure of the 93D locus to be induced by heat shock in some mutants of *D. melanogaster* was also accompanied by an unequal induction of the 87A and 87C puffs (Lakhotia *et al.* 1990). It is notable that the unequal puffing of the 87A and 87C heat shock loci associated with non-induction of the 93D locus under the various conditions varied in a characteristic fashion: under certain conditions, the 87A puff was always more active than the 87C, while the reverse was obtained under other conditions (see Lakhotia 1987, 1989). These findings raised the possibility that transcriptional activity of 93D influenced the 87A and 87C activity (reviewed in Lakhotia 1987, 1989). The earlier [^{3}H]uridine labeled transcription autoradiograms could not resolve (i) if the different treatments resulted in different sets of transcripts at the 93D locus and (ii) if the unequal puffing of the 87A and 87C sites after the above-noted combination treatments was accompanied by changes, if any, in hsp70 and/or the alpha–beta transcripts. In the present set of studies, therefore, we used antisense RNA probes for hsr-omega, the hsp70 and for the alpha–beta transcripts for quantitative *in situ* hybridization to RNA on polytene bands/puffs to permit a relative quantitation (reflecting synthesis and/or turnover) of the different hsr-omega, hsp70 and alpha–beta transcripts *in situ* in variously treated polytene nuclei of *D. melanogaster*. The results of hybridization with the various hsr-omega probes are presented in this paper, while those with the hsp70 and alpha–beta probes are presented elsewhere (Sharma & Lakhotia 1994).

Materials and methods

Clones

The *pJG10*, *pDRM30* and *DRM32* clones for the 93D locus were obtained from Professor M.L. Pardue and were either used directly (*pJG10* and *pDRM30*) for generating region specific antisense labeled RNA probes or further subcloned (*pDRM32*) before use.

pJG10 is a 93D exon-specific clone containing approximately 1.2 kb of cDNA in *pSP65* vector. SP6 RNA polymerase was used to transcribe *in vitro* exon-specific antisense RNA probe. *pDRM30* is a repeat-specific clone containing approximately a 280 bp *Asu II* fragment of the 93D repeat region in *pGEM3* vector. It was transcribed *in vitro* using T7 RNA polymerase to produce a repeat-specific antisense RNA probe.

The *pDRM32* clone contains 0.2, 0.7 and 0.02 kb, respectively, of exon I, intron and exon II of hsr-omega in *pGEM3* vector. To remove the 0.2 kb of exon I from this, an approximately 0.6 kb-long *Eco*RI–*Pst*I fragment from *pDRM32* was subcloned in *pSPT18* vector at the *Eco*RI and *Pst*I sites. The resulting *pDRM18* clone included a major portion of the intron (~570 bp) and only 20 bp of exon II sequence, and, therefore, was used to generate intron-specific antisense transcripts with T7 RNA polymerase.

In addition to the above hsr-omega-specific clones, clones for hsp70 (*pPW18*) and the alpha–beta (*pAB18*) were also used for generating antisense probes (for details see Sharma & Lakhotia 1994).

In vitro transcription

Aliquots of 500 ng of gel-purified, linearized, template-cloned DNAs (*pJG10*, *pDRM18*, *pDRM30*, *pPW18* and *pAB18*) were transcribed *in vitro* for 2 h at 37°C in a total reaction volume of 10 µl containing 40 mM Tris–Cl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 20 units of SP6 or T7 RNA polymerase (Boehringer Mannheim) as desired (see above), 170 µCi of [^{35}S]ATP (Amersham; sp. act. ~600 Ci/mmol), 100 pmol each of CTP, GTP and UTP and 20 units of RNAse inhibitor (from human placenta, Boehringer Mannheim). After synthesis, the template DNAs were digested with 10 units of RNAse-free DNAse I (Boehringer Mannheim) for 15 min at 37°C. The reactions were stopped by adding EDTA (pH 8) to a final concentration of 20 mM and the labeled RNAs were ethanol precipitated with 400 mM LiCl. The precipitated RNAs were dissolved in 50 µl of diethyl pyrocarbonate (DEPC)-treated water, supplemented with 5 units of RNAse inhibitor, and stored at −20°C till use. Specific activities of the various 93D RNA probes as c.p.m. per µg of template DNA were as follows: *pJG10*, 1.6×10^7; *pDRM18*, 6×10^7; *pDRM30*, 3.2×10^6.

Flies and culture conditions

A wild-type Oregon R^+ strain of *Drosophila melanogaster* was used. Flies were maintained in milk bottles on medium containing agar, yeast, maize powder and sugar at 22° ± 10°C. For studies on late third instar larvae, eggs were collected at hourly intervals in food-filled Petri dishes supplemented with additional yeast suspension for healthy growth.

In vitro treatments of salivary glands

Salivary glands from actively migrating late third instar larvae were dissected in the inorganic salt constituents of Poels' tissue culture medium (PSS, Lakhotia & Mukherjee 1980) and immediately subjected to the following treatments:

Control (C): incubated in PSS at 24°C for 30 min;

Heat shock (HS): incubated in PSS at 37°C for 40 min;

Benzamide (BM): incubated at 24°C for 20 min in PSS containing benzamide (BDH, Poole, UK) at a concentration of 1.0 mg/ml;

Colchicine (Col): incubated at 24°C for 40 min in PSS containing colchicine (Russel Doglas, France) at a concentration of 0.1 mg/ml;

Heat shock followed by benzamide (HS→BM): following heat shock at 37°C for 30 min, the glands were treated with benzamide (1 mg/ml) at 24°C for 10 min; and

Heat shock in the presence of colchicine (Col + HS): colchicine (0.1 mg/ml) treatment was applied for 40 min at 37°C.

Polytene chromosome squash preparations

After the desired treatments, salivary glands were fixed in 45% acetic acid for 2–3 min and were then squashed using acid-cleaned, baked cover glasses and slides. The cover glasses were removed with a razor blade after freezing at −70°C. The slides were dehydrated through ascending grades of ethanol and air dried. To avoid denaturation of chromosomal DNA, the hot 2 × SSC treatment (Pardue 1986) was not applied.

In situ hybridization to polytene RNA

Hybridization was carried out in 5 × SSC, 50% formamide, 5% blocking reagent (Boehringer Mannheim) at 37°C for 36 h using the above [^{35}S]ATP-labeled antisense RNA probes. The total activity of each probe used per slide for hybridization was as follows: *pJG10*, 2×10^5 c.p.m.; *pDRM18*, 3×10^5 c.p.m.; *pDRM30*, 3×10^3 c.p.m. (these activities were selected on the basis of a series of pilot *in situ* hybridizations to generate a sufficient, but non-saturating, number of silver grains after 6 days' autoradiographic exposure). It may be noted that along with the hsr-omega probe, ^{35}S-labeled antisense RNA probe for either hsp70 or the alpha–beta was also mixed for simultaneous hybridization to chromosomal transcripts. (The results of the hsr-omega probe hybridization are presented in this paper, while those of the hsp70 and alpha–beta probes are presented in the accompanying paper, Sharma & Lakhotia 1994.) After hybridization, the slides were cooled to 4°C, washed twice for 30 min each in 1 × SSC and 0.5 × SSC at 60°C, treated with 20 µg/ml RNAse A (Boehringer Mannheim) in 2 × SSC at 37°C for 2 h, rewashed in 2 × SSC, 1 × SSC and 0.5 × SSC for 15 min each, at 60°C, dehydrated through ethanol grades and processed for autoradiography with Kodak NTB2 nuclear emulsion. The exposure time in all cases was 6 days at 4°C in dark.

In situ hybridization to polytene DNA

Polytene chromosome squashes from heat-shocked glands, prepared as above, were treated with 100 µg/ml RNAse A (Boehringer Mannheim) in 2 × SSC for 2 h at 37°C, washed three times in 2 × SSC for 30 min each, dehydrated through graded alcohol and air dried. For denaturation of chromosomal DNA, the preparations were kept in 0.07 N NaOH for 3 min at room temperature, dehydrated and air dried. Hybridization of the various antisense RNA probes to polytene chromosome DNA *in situ* was carried out using the same amount of the different labeled probes under exactly the same conditions as for RNA–RNA hybridization above. Washing and autoradiographic steps were the same as above.

Specificity of *in situ* hybridization to transcripts on polytene chromosomes for quantitative analysis.

Since polytene chromosomes contain many template DNA strands, it is possible that some of the hybridization seen under conditions favoring hybridization to chromosomal RNA (no RNAse and denaturion pretreatment steps) may nevertheless be to DNA, which may remain denatured either because of the structural features of polytene chromosomes or as a result of the preparative steps. To check this possibility, ^{35}S-labeled antisense RNA transcribed from the *pDRM30* (repeat-specific) clone was hybridized *in situ* to polytene chromosomes that were treated with RNAse-free DNAse I (Boehringer Mannheim; 1 U/µl in 10 mM Tris-Cl, pH 7.5 and 10 mM $MgCl_2$ at 37°C for 2 h) and/or RNAse A (Boehringer Mannheim; 100 ng/µl in 2 × SSC at 37°C for 2 h) prior to hybridization of the probe to chromosomal RNA (no denaturation step). To confirm the efficiency of the DNAse and RNAse treatments, polytene chromosomes labeled with [^{3}H]thymidine or [^{3}H]uridine were also digested with DNAse or RNAse, respectively, prior to autoradiographic detection of the incorporated label. It was seen (data not presented) that [^{3}H]thymidine or [^{3}H]uridine incorporation was completely abolished by the DNAse or RNAse treatment respectively. DNAse treatment prior to *in situ* hybridization of the antisense probe to chromosomal RNA, on the other hand, had no effect on the signal. However, RNAse treatment prior to hybridization to chromosomal RNA did not completely abolish the autoradiographic signal since about 30% of the control signal (without RNAse pretreatment) was still detectable in all cases. Possible reasons for the 'RNAse-resistant' signal are considered in the Discussion. Nevertheless, since the DNAse pretreatment did not affect the signal, we believe the *in situ* hybridization of antisense RNA probes to transcripts on polytene chromosomes to be specific.

Results

In situ hybridization of 93D exon-, intron- and repeat-specific antisense RNA probes to chromosomal DNA and to transcripts

In situ hybridization to polytene RNA as well as to polytene DNA was carried out with ^{35}S-labeled antisense RNA probes. Each of the three probes specifically hybridized only to DNA at the 93D site; in the case of

hybridization to chromosomal RNA, in addition to the 93D site a general background labeling of nucleoplasm and cytoplasm was also evident. However, except for this general background, no significant labeling of any chromosomal site, other than the 93D locus, was seen (representative examples of *in situ* hybridization to polytene DNA and puff RNA are presented in Figures 1–5). The numbers of autoradiographic silver grains over the 93D site were counted. The intra- and inter-preparation variability in the numbers of silver grains on the target site in different nuclei with a given probe and given condition of treatment were within statistical limits. As a measure of the background labeling, silver grains on the segment 100B–100F of 3R in each nucleus were also counted. The mean numbers of silver grains on this segment after different treatments and hybridization with different probes varied between 2 and 7 (detailed data not presented). Since compared with the grain counts on the 93D site of the given nucleus the background grain counts on the larger chromosome segment (100B–100F) were at least 10–15 times less, it was not necessary to correct the grain counts on the 93D puff site for background. The data on mean grain counts on the 93D site following hybridization of the three probes to DNA and to RNA are presented in Tables 1 & 2 respectively. Since the conditions of hybridization to chromosomal DNA and RNA were identical (except for the preceding RNAse and alkali treatments in the case of hybridization to DNA; see Materials and methods), these two sets of data were used to obtain estimates of transcripts hybridizable to the three probes on a per unit DNA basis to permit direct comparisons between the different treatments and the three probes. For this purpose, the numbers of silver grains seen in different nuclei after hybridization of a given probe to chromosomal RNA were individually divided by the mean number of silver grains observed after hybridization to chromosomal DNA with that probe to give RNA per unit DNA values. The means of RNA per unit DNA for the three probes are shown in Table 3. These data showed that the *in situ* hybridizable levels of RNA per unit of *hsr-omega* DNA varied between the three probes and also for each probe between control and the variously treated glands.

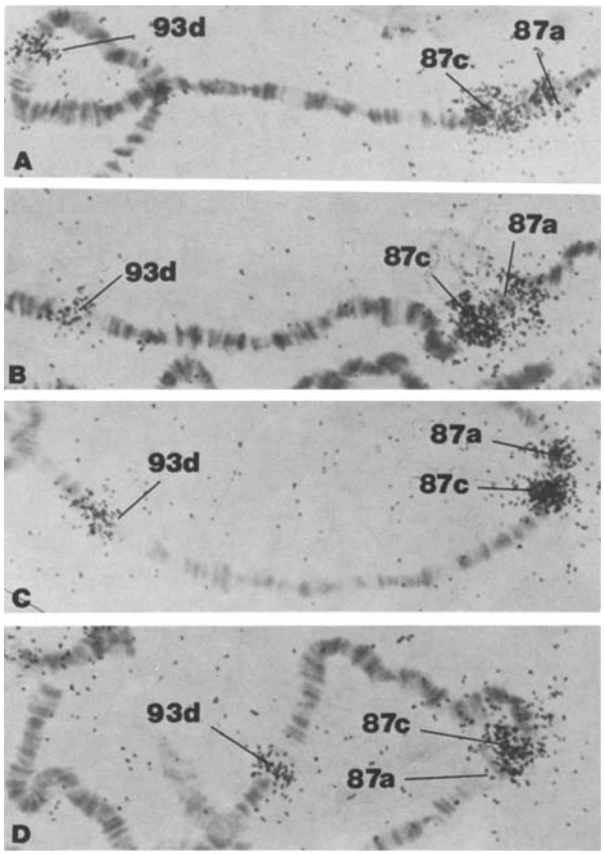

Figure 1. *In situ* hybridization of different ^{35}S-labeled antisense RNA probes to DNA on polytene chromosomes of heat-shocked salivary glands: **A** pJG10 (93D exon) and pPW18 (hsp70). **B** pDRM18 (93D intron) and pPW18 (hsp70). **C** pDRM30 (93D repeat) and pPW18 (hsp70). **D** pDRM18 (93D intron) and pAB18 (alpha–beta). The 93D, 87C and 87A puff sites are indicated. ×1050.

Estimation of relative quantities of the three hsr-omega transcripts under different treatment conditions

The relative quantities of omega-n, omega-pre-c and omega-c RNA species present at 93D locus after the various treatments were calculated from the RNA per DNA unit values presented in Table 3. The basis for these estimations was as follows.

As stated in the Introduction, the *hsr-omega* locus transcribes two primary RNA species, namely omega-n (comprising the two exons, the intron and the tandem repeats) and omega-pre-c (comprising only the two exons and the intron), and if they were present at the transcribing puff site in equimolar quantities the RNA per unit DNA values for exon-, intron- and repeat-specific probes should be in the ratio 2:2:1 since, of the two hsr-omega primary transcripts, the repeat probe (pDRM30) can hybridize only to the omega-n, while the exon (pJG10) and intron (pDRM18) probes can hybridize equally to both of them. Therefore, the mean RNA per unit DNA value obtained for the repeat probe in control glands (Table 3) was taken as representing a unit quantity of hsr-omega-n and all the other values in Table 3 were normalized to this by dividing by 0.33 (the mean for the repeat probe, pDRM30, in control in Table 3). The normalized values so obtained are presented in Table 4. Estimates of relative abundance of the various hsr-omega transcripts (Table 5) were then arrived at in the following manner:

1. Values for 'repeat' in Table 4 were considered to be representative of that many units of omega-n transcripts (a in Table 5).

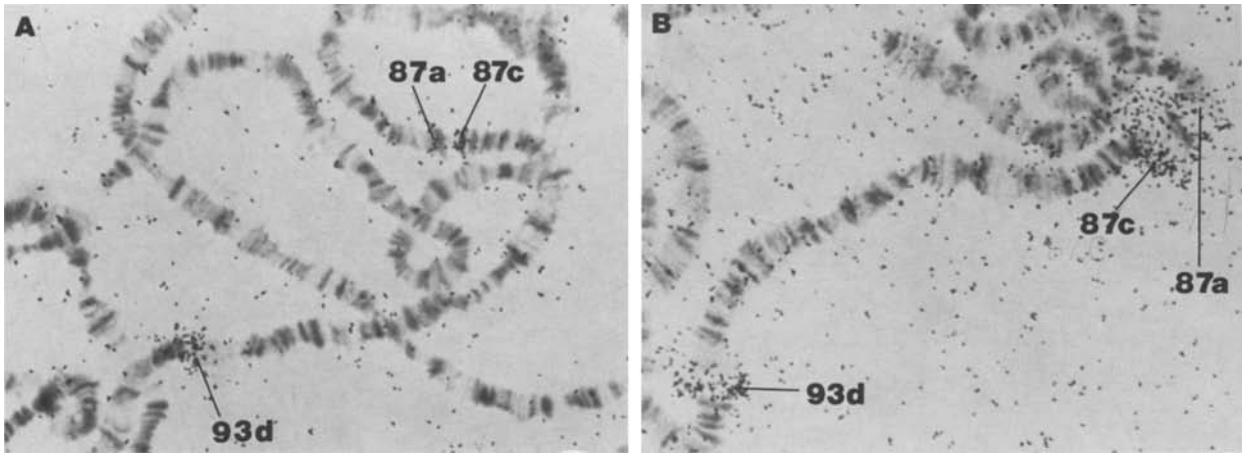

Figure 2. *In situ* hybridization of ^{35}S-labeled 93D exon and hsp70 antisense RNA probes to RNA on polytene chromosomes in control (**A**) and heat-shocked (**B**) salivary glands. ×1050.

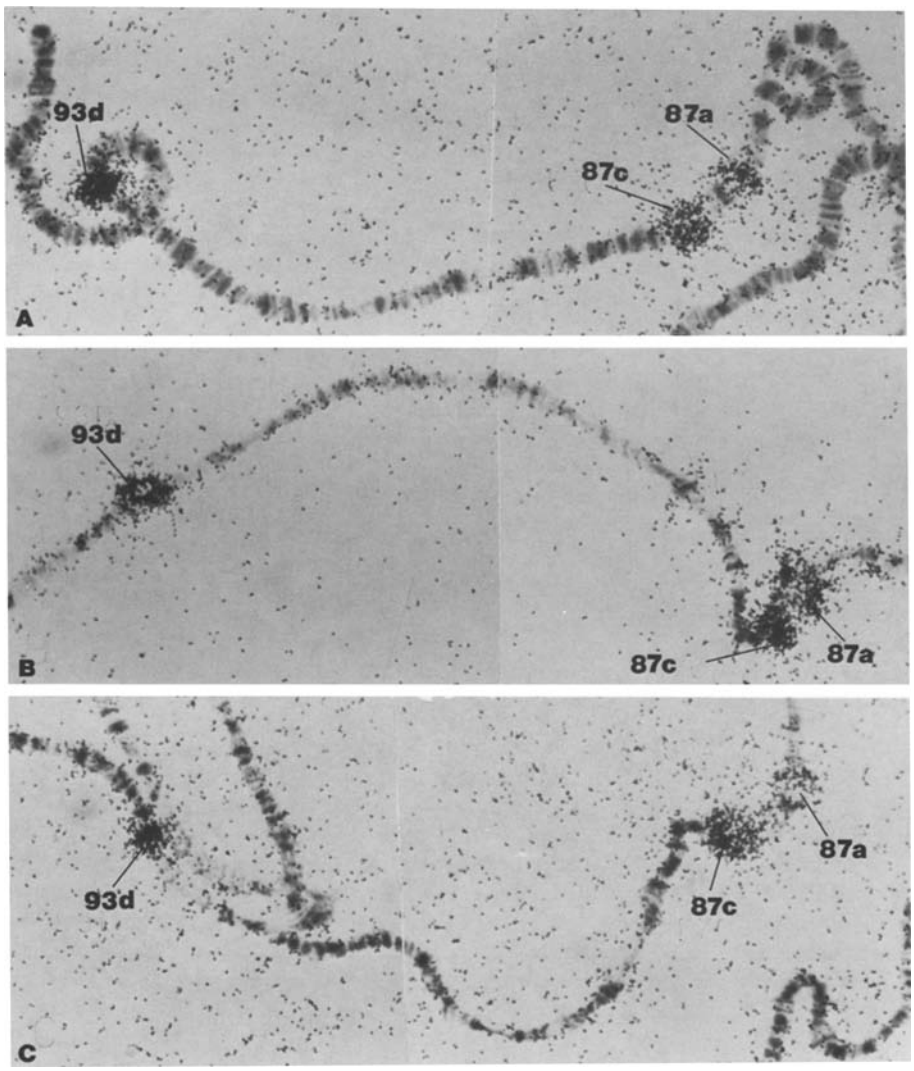

Figure 3. *In situ* hybridization of ^{35}S-labeled 93D intron and hsp70 antisense RNA probes to RNA on polytene chromosomes after (**A**) heat shock, (**B**) heat shock followed by benzamide and (**C**) heat shock in the presence of colchicine to salivary glands. ×1050.

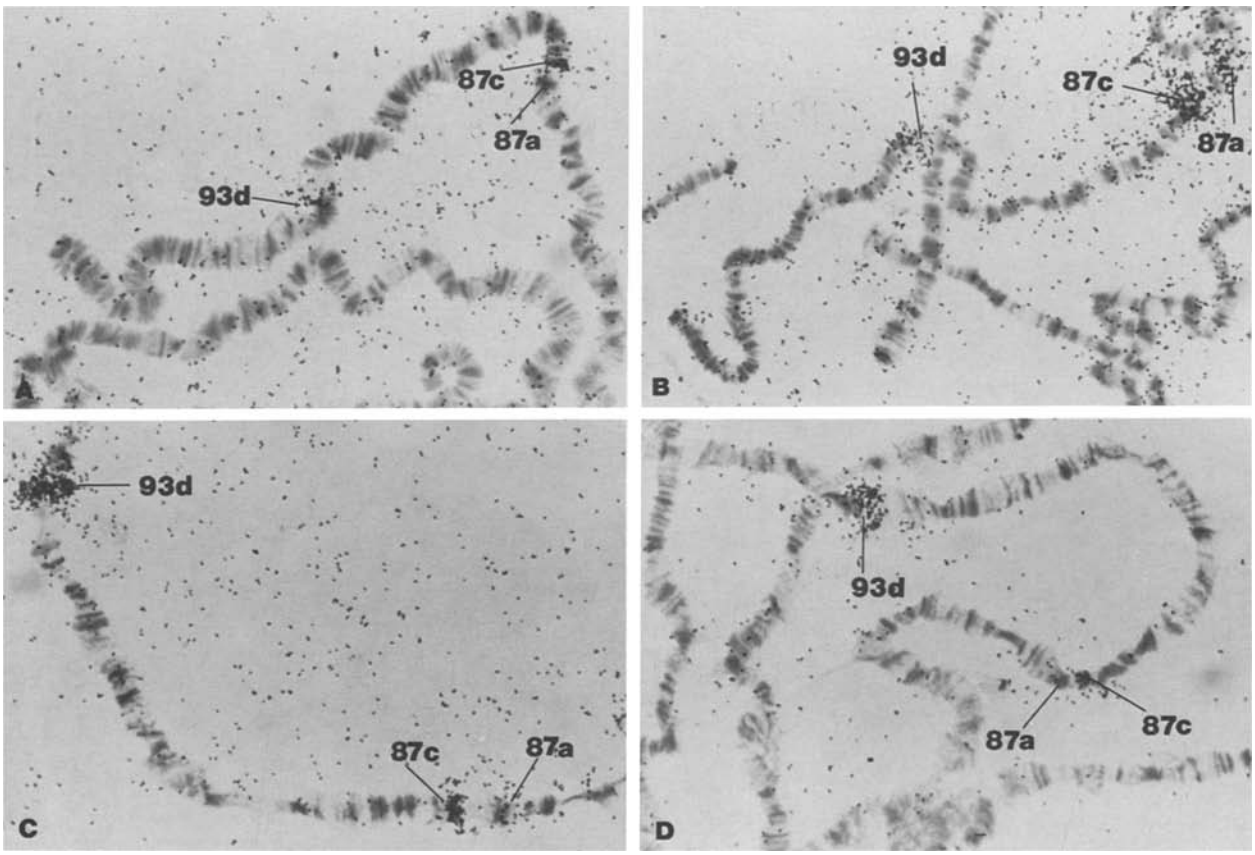

Figure 4. *In situ* hybridization of ^{35}S-labeled 93D repeat and hsp70 antisense RNA probes to RNA on polytene chromosomes in control salivary glands (**A**) or salivary glands treated with heat shock (**B**), benzamide (**C**) or colchicine (**D**). ×1050.

Table 1. *In situ* hybridization to DNA on polytene chromosomes using various hsr-omega (pJG10, pDRM18 and pDRM30) antisense RNA probes

Mean no. (± SE) of silver grains on the 93D site		
pJG10	pDRM18	PDRM30
64.83 ± 3.04 (n = 12)	68.39 ± 2.92 (n = 18)	109.67 ± 6.96 (n = 12)

Numbers in parentheses indicate the total number of nuclei observed.

2. The difference between the RNA per unit DNA values for the intron and repeat probes was taken to represent the 'total' hsr omega-pre-c (b in Table 5).
3. Since, unlike the expected 1:1 ratio for the intron and exon probes, the observed hybridization signal for the intron probe was always significantly greater than that for the exon probe (Tables 2–4), it was inferred that splicing of the hsr-omega-pre-c occurred at the 93D site and that, while the 'free' intron (termed hsr-omega-fi) accumulated *in situ*, the hsr-omega-c was rapidly transported away to cytoplasm (see Discussion). Therefore, the differences between the RNA per unit DNA signal for intron and exon probes were taken to represent the relative levels of the omega-fi (c in Table 5) and indirectly the levels of the omega-c (transported away) in each case.
4. The difference between the total hsr-omega-pre-c (b in Table 5) and the omega-fi (c in Table 5) was indicative of the unspliced hsr-omega-pre-c (d in Table 5) accumulating at the site.
5. The ratio of omega-n/omega-pre-c (e in Table 5) indicated the relative levels of the two primary transcripts *in situ*.

As seen from the data in Table 5, compared with the value in controls, all five treatments resulted in a significant increase in the *in situ* levels of omega-n, with HS causing the least and HS→BM the maximum increase. While HS led to an increase in the level of 'total' omega-pre-c also, the *in situ* levels of this transcript after the BM and Col treatments were lower than in control glands; on the other hand, the two combination treatments (HS→BM and Col+HS) only slightly affected the control levels of omega-pre-c. The level of omega-fi (or omega-c) was greatly increased after HS and to some extent after HS→BM, but the other three treatments resulted in a slight (Col and Col+HS) or severe (BM treatment) decline in its levels.

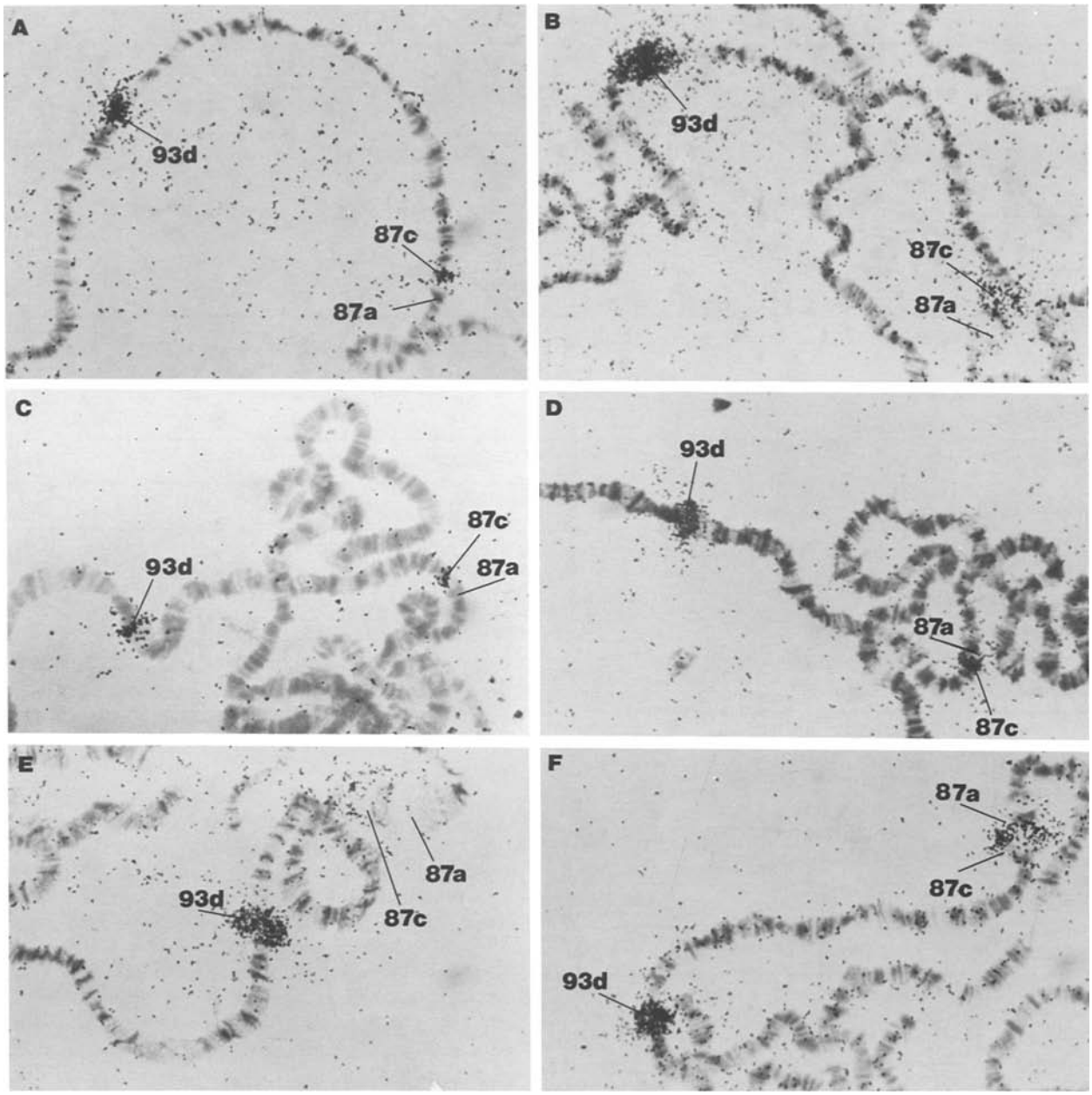

Figure 5. *In situ* hybridization of ^{35}S-labeled 93D intron and alpha–beta antisense RNA probes to RNA on polytene chromosomes in (**A**) control or after (**B**) heat shock, (**C**) benzamide, (**D**) colchicine, (**E**) heat shock followed by benzamide and (**F**) heat shock in presence of colchicine treatments to salivary glands. ×1050.

The values under e in Table 5 (omega-n/'total' omega pre-c ratio) showed that while, after HS, the proportion of these two transcripts relative to each other remained the same as in control glands, the BM and Col treatments altered the ratio in favor of omega-n. The HS→BM combination treatment caused a still greater shift in favor of omega-n. The Col+HS combination treatment resulted in this ratio being intermediate between those obtained after HS and Col treatments.

Discussion

In situ localization of transcripts by hybridization of DNA or RNA probes to cellular RNA is increasingly being used to obtain information that is not obtainable through conventional transcription autoradiography or by Northern analysis (Foley *et al.* 1993). *In situ* hybridization to transcripts on polytene chromosomes offers very high-resolution localization and quantitation of specific transcripts at the site of synthesis. However, in

Table 2. *in situ* hybridization of the various *hrs-omega* antisense RNA probes to RNA on the 93D site of polytene chromosomes following the different treatments of salivary glands

Probe	Mean no. (± SE) of silver grains on the 93D site					
	C	HS	BM	Col	HS → BM	Col+HS
pJG10 (exon)	47.6± 3.5 (*n* = 44)	78.5 ± 6.9 (*n* = 17)	110.7 ± 9.7 (*n* = 35)	55.1 ± 1.3 (*n* = 29)	86.2 ± 7.6 (*n* = 24)	87.5 ± 9.1 (*n* = 23)
pDRM18 intron	112.8 ± 6.7 (*n* = 48)	197.3 ± 10.51 (*n* = 43)	124.6 ± 9.3 (*n* = 56)	111.8 ± 9.2 (*n* = 52)	167.4 ± 10.2 (*n* = 36)	146.8 ± 12.9 (*n* = 44)
pDRM30 repeat	36.1 ± 4.1 (*n* = 27)	70.7 ± 6.1 (*n* = 17)	97.1 ± 8.6 (*n* = 38)	84.7 ± 8.5 (*n* = 25)	143.35 ± 17.7 (*n* = 19)	79.6 ± 8.1 (*n* = 16)

Numbers in parentheses indicate numbers of nuclei observed.

Table 3. *In situ* hybridization of the different *hsr-omega* probes to the 93D site expressed as RNA per unit DNA (see text for details)

Probe	Mean no. (± SE) RNA per unit DNA value					
	C	HS	BM	Col	HS→BM	Col+HS
pJG10 (exon)	0.73 ± 0.05 (*n* = 44)	1.21 ± 0.11 (*n* = 17)	1.71 ± 0.15 (*n* = 35)	0.85 ± 0.04 (*n* = 29)	1.33 ± 0.12 (*n* = 24)	1.35 ± 0.14 (*n* = 23)
pDRM18(93D) intron	1.65 ± 0.10 (*n* = 48)	2.89 ± 0.15 (*n* = 43)	1.82 ± 0.14 (*n* = 56)	1.63 ± 0.15 (*n* = 52)	2.45 ± 0.15 (*n* = 36)	2.16 ± 0.19 (*n* = 44)
pDRM30(93D) (repeat)	0.33 ± 0.03 (*n* = 27)	0.65 ± 0.6 (*n* = 17)	0.89 ± 0.08 (*n* = 38)	0.77 ± 0.08 (*n* = 25)	1.32 ± 0.16 (*n* = 19)	0.73 ± 0.07 (*n* = 16)

Numbers in parentheses indicate the total number of nuclei observed.

Table 4. Relative hybridization of different *hsr-omega* probes expressed as RNA per unit DNA after normalization to the repeat probe (see text for details)

Probe	Treatment					
	C	HS	BM	Col	HS→BM	Col+HS
Exon	2.21	3.66	5.18	2.57	4.03	4.09
Intron	5.00	8.75	5.51	4.93	7.42	6.54
Repeat	1.00	1.96	2.69	2.33	4.02	2.21

view of the presence of a large number of template DNA strands in polytene chromosomes, earlier reports (Artavanis-Tsakonas *et al.* 1979, Izquiredo *et al.* 1981, Vlassova *et al.* 1991) gave conflicting views about the specificity of hybridization *in situ* to transcripts only. Our own results, briefly mentioned in the Materials and methods section, showed that about 30% of the signal in RNA–RNA hybridization preparations was insensitive to a pretreatment with RNAse. It is known that the RNAse treatment of fixed chromosomes preceding *in situ* hybridization causes denaturation of about 25% of chromosomal DNA (Kurnit 1974, Peretti & Mezzanotte 1993). Thus the so-called 'RNAse-resistant' signal may actually be a consequence of the RNAse treatment itself. In addition, it is also possible that some RNA on chromosomes may be less sensitive to RNAse owing to its being complexed with proteins for processing or transport, etc. Since the DNAse pretreatment had no effect on hybridization under similar conditions, we believe that in our RNA–RNA *in situ* hybridization preparations, which did not involve any RNAse pretreatment, undesired hybridization to polytene DNA was negligible. Even if a small proportion of silver grains in the autoradiograms of RNA–RNA *in situ* hybridization preparations were due to hybridization to chromosomal DNA, this would not seriously affect the present results since this 'residual' hybridization would remain common to the control as well as to the variously treated chromosomes and would be effectively nullified in a comparative analysis. Furthermore, as shown in the accompanying paper (Sharma & Lakhotia 1994), *in situ* hybridization of ^{35}S-labeled hsp70 antisense RNA probe to DNA in polytene chromosomes generated silver grains at the 87A and 87C sites in the same ratio as the numbers of *hsp70* gene copies at these two sites (Hellmund & Serfling 1984). This concordance between the actual gene copy numbers and the signal obtained with *in situ* hybridization clearly demonstrated the quantitative nature of our experimental conditions.

The *hsr-omega* locus is transcriptionally induced by a variety of treatments and produces at least three major

Table 5. Estimates of the relative abundance of different *hsr-omega* transcripts after the various treatments (see text for details)

RNA	Treatment					
	C	HS	BM	Col	HS→BM	Col+HS
hsr-omega-n	1.00	1.96	2.69	2.33	4.02	2.21
'total' hsr-omega-pre-c	4.00	6.79	2.82	2.6	3.40	4.33
'free' intron (hsr-omega-fi)	2.79	5.09	0.33	2.36	3.39	2.45
'unspliced' hsr-omega-pre-c	0.21	1.70	2.49	0.24	0.01	1.88
hsr-omega-n/'total' omega-pre-c ratio	0.25	0.28	0.95	0.89	1.29	0.51

transcripts (see Lakhotia 1987, 1989, Bendena *et al.* 1989, Hogan *et al.* 1994). To assess the relative levels of the different transcripts hybridizable to different hsr-omega probes *in situ* following the various treatments, it was necessary to normalize the silver grains obtained with different probes (of varying sizes and specific activities) to a common denominator. Since the same antisense RNA probes were used under identical conditions of hybridization to polytene DNA and to RNA, and since the variability in the observed numbers of grains at a given site under a given set of conditions was within statistical limits, the values obtained for each RNA–RNA hybridization were normalized against the mean numbers of silver grains obtained for the RNA–DNA hybridization with each probe. These normalized values, expressed as RNA per unit DNA, allowed estimation of relative levels of the different omega transcripts.

In agreement with the results of an earlier Northern analysis of the different hsr-omega transcripts induced by HS, BM or Col in dividing cells of *Drosophila melanogaster* (Bendena *et al.* 1989), the present results showed that, in polytene cells also, the different inducers (HS, BM and Col) of the 93D locus resulted in an inducer-specific profile of the various hsr-omega transcripts *in situ* (Table 5). While Northern analysis provided information on the total RNA present in cells, the present *in situ* analysis allowed us to monitor changes in transcript population at the site of synthesis itself. Our results revealed that, although at the puff level the activity induced in response to HS, BM and Col treatments appeared similar (Lakhotia 1987), the three inducers caused different *in situ* profiles of different hsr-omega transcripts. While HS enhanced levels of all the three hsr-omega transcripts (omega-n, -pre-c and -c), BM and Col resulted in a greater induction of omega-n but a decline in the levels of 'total' omega-pre-c (Table 5). HS maintained the ratio between omega-n and the other two hsr-omega transcripts at 0.25 found in controls, but the two drugs changed this ratio greatly in favor of omega-n. From the point of view of cellular activities, a major difference between C-, HS-, BM- and Col-treated cells is that, whereas C- and HS-treated cells need continued translation of newly synthesized normal and/or heat-induced transcripts, BM and Col treatments do not necessitate translation of novel transcripts since both drugs inhibit general chromosomal transcription except that of the non-coding *hsr-omega* locus (Lakhotia & Mukherjee 1980, 1984). Since the levels of hsr-omega-pre-c were high only in C- and HS-treated cells, its spliced product (omega-c) appears to have cytoplasmic function(s) related to protein synthesis. HS, BM and Col treatments all induced omega-n irrespective of induction or suppression of other *omega* transcripts. Since all three treatments inhibit general chromosomal transcription (see Lakhotia 1987), omega-n appears to have roles in general transcription, RNA processing and/or turnover/transport of RNA. These functions are also supported by the reported accumulation of hsr-omega-c or of hsr-omega-n following inhibition of translation or transcription respectively (Bendena *et al.* 1989, Hogan *et al.* 1994). The results of another study (Sharma & Lakhotia 1994) showed that the elevated *in situ* levels of hsr-omega-n transcripts following combination treatments were accompanied by altered rates of transcription and/or turnover of hsp70 and alpha–beta transcripts at 87A and 87C sites. Hogan *et al.* (1994) also found a change in turnover of hsp83 mRNA along with the reduced turnover of hsr-omega-n after actinomycin D treatment. Therefore, it is very likely that the hsr-omega-n RNA has some specific role(s) in RNA turnover and transport. It is notable in this context that the cytoskeleton, which plays an important role in intracellular transport and localization of diverse RNAs (Singer 1992), is known to be affected by agents (colchicine, benzamide, heat shock, etc.) that induce the 93D locus (see Lakhotia 1987, 1989).

Compared with the exon or the repeat probe, the significantly higher signal obtained with the intron probe in all cases (Tables 2 and 3) was most likely due to splicing occurring at the site, with the spliced product, omega-c, being transported away and the spliced-out intron accumulating. Splicing at the site of transcription is known (Osheim *et al.* 1985, Beyer

& Osheim 1988, LeMaire & Thummel 1990, Zacher *et al.* 1993, Xing *et al.* 1993). An earlier Northern study (Bendena *et al.* 1989) has also shown spliced-out hsr-omega intron RNA to be unusually stable. We designate the free intron as hsr-omega-fi since its accumulation at the 93D locus itself is significant and reflects yet another intriguing feature of this locus. Our results revealed that the *in situ* levels of hsr-omega-fi varied in characteristic fashion depending upon the treatment.

Dangli *et al.* (1983) showed the presence of unique antigens associated with the unusually large ribonuclear protein (RNP) particles at the 93D puff of *D. melanogaster.* Laran *et al.* (1990) reported association of hsp70 protein with the *hsr-omega* locus (2–48C) of *D. hydei,* and Morcillo *et al.* (1993) showed binding of hsp83 protein with the heat shock-induced 93D puff of *D. melanogaster.* It is not known if any or all of these protein associations are related to *in situ* splicing and accumulation of the spliced out intron at the 93D site. This needs further studies.

Earlier studies (see Lakhotia 1987 for a review) had shown that heat shock in combination with BM or Col resulted in non-induction of the 93D puff and a reduced [^{3}H]uridine incorporation at this site. Therefore, the presence of hsr-omega transcripts *in situ* at high levels following these two combination (HS→BM and Col+HS) treatments, as found in this study, was rather unexpected. This difference between [^{3}H]uridine labeling and *in situ* hybridization to various hsr-omega transcripts at the 93D locus is apparently due to a significantly reduced turnover of hsr-omega transcripts from the site of synthesis in glands exposed to the combination treatments. These two combination treatments resulted mainly in accumulation of hsr-omega-n transcripts in excess of the newly synthesized ones. Since it is known (Hogan *et al.* 1994) that inhibition of transcription with actinomycin D stabilizes omega-n transcripts and that turnover of this RNA requires ongoing transcription, the accumulation of the hsr-omega-n transcripts *in situ* following the combination treatments also appears to be due to the strong inhibition of chromosomal transcription by these treatments (Lakhotia & Mukherjee 1980, 1984). The phenomenon of puffing in polytene chromosomes is dependent upon several factors such as the extent of ongoing transcription, size of the transcripts, turnover of the newly synthesized RNA, etc. (Bonner & Pardue 1977, Simon *et al.* 1985). It appears that a major determinant of puffing at the 93D locus is a high rate of transcription of omega-n.

The differential puffing [^{3}H]uridine incorporation at 87A and 87C under certain conditions of heat shock was previously correlated with the decreased 93D activity, and it was proposed that 93D activity somehow regulated the relative expression of 87A and 87C puff sites (Lakhotia, 1987, 1989, Lakhotia *et al.* 1990). Hochstrasser (1987), on the other hand, suggested that activity at the 93D locus may not be responsible for the differential puffing of 87A and 87C. The present results provide direct support for the view of Lakhotia *et al.* (1990) that cytological puffing of the 93D locus did not allow one to ascertain the profile of the transcript population induced at this site and, therefore, all situations where the 93D locus was cytologically puffed need not be equivalent in terms of the set of 93D transcripts actually available in the cell. It was found that the different inducers of puffing at 93D induced different sets of hsr-omega transcripts; furthermore, although both the combination treatments resulted in non-puffing of the 93D locus, this was associated with very different effects on the relative levels of the two primary hsr-omega transcripts. Therefore, the specific metabolism of the hsr-omega transcripts appears to influence puffing at the 87A and 87C loci (see Sharma & Lakhotia 1984).

Earlier studies on induced puffing and [^{3}H]uridine incorporation suggested that regulation of the 93D locus is complex and distinct from other heat shock loci and that perhaps different regulatory elements mediate the response of this locus to various conditions (Lakhotia 1987, 1989, Lakhotia *et al.* 1990, Vazquez *et al.* 1993). The present findings of an inducer-specific profile of synthesis/accumulation of the various hsr-omega transcripts at the 93D site and inhibition of new synthesis and turnover of hsr-omega transcripts after combination treatments reveal further complexities of regulation of this locus.

Acknowledgements

This work was supported by a research grant from the Department of Science and Technology, Government of India, New Delhi, to S.C.L. A.S. was supported by a Junior Research Fellowship from the University Grants Commission, New Delhi. We also thank Professor Mary Lou Pardue, Department of Biology, MIT, Cambridge, USA, for providing the 93D clones.

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