Forty years of the 93D puff of *Drosophila melanogaster*

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The 93D puff of *Drosophila melanogaster* became attractive in 1970 because of its singular inducibility by benzamide and has since then remained a major point of focus in my laboratory. Studies on this locus in my and several other laboratories during the past four decades have revealed that (i) this locus is developmentally active, (ii) it is a member of the heat shock gene family but selectively inducible by amides, (iii) the *93D* or *heat shock RNA omega* (*hsrω*) gene produces multiple nuclear and cytoplasmic large non-coding RNAs (hsrω-n, hsrω-pre-c and hsrω-c), (iv) a variety of RNA-processing proteins, especially the hnRNPs, associate with its >10 kb nuclear (hsrω-n) transcript to form the nucleoplasmic omega speckles, (v) its genomic architecture and hnRNP-binding properties with the nuclear transcript are conserved in different species although the primary base sequence has diverged rapidly, (vi) heat shock causes the omega speckles to disappear and all the omega speckle associated proteins and the hsrω-n transcript to accumulate at the 93D locus, (vii) the hsrω-n transcript directly or indirectly affects the localization/stability/activity of a variety of proteins including hnRNPs, Sxl, Hsp83, CBP, DIAP1, JNK-signalling members, proteasome constituents, lamin C, ISWI, HP1 and poly(ADP)-ribose polymerase and (viii) a balanced level of its transcripts is essential for the orderly relocation of various proteins, including hnRNPs, RNA pol II and HP1, to developmentally active chromosome regions during recovery from heat stress. In view of such multitudes of interactions, it appears that large non-coding RNAs like those produced by the *hsrω* gene may function as hubs to coordinate multiple cellular networks and thus play important roles in maintenance of cellular homeostasis.

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1. Genesis of the 93D puff

The 93D puff has occupied a major part of my research career. I became fascinated by the 93D puff sometimes in 1969 and this fascination has increased since then. A personalized narrative of how the story of 93D puff developed during the past 40 years is presented on the occasion of completion of 65 years of my life.

Following the completion of a master's degree in zoology and comparative anatomy with specialization in parasitology, I entered the newly established genetics laboratory of Dr AS Mukherjee in 1967 for doctoral studies. Dr Mukherjee, a young and remarkably stimulating teacher, joined the Department of Zoology at the University of Calcutta (now University of Kolkata) in 1965 after his PhD under Prof Curt Stern (University of California Berkeley, USA) and post-doctoral work with Prof W Beermann (Max-Planck Institute, Tubingen, Germany). I opted to undertake research on dosage compensation of the X-linked genes of *Drosophila*. Mukherjee and Beermann (1965) had suggested that dosage compensation operates through hyperactivation of the X-linked genes in somatic cells of males such that the transcriptional activity of the single X-chromosome in male equaled that of the two X-chromosomes in female somatic cells. The dual expertise of Dr Mukherjee in *Drosophila* genetics and in polytene chromosome cytology was a great starting point for me. My studies, besides reconfirming the hyperactivity (increased rate of transcription and faster completion of a polytene replication cycle of the X-chromosome in salivary glands of male larvae; *see* Lakhotia and Mukherjee 1969, 1970a, b), also showed that the hyperactive state of the single X-chromosome in males was cell autonomous (Lakhotia and Mukherjee 1969). Further, in agreement with the proposal of Muller and Kaplan (1966) that, unlike the whole X-chromosome inactivation in female mammals (Lyon 1961),

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the different X-linked genes in *Drosophila* were individually regulated for dosage compensation ('piecemeal' regulation), it was shown that the hyperactive state of the male X-chromosome did not spread to an inserted autosomal segment (Lakhotia 1970).

The evidence for the hyperactive state of the male X-chromosome was based entirely on its morphology, its faster completion of a replication cycle in polytene cells and relatively greater incorporation of ^{3}H-uridine along its length compared with that on the autosomes in the larval salivary gland polytene chromosomes of *Drosophila melanogaster*. We reasoned that demonstration of a greater sensitivity of the rate of RNA synthesis on the male X-chromosome to transcriptional inhibition would more firmly establish the hyperactive-X model of Mukherjee and Beermann (1965). In the 1960s, actinomycin D was the commonly used inhibitor of eukaryotic transcription. However, its primary target was nucleolar (ribosomal) rather than chromosomal transcription, which was inhibited only at higher dosages of the inhibitor (Perry and Kelley 1968). α-Amanitin was yet to be identified as a potent inhibitor of chromosomal or RNA-pol-II-dependent transcription (Lindell *et al.* 1970). Since actinomycin would inhibit chromosomal transcription only at a higher concentration, I avoided its use for testing the predicted greater sensitivity of the male X-chromosome transcription. A search of the literature revealed that some benzimidazole derivatives and benzamide (BM) were already used (Sirlin and Jacob 1964) to specifically inhibit chromosomal transcription, without much effect on the nucleolar transcription, in polytene nuclei of a *Smittia*, a member of the Chironomidae family. Therefore, these inhibitors appeared attractive for our experiments. Because of the high cost of benzimidazole derivatives, I decided to try BM to see if it primarily blocked chromosomal transcription in *Drosophila* polytene nuclei as well, and if the X-chromosomal transcription in male nuclei was indeed more sensitive to such inhibition. A brief treatment of salivary glands with 1.3 mg/ml (~10 mM) concentration of BM did inhibit uptake of ^{3}H-uridine at chromosomal sites without affecting the nucleolar incorporation and, as expected on the hyperactive-male-X model of dosage compensation (Mukherjee and Beermann 1965), it did reveal the X-chromosomal transcription in male cells to be more sensitive to the inhibition (Lakhotia 1970). However, a completely unexpected finding was that in the BM-treated polytene nuclei, one of the chromosome sites was highly puffed and showed several-fold higher incorporation of ^{3}H-uridine amidst the otherwise poorly labelled polytene chromosome arms (figure 1). This cytological region, visible as a large puff with very high incorporation of ^{3}H-uridine, was identified on the maps of polytene chromosomes prepared by Bridges as the 93D cytogenetic region on the right arm of chromosome 3. This serendipitous observation was the starting point for my fascination of the 93D puff (Lakhotia and Mukherjee 1970b).

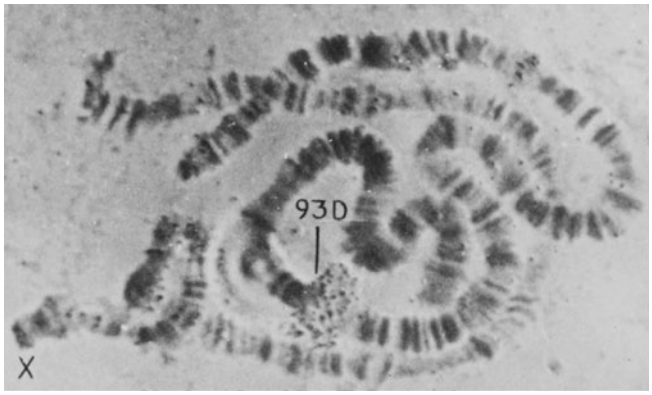

Figure 1. Specific induction of 93D puff by 10 min treatment of salivary glands of *D. melanogaster* larva (male) with 1.3 mg/ml benzamide at 24°C. While most of the chromosomal incorporation of ^{3}H-uridine, visualized as small black granules in the autoradiogram, is blocked, that at the 93D site, which also forms a large puff, is enhanced. Image reproduced from Lakhotia (1970).

Classical studies in the 1960s utilizing polytene chromosomes provided much of the direct evidence for correlation of chromatin organization, gene activity and transcription. An elegant study (Edstrom and Beermann 1962) using microdissected Balbiani rings and chromosomes to analyse the base composition of DNA and RNA isolated from specific regions provided evidence that one of the two DNA strands of a given Balbiani ring produces its distinct transcript. This and other evidences then available established that puffs are sites of transcription and, therefore, of gene activity (reviewed by Berendes and Beermann 1969). However, a similar direct correlation between a given puff and the expected protein product was still not available in the 1960s, except for indirect evidence from studies like the observed specific difference in puffing of a given chromosome region and its correlation with the presence or absence of a secretory protein in salivary glands of two related species of *Chironomus* (Grossbach 1969). Against this background, the singular induction of the 93D puff in larval salivary glands of *D. melanogaster* following brief treatment with BM appeared to provide a unique and exciting opportunity to examine the correlation between a puff, its RNA transcript and the final protein product. Consequently, I decided to investigate the 93D puff further as and when I had the opportunity to take up my independent research programme.

By the end of my doctoral research, besides the desire to study the 93D puff in greater detail, I had interests in the processes of replication and transcription at chromosomal level and in heterochromatin. I did not, however, initiate work on the 93D puff for a few years after completing my doctorate in 1970. After the fly experience, I decided to gain some

experience in mammalian cytogenetics and joined Dr SRV Rao's laboratory at the Department of Zoology, Delhi University, as a post-doctoral fellow, where I participated in the description of karyotypes of a few as yet chromosomally uncharacterized rodent species. I took up a faculty position at the Department of Zoology of Burdwan University (Burdwan) in 1971 but moved to Gujarat University (Ahmedabad) in June 1972. As a legacy of my doctoral research, I was interested in knowing if replication and transcription could occur simultaneously at a given chromosome region and had imagined that electron microscope (EM) autoradiographic studies on polytene chromosomes doubly labelled with ^{14}C-thymidine and ^{3}H-uridine would be ideal to address this question. While at Delhi in 1970–1971, I met Dr J Jacob of the Institute of Animal Genetics, Edinburgh, who had considerable experience in EM autoradiography and who also happened to be one of the authors of the paper (Sirlin and Jacob 1964) that introduced me to BM. During one of our discussions, he indicated that if I could get a fellowship to come to Edinburgh, I could use his EM facility to examine the relationship between transcription and replication. Keeping this in mind, I went to the Institute of Animal Genetics (Edinburgh) in November 1972, having been selected for the Overseas Scholarship of the Royal Commission for the Exhibition of 1851 (UK). Although the experimental design for examining simultaneous transcription and replication at a given chromosome region at the EM level did not work out, during the 11 months' stay at Edinburgh, I obtained strong evidence for (i) active transcription in the classical chromocentric heterochromatin in polytene nuclei of *D. melanogaster* (Lakhotia and Jacob 1974b), which contradicted the then 'established fact' of heterochromatin being completely inert, (ii) absence of any replication of the α-heterochromatin during polytenization (Lakhotia 1974) and (iii) initiation of replication in chromatin regions that are not associated with the nuclear envelope (Lakhotia and Jacob 1974a). Based on these studies, my interest in heterochromatin and chromosomal replication in *Drosophila* also became more firmly rooted. After returning to Gujarat University, I decided to start studying the 93D puff, heterochromatin and replication in *Drosophila* chromosomes. While active research in the 93D puff has continued till date, I had to give up research relating to heterochromatin and chromosomal replication after several years of active involvement and many research papers, because of my increasing interest in the 93D puff and, emanating from it, the regulation and functions of other heat shock genes.

2. The 93D puff is a member of heat shock gene family but behaves differently from the other heat shock puffs

I initiated research on the 93D puff in 1974 at Gujarat University and one of the experiments, which appeared straightforward, was to see if following the BM treatment, a new protein was synthesized in the larval salivary glands, which could be correlated with the BM-induced enhanced transcription at the 93D puff. However, this rather simple experiment proved to be quite frustrating for several years because of the extremely limited laboratory and financial resources. During this period, two seminal papers (Tissieres *et al.* 1974; Lewis *et al.* 1975) on heat-shock-induced synthesis of new proteins in *Drosophila* tissues were published. The 93D puff was already identified to be one of the major heat-shock-induced puffs in salivary glands of *D. melanogaster* (Ashburner 1970). Thus, our task appeared narrowed down to identifying which of the heat-shock-induced proteins was also induced by BM treatment, presuming *prima facie* that the same gene was induced by the two treatments, which was subsequently indeed proven to be a correct surmise (sections 3 and 4). However, our protein synthesis studies were not making any headway because of the inability to get a vertical slab gel electrophoresis apparatus and a gel drier. We tried various 'homemade' alternatives for running thin polyacrylamide slab gels without success.

I moved to the Department of Zoology at Banaras Hindu University in September 1976 and, after settling down, continued with my research interests in the 93D puff, heterochromatin and chromosomal replication in *Drosophila.* Tapas Mukherjee became my PhD student in 1977, the first to work on the 93D puff. By this time, I had two research grants but still not enough funding to buy a vertical slab gel electrophoresis apparatus and gel drier. Because the protein synthesis study was still not feasible, Tapas started examining the heat-shock- and BM-induced puffing.

An early interesting observation was that the relative level of heat-shock-induced transcriptional activity of the 93D puff varied independently of the other major heat shock puffs, which were induced in concert like a 'battery' (Mukherjee and Lakhotia 1979). It was reported by Compton and McCarthy (1978) that incubation of isolated polytene nuclei of *D. melanogaster* in cytoplasm from heat-shocked Kc cells resulted in only a weak induction of the 93D puff while the other heat shock puffs were strongly induced as after heat shock to intact salivary glands. Following this report, we (Mukherjee and Lakhotia 1981) found that incubation of intact salivary glands, rather than isolated polytene nuclei, in a homogenate of heat-shocked salivary glands resulted in specific activation of only the 93D puff. These observations indicated that the 93D puff was regulated differently from the other heat shock puffs, a surmise that found support in subsequent reports that the binding of the heat shock factor to the 93D site following a typical heat shock was very transient compared to that on the other heat shock induced puff sites (Westwood *et al.* 1991). We also found

that although BM and heat shock both induced the 93D puff, a combination of the two inducers (one following the other or both applied together) results in inhibition of puffing and transcriptional activity of the 93D puff (Lakhotia and Mukherjee 1980). Likewise, recovery from anoxia at 24°C results in strong induction of 93D and other heat shock puffs; the same at 37°C fails to induce the 93D although the other heat shock puffs are activated (Mukherjee and Lakhotia 1982). Another intriguing observation was that the non-induction of the 93D puff following heat shock in combination with BM or recovery from anoxia at 37°C is accompanied by differential activation (Lakhotia and Mukherjee 1980; Mukherjee and Lakhotia 1982) of the otherwise nearly equally induced 87A and 87C puffs, which were by then known to be duplicated loci encoding Hsp70 (Livak *et al.* 1978). The observation relating to the unequal puffing of the 87A and 87C puffs when the 93D puff is not induced by heat shock has been a recurring theme in later studies (see section 5), and this aroused my interest in the other heat shock genes and thus in stress biology.

Some studies from Mary-Lou Pardue's laboratory in late 1970s had also suggested unusual properties of the 93D puff. In a remarkable study, Spradling *et al.* (1977) used electrophoretically separated fractions of radioactively labelled newly synthesized RNA from heat-shocked cells of *Drosophila* to hybridize *in situ* with polytene chromosomes (for the present day molecular or genomic biologists, the approach used in this study would be analogous to identification of *newly synthesized RNA* species using a whole genome microarray analysis). They found that none of the newly synthesized polyA+ RNA fractions from heat-shocked cells hybridized to the 93D puff. On the other hand, Bonner and Pardue (1976) had earlier shown that, unlike the other heat shock sites, the 93D puff was activated to very different levels depending upon the conditions of heat shock. In yet another study, Lengyel *et al.* (1980) examined metabolism of RNA at the 93D puff in heat-shocked cells and showed that this RNA may contain repeated sequences and only a part of the sequences transcribed from 93D were exported from the nucleus to cytoplasm, while a greater part stayed in the nucleus itself.

I first met Mary-Lou Pardue in the late 1970s during a meeting at Tata Institute of Fundamental Research, Mumbai. Following this meeting, we started regular and long-lasting exchange of our results and ideas. She visited my laboratory at Varanasi in the 1980s several times, not only because of our common interest in the 93D puff but also because Varanasi was on her way to Kathmandu for trekking in the Himalayas. I also visited her laboratory in 1985 for 3 months. These exchanges resulted in two joint publications in 1989 (section 7).

3. A homolog of the 93D puff is present in different species of *Drosophila* but does not seem to encode a protein

It was clear from the publications in the late 1970s that the 93D locus differed from the other heat shock loci in features that warranted additional study, especially when the heat shock response was becoming 'hotter' for molecular biological studies on gene expression and regulation. Given this background, it was important for us to identify the protein product that was expected to be encoded by the 93D gene. In the absence of a commercial vertical slab gel apparatus and a gel drier, Tapas decided to fabricate these apparatuses himself and, remarkably, he succeeded. However, when we finally performed this experiment with the 'home-made' gel system, we found no detectable difference in protein synthesis in BM-treated and control salivary glands and we suggested that the heat shock and BM-inducible 93D puff may not encode a protein (Lakhotia and Mukherjee 1982; figure 2). The above-noted findings of Lengyel *et al.* (1980) on metabolism of this puff's transcripts also seemed to indicate such a possibility.

The apparent non-coding nature of this gene was generally disappointing in view of the emerging concept of 'selfish' or 'junk' DNA (Doolittle and Sapienza 1980; Orgel and Crick 1980). However, our interest in this gene grew because we had just found that a BM- and heat-shock-inducible puff was present in every species of *Drosophila* that we examined (Lakhotia and Singh 1982; figure 3). Having a strong faith in the power of natural selection, I continued to believe in functional requirement of the 93D puff and its homologs in *Drosophila*, even if these loci were not coding for a protein.

Identification of the 2-48B (also sometimes named as 2-48C) puff of *Drosophila hydei* as a homolog of the 93D puff of *D. melanogaster* (Lakhotia and Singh 1982) was especially interesting since the 2-48B puff was shown by the group at Nijmegen (the Netherlands), led by Dr HD Berendes, to be singularly induced by vit-B6 or pyridoxine (Leenders *et al.* 1973). While on a brief visit to the Nijmegen laboratory in 1973, I asked Dr HJ Leenders if his group had ever examined inducibility of the 2-48B puff of *D. hydei* with BM. Dr Leenders offered to check this inducibility and, shortly thereafter, informed me in the negative. However, it soon became apparent that he tested benzidine rather than BM; because the Nijmegen laboratory did not have BM, the issue was not followed further. Only 9 years later we identified the *D. hydei* 2-48B puff as BM inducible (Lakhotia and Singh 1982). Interestingly, the Nijmegen group had attempted to find homology of different heat shock puffs in *Drosophila* species through *in situ* hybridization of transcripts

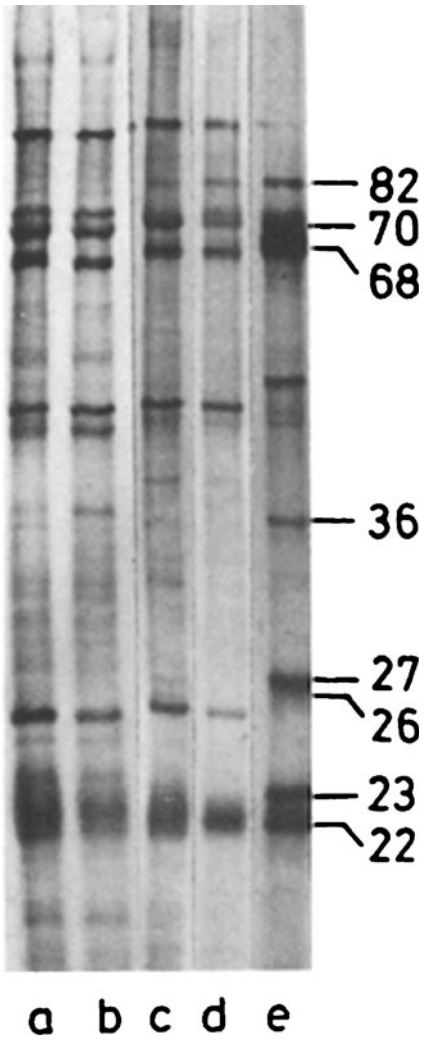

Figure 2. Specific induction of the 93D puff by BM is not accompanied by synthesis of a new polypeptide. Autoradiogram of ^{35}S-methionine-labelled polypeptides separated by SDS-polyacrylamide gel electrophoresis reveals similar patterns of protein synthesis in control (lanes a and c) and in corresponding sister glands treated with benzamide at 24°C for 20 min (lanes b and d). Lane e shows protein synthesis pattern in heat-shocked glands. Note the absence of labelling of any novel polypeptide in glands treated with BM (lanes b and d). Image reproduced from Lakhotia and Mukherjee (1982)

induced in response to heat shock and vit-B6. Peters *et al.* (1980) reported that the nuclear transcripts from the 2-48B puff of *D. hydei* did not hybridize with any of the chromosome sites in *D. melanogaster* or in *D. virilis* and concluded that while the 20CD puff of *D. virilis* was an homolog of the 2-48B puff because of their common vit-B6 inducibility, a homolog of 2-48B did not exist in *D. melanogaster* because vit-B6 did not induce any puff in this species. Although the inference of Peters *et al.* (1980) that a 2-48B homolog was absent in *D. melanogaster* turned out to incorrect, their study did portend the subsequent discovery (section 5) of the rather rapid DNA base sequence divergence of the 93D and its homologs in other species. A later study in my lab (Singh and Lakhotia 1983) found that while the homogenate of heat shocked salivary glands of *D. melanogaster* retained its ability to specifically induce the 93D puff even after being heated to 100°C, that of *D. hydei* lost its 2-48B inducing ability when heated. The reason for the 93D puff of *D. melanogaster* remaining refractory to vit-B6 treatment as well as the nature and identity of 93D-inducing factors in the homogenate of heat-shocked cells remain unknown.

In view of a correlated increase in synthesis of a 40 kDa tyrosine aminotransferase following heat-shock- or pyridoxine-induced activation of the 2-48B puff of *D. hydei*, Belew and Brady (1981; also see Brady and Belew 1981) suggested that the cytoplasmic transcript of this gene (Peters *et al.* 1980) may encode the tyrosine aminotransferase. However, this has not been confirmed subsequently. Likewise, Scalenghe and Ritossa (1977) suggested that the 93D puff of *D. melanogaster* may encode subunit I of glutamine synthetase, but this paper was subsequently withdrawn.

With a view to identify a 93D-like locus in other dipterans, we examined heat-shock- and BM-inducible puffs in *Anopheles* and *Chironomus* (Nath and Lakhotia 1991). Although a BM- or colchicine-inducible puff was not detected in these two species, the nature of transcripts, base sequence evolution and binding of hnRNPs and Hsp90 at one of the heat-shock-inducible telomeric balbiani ring TBR-III in *Chironomus thummi* (Santa-Cruz *et al.* 1984; Carmona *et al.* 1985, Lakhotia 1989; Nath and Lakhotia 1991; Morcillo *et al.* 1994) suggested that this locus could be functionally similar to the 93D puff.

4. Cloning and sequencing of the 93D puff and its homologs in other species confirm its non-coding nature as well as rapid sequence divergence

Cloning of some sequences from the 2-48B puff of *D. hydei* (Peters *et al.* 1980) indicated certain unusual features of this puff, which were further confirmed by a more detailed characterization of several clones from this region (Peters *et al.* 1984). In the same year, cloning of the 93D puff sequences using micro-dissection of polytene chromosomes of *D. melanogaster* was reported (Walldorf *et al.* 1984). These studies also indicated that the nuclear transcripts of these puffs were not translated since they appeared to be mostly made up of repeated sequences with

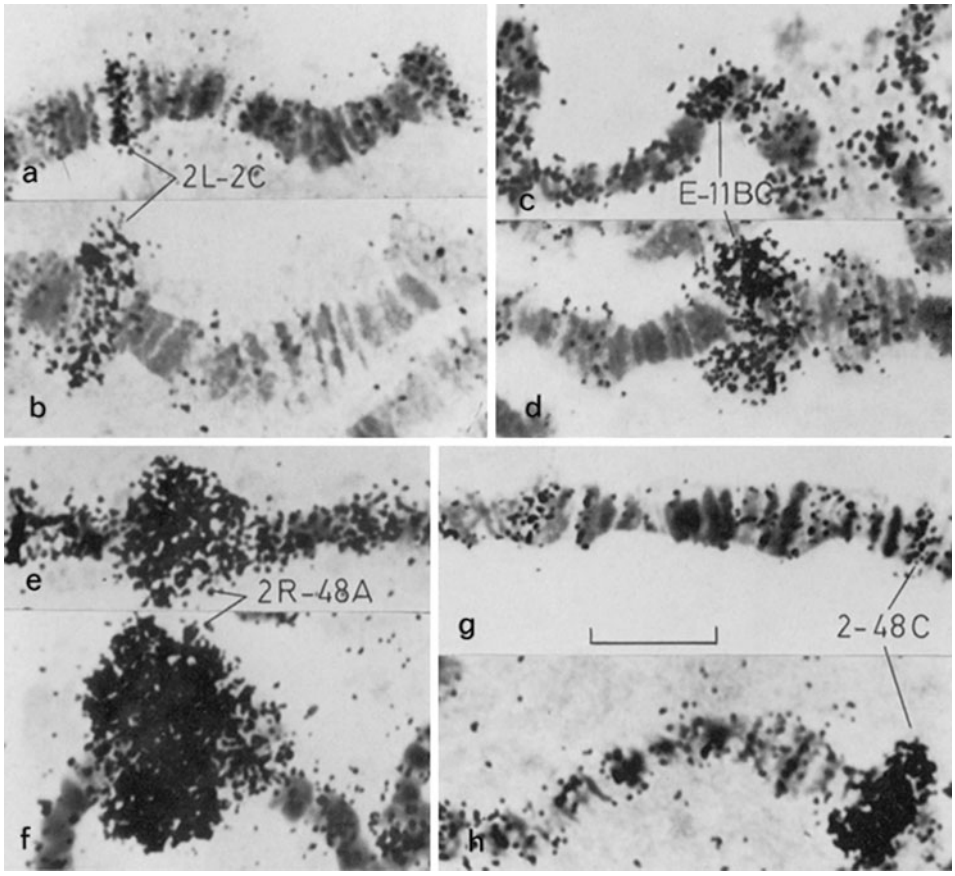

Figure 3. 93D homologs in other species of *Drosophila* identified through ^{3}H-uridine labelling of the benzamide-inducible heat shock puff site: control (**a**, **c**, **e** and **g**) and BM-treated (**b**, **d**, **f** and **h**) salivary glands of (**a**–**b**) *D. ananassae,* (**c**–**d**) *D. kikkawai,* (**e**–**f**) *D. nasuta* and (**g**–**h**) *D. hydei*. Reproduced from Lakhotia and Singh (1982).

very limited coding potential. Subsequent cloning and sequencing studies in the laboratories of Pardue (Garbe *et al.* 1986; Garbe and Pardue 1986; Fini *et al.* 1989) and B Hovemann (Ryseck *et al.* 1985, 1987; Hovemann *et al.* 1986) provided data for comparison of the base sequences of the 93D and its homologs in *D. hydei* and *D. pseudoobscura*; a 93D-like puff was identified by Burma and Lakhotia (1984) in the latter species also. These data not only confirmed the earlier predictions (Lengyel *et al.* 1980; Peters *et al.* 1980; Lakhotia and Mukherjee 1982) that this locus may not encode a protein but also revealed production of multiple transcripts by this locus and its unexpected rapid sequence divergence in spite of the rather conserved architecture in *Drosophila* species (reviewed in Lakhotia 1987; Bendena *et al.* 1989a; Pardue *et al.* 1990). On the basis of comparison of the base sequence data for three species, *D. melanogaster*, *D. hydei* and *D. pseudoobscura*, the following unconventional features of the organization and transcripts of this locus emerged (figure 4). It may be mentioned that a bioinformatic analysis (Eshita Mutt and Lakhotia, unpublished) of the genomic sequences of different *Drosophila* species available on the Flybase (*http://flybase.org*) has confirmed the below noted architectural features of the 93D-like loci in all the species:

a. The 93D locus and its homologs in all other species have ~10- to 15-kb-long transcription unit with the proximal part (~2.6 kb) comprising of unique sequence and distal region (>5 kb) comprising entirely of tandem repeats of a short sequence, unique to this locus.
b. Two primary and independent transcripts, both limited to nucleus, are produced by this locus: the longer transcript (usually >10 kb) represents the

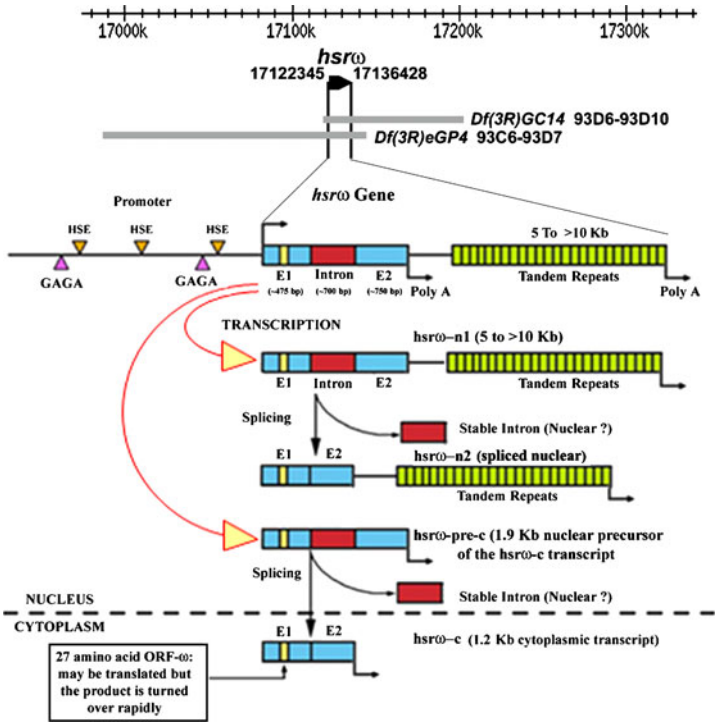

Figure 4. Architecture of the *hsrω* locus in *D. melanogaster*. On the top are shown the genomic coordinates of the *hsrω* gene with the numbers on a scale reflecting the base numbers on chromosome 3; the two parallel vertical lines indicate the span of the *hsrω* gene with the arrowhead showing the direction of transcription. The base numbers of transcription start and termination sites, with reference to the genomic sequence of chromosome 3 are also indicated (data from *http://www.flybase.org*). Spans of two deletions, *Df(3R)GC14* and *Df(3R)e*^{Gp4}, used in several studies described in the text are indicated by the gray horizontal bars. Some of the regulatory elements in the proximal promoter regions, as identified by Mutsuddi and Lakhotia (1995) and the transcribed region comprising of two exons (E1 and E2), one intron, and the long stretch (5 to >10 kb) of tandem repeats (280 bp repeat unit) are shown in the middle. Two primary transcripts, hsrω-n1 and hsrω-pre-c are produced, each of which is spliced to remove the ~700 b long intron. The hsrω-n1 and hsrω-n2 transcripts remain within the nucleus while the 1.2 kb hsrω-c transcript moves to cytoplasm where its ORF-ω may be translated. The hsrω homologs in other species of *Drosophila* also show a comparable architecture and transcript pattern.

entire transcription unit, while the shorter one is about 1.9 kb and corresponds to part of the proximal unique region till the first poly-A signal, which is about 700 bp prior to the beginning of the stretch of repeats.

c. The shorter 1.9 kb nuclear transcript is spliced to remove the single intron (~700 bases long) and the 1.2 kb transcript is transported to cytoplasm.

d. Neither the unique part nor the repeats show any significant degree of homology between the species except for a remarkably conserved stretch of about 15 and 59 bases at the 5′ and 3′ exon–intron junctions, respectively.

e. Amidst the high sequence divergence of the repeat units at the 3′ end of the gene in the two species (*melanogaster* and *hydei*), a nonamer (ATAGGTAGG) appears to be conserved so that it is present at nearly equal intervals in the repeat part of the large nuclear transcript.

f. The ~1.2 kb cytoplasmic transcript is associated with mono- or di-ribosomes and carries a potentially translatable open reading frame (ORF-ω) that may encode 23–27 amino acids in different species (Garbe *et al.* 1986) but whose translational product is not detectable (Fini *et al.* 1989). Intriguingly, the amino acid sequence potentially encoded by the ORF-ω in different species shows little conservation (Garbe *et al.* 1986).

Since the 93D locus was heat shock inducible but yielded only RNA as the final product, the gene was named *hsrω* or *heat shock RNA omega* (Bendena *et al.* 1989a), and the

three transcripts were named (Hogan *et al.* 1994) as hsrω-n (>10 kb, nuclear), hsrω-pre-c (1.9 kb, nuclear and precursor to the cytoplasmic transcript) and hsrω-c (1.2 kb, cytoplasmic). These three transcripts have also been named as ω-1, ω-2 and ω-3 (Garbe *et al.* 1989) or Hsrω-RB, Hsrω-RC and Hsrω-RA (*http://flybase.org*), respectively. It may be noted that until recently it was believed that the >10 kb hsrω-n transcript is not spliced. However, we (Mallik and Lakhotia 2011) recently found that this transcript is also spliced and that the unspliced as well as spliced forms, named, respectively, as hsrω-n1 and hsrω-n2, exist in the nucleus.

The realization that the 93D or the *hsrω* locus does not produce any detectable protein product was a disappointment in the 1980s, when biology was undergoing revolutionary changes ushered in by the increasingly efficient recombinant DNA methods and at a time when any DNA sequence not involved in production of proteins was generally considered 'junk' or 'selfish' and, therefore, not worthy of funding. The rapid sequence divergence of *hsrω* locus in *Drosophila* species also appeared to agree with its 'selfish' nature, a view further supported by the failure to isolate any point mutation in the *hsrω* locus (Mohler and Pardue 1982, 1984). As a consequence, both the Hovemann as well as the Pardue laboratories gradually stopped an active pursuit of this locus in the 1990s.

5. Diverse inducers or modifiers of its induction exist but the mechanism is not understood

We could not initiate typical molecular biological studies of the locus for several years because of the limited resources and facilities. In the absence of any mutant allele of the *hsrω* gene, a direct functional analysis of this non-coding gene was also not possible. Consequently, 93D puffing kept us engaged for some years and most of our studies during the 1980s and early 1990s essentially addressed the phenomena rather than the mechanisms in the hope that studies of the phenomena would give us some insight into functional significance of the 93D puff and the underlying mechanisms.

5.1 *Amides as specific inducer of 93D puff*

A review on heat-shock-induced gene activity by Ashburner and Bonner (1979) cited a publication by Gubenko and Baricheva (1979) in which colchicine was reported as a specific inducer of the 20CD puff of *D. virilis*, which on the basis of the above-noted studies of Peters *et al.* (1980) was a homolog of the 2-48B puff of *D. hydei*. Since the 2-48B puff was identified as a homolog of the 93D puff, we wanted to test if colchicine would also be a singular inducer of the 93D puff. We expected that colchicine, being a well-known disruptor of microtubules (Dustin 1978), may provide a better understanding of the significance of the 93D puff than BM, whose cellular effects were little understood. Colchicine as well as colcemid indeed mimicked BM in selective induction of the 93D puff (Lakhotia and Mukherjee 1984). To confirm the involvement of microtubules in 93D puff induction, Singh and Lakhotia (1984) examined cold shock and several other known microtubule poisons such as chloral hydrate, diamide, podophyllotoxin, vinblastin, nocodazole and griseofulvin to see if they induced the 93D puff or its homologs in other species. Surprisingly, none of these, except the cold shock, induced the 93D or other heat shock puffs (Singh and Lakhotia 1984). Thus, the disruption of microtubule organization did not appear to be the cause for selective induction of the 93D puff and its homologs in other species. Subsequent studies suggested that the induction of 93D puff by colchicine was due to its being an amide (*see* below).

Behnel (1982) reported that thiamphenicol specifically induced only the 93D puff, while Srivastava and Bangia (1985) noted that paracetamol also specifically induced the 93D puff. A structural analysis of these 93D-inducing chemicals (benzamide, colchicine, colcemid, thiamphenicol and paracetamol) with very different known effects on cellular metabolism revealed that all of them were amides (Tapadia and Lakhotia 1997). Therefore, we tested several other amides (nicotinamide, acetamide, formamide and 3-aminobenzamide) for their 93D-inducing activity, and indeed, all of them had the same effect as BM (Tapadia and Lakhotia 1997). Thus specific induction of the 93D puff and its homologs in other species by these diverse chemicals seem to be dependent upon their being amides. By this time, 3-aminobenzamide, a derivative of BM, was widely used as an inhibitor of poly-ADP ribose polymerase, or PARP (Sims *et al.* 1983), especially in DNA repair studies. In our paper (Tapadia and Lakhotia 1997) we discounted the possibility that the specific induction of the 93D puff may be related to the well-known inhibition of PARP by several of these amides because while that enzyme was inhibited with as little concentration of the amides as 3 mM, the 93D-inducing activity required 10 mM concentration. However, this conclusion needs re-examination in view of the recent report (Ji and Tulin 2009) of interactions between PARP and different hnRNPs, which in turn interact with the hsrω-n transcripts (section 9).

5.2 *Developmental conditions affect 93D inducibility*

Lakhotia and Singh (1985) reported that the 93D puff was refractory to induction by heat shock (37°C) or BM

or colchicine in salivary glands of *D. melanogaster* late third instar larvae that were reared at 10°C since hatching. However, transferring these glands from 10°C to 24°C by itself induced the 93D puff, without induction of the other heat shock puffs. Another intriguing observation was that ingredients in the fly food may affect the BM or colchicine inducibility of the 93D puff (Lakhotia 1989). Significance and mechanism of the effect of developmental conditions on 93D puff inducibility have remained unexplored.

5.3 *β-Alanine as a modifier of heat shock inducibility of 93D*

Based on the consistency and reproducibility of effects of non-induction of the 93D puff under certain conditions of heat shock on the 87A and 87C puffing after heat shock, we felt that the state of active transcription at the 93D puff has some role to play in the relative puffing of the two Hsp70-coding loci (Lakhotia 1987). However, Hochstrasser (1987) questioned this because he found unequal puffing at 87A and 87C even when the 93D puff appeared to be typically induced by heat shock. In this context, we used the *T(1;3)eH*^{2}*, red e* chromosome which displayed position effect variegation at the *ebony* locus (Henikoff 1980; Lakhotia *et al.* 1990). We expected the 93D locus, being close to the *ebony* locus, to also show variegated expression in this chromosome, and therefore, we hoped to examine the effect of such variable induction of the 93D puff on the relative levels of the 87A and 87C puffs in different cells of the same gland. Although a variegated induction of the 93D puff was not observed, these studies revealed an unexpected and intriguing effect of mutant alleles of the *ebony* or the *black* locus on the heat-shock-induced activity of the 93D puff (Lakhotia *et al.* 1990). Loss of function alleles of the *ebony* enhance while those of the *black* reduce levels of β-alanine in larval hemolymph (Wright 1987). Both these mutant conditions were found to inhibit induction of 93D puff by heat shock (Lakhotia *et al.* 1990). Feeding wild-type larvae with excess β-alanine also caused the 93D puff to be refractory to heat shock, but feeding the *black* homozygous mutant larvae on β-alanine restored heat-shock-induced puffing of the 93D locus (Lakhotia *et al.* 1990). Interestingly, the changes in levels of β-alanine due to mutation or feeding had no effect on induction of the 93D puff by BM. The mechanism of the inhibition of 93D puffing after heat shock by altered levels of β-alanine remains unexplored. In the context of the effect of 93D puffing during heat shock on 87A and 87C activity, the absence of 93D puff during heat shock following changes in β-alanine levels was also found to be associated with unequal puffing of the 87A and 87C sites (Lakhotia *et al.* 1990).

5.4 *Dosage compensation for developmental expression of 93D*

During their screen for mutations at the 93D locus, Mohler and Pardue (1982, 1984) generated two small deficiencies (*Df(3R)e*^{Gp4} and *Df(3R)GC14*) spanning the 93D region such that their overlap homozygously deleted the 93D heat-shock- and BM-inducible locus (Burma and Lakhotia 1986) while the flanking genes were believed to be present at least in one copy in the *Df(3R)e*^{Gp4}*/Df(3R)GC14* trans-heterozygotes (figure 4). The *93D* or *hsrω*-null *Df(3R)e*^{Gp4}*/Df(3R)GC14* trans-heterozygotes have poor viability, with nearly 80% dying as embryos or early larvae (Mohler and Pardue 1984; Lakhotia and Ray 1996) while the survivors emerge as very weak short-lived flies, suggesting an essential developmental requirement of this non-coding gene. Studies on the heat shock and benzamide inducibility of heat shock puffs in these deficiency chromosomes revealed another interesting property of the 93D puff. Burma and Lakhotia (1986) found that the developmental or BM-induced rates of transcription at the 93D locus in *Df(3R)e*^{Gp4}*/+* or *Df(3R)GC14/+* salivary glands, with only one copy of the locus, were equal to those in corresponding wild-type glands, which suggested that the developmental or BM-induced activities of the 93D puff were dosage compensated. On the other hand, heat shock caused regression of this puff in either of these genotypes, which was accompanied by unequal puffing of the 87A and 87C sites. Surprisingly, in *hsrω*-null *Df(3R)e*^{Gp4}*/Df(3R)GC14* trans-heterozygotes, the 87A and 87C puffs in about 50% of the glands were equally active, while in the remainder, the 87C puff was less active than the 87A (Burma and Lakhotia 1986).

6. Non-induction of the 93D puff during heat shock affects the Hsp70-encoding twin puffs at 87A and 87C

We were intrigued by the recurring observation that whenever the 93D puff was not induced during heat shock, the puffing activity at the 87A and 87C puffs, the twin loci harboring two and three copies, respectively, of *Hsp70* genes (Livak *et al.* 1978), was differential instead of being nearly equal as seen after a simple heat shock to larval salivary glands (reviewed in Lakhotia 1987, 1989). The 87C site also carries heat-inducible alpha-beta (αβ) repeats (Livak *et al.* 1978). To assess the actual levels of the hsp70 and αβ transcripts at the two sites following the different experimental conditions, we (Sharma and Lakhotia 1995) quantified their levels at the 87A and 87C heat shock loci of *D. melanogaster* by *in situ* hybridization of ^{35}S-labelled antisense RNA probes with polytene chromosome squashes of larval salivary glands treated with heat shock,

benzamide, colchicine, heat shock followed by benzamide or heat shock in the presence of colchicine. These results (figure 5) revealed that the larger or smaller size of 87A and 87C puffs under different conditions of heat shock correlated with altered levels of the hsp70 transcripts at the two sites and αβ-transcripts at the 87C site. Interestingly, colchicine, but not benzamide, treatment itself also enhanced αβ transcripts at the 87C site, without any induction of the hsp70 transcripts at either of the puff sites. Parallel studies (Lakhotia and Sharma 1995, see section 7) showed that the hsrω transcript profile at the 93D puff in these different conditions also varied. These results seem to establish a causal relationship between hsrω transcripts synthesized at the 93D locus and RNA metabolism at the 87A and 87C sites, although the underlying mechanism remains unknown. In this context, it is interesting to note that another study (Kar Chowdhury and Lakhotia 1986) compared the 87A and 87C puffs of *D. simulans* and *D. melanogaster* sibling species; it was found that the 87A and 87C puffs of *D. simulans,* which does not carry αβ-repeats at the 87C site, were equally induced by heat shock even when the 93D puff was not induced due to combined treatment with BM or colchicine. Interestingly, in the interspecific hybrid polytene cells, the 87A and 87C loci on the two species' homologs behaved in their parental species-specific manner. This difference between the two species appeared to correlate with the absence of the αβ repeats at the 87C locus in *D. simulans* (Kar Chowdhury and Lakhotia 1986). Significance of the heat-shock-inducible αβ repeats at the 87C site of *D. melanogaster* and their interaction with the hsrω transcripts remains unknown.

Our recent unpublished studies show that conditions under which the optimal levels of hsrω transcripts are not available in the cell during or after heat shock, association of some of the hnRNPs with the 87A and 87C puff sites is affected. Further studies will help us understand this relationship.

7. Inducer-specific transcript profile of the *hsrω* gene

I visited the Pardue laboratory at MIT (USA) for 3 months in 1985 on a Fulbright fellowship and initiated studies to examine the nature of transcripts induced by BM and colchicine treatments. This collaboration resulted, some years later, in a research paper (Bendena *et al.* 1989b) and a review (Bendena *et al.* 1989a). It was found that BM and colchicine indeed enhance levels of different hsrω tran-

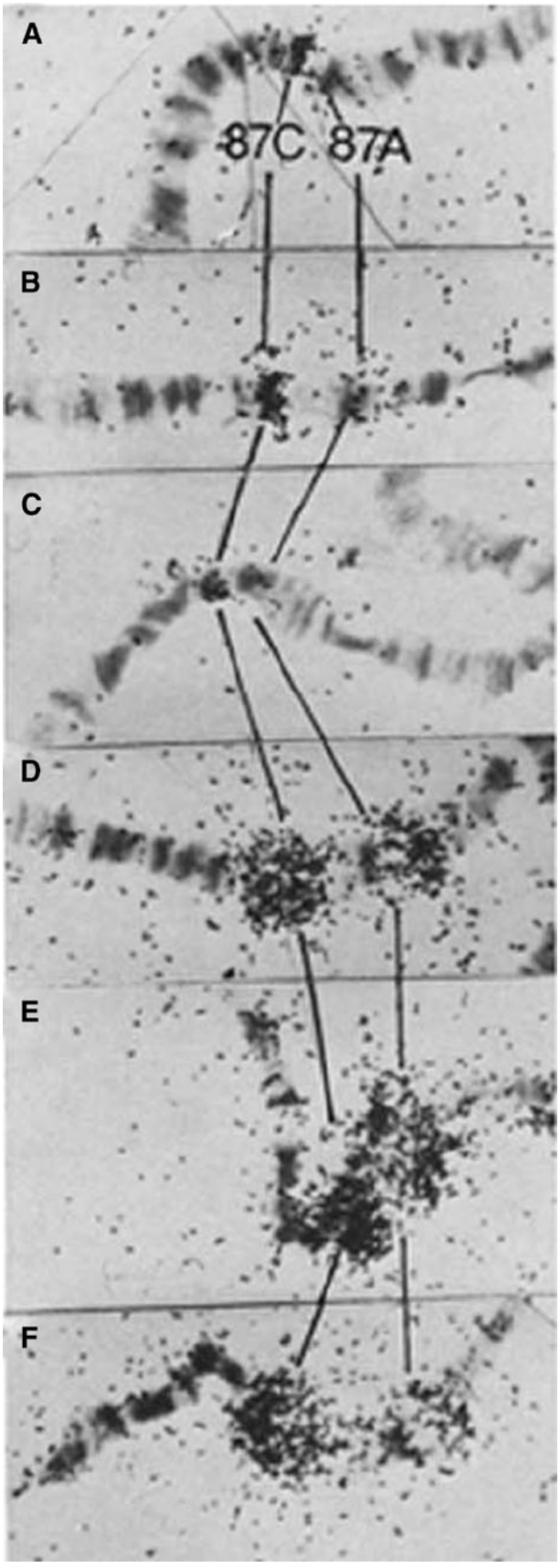

Figure 5. *In situ* hybridization of ^{35}S-labelled hsp70 antisense RNA probe to RNA on the 87A and 87C sites of polytene chromosomes in (**A**) control salivary glands or after treatment with (**B**) benzamide, (**C**) colchicine, (**D**) heat shock, (**E**) heat shock followed by benzamide and (**F**) heat shock in the presence of colchicine. Reproduced from Sharma and Lakhotia (1995).

scripts in diploid Schneider cells as well, but the different hsrω transcripts are affected in an inducer-specific manner. While heat shock causes elevation of all the hsrω transcripts, BM and colchicine elevate only the large nuclear hsrω-n transcript. These studies also revealed interesting differences in kinetics of accumulation and degradation of the different hsrω transcripts in an inducer-specific manner. The developmentally expressed hsrω-n transcripts had a slow turnover, but those induced by heat shock, BM or colchicine turned over rapidly; however, this turnover appeared to be dependent upon continued transcription since in the presence of actinomycin D, the hsrω-n transcript levels remained high even after withdrawal of BM (Bendena *et al.* 1989b). In contrast, the hsrω-pre-c and hsrω-c transcripts had very short half-lives but were stabilized by inhibition of translation. The differential effects of heat shock, BM, colchicine and protein synthesis inhibitors (Bendena *et al.* 1989b) suggested that while the large hsrω-n transcript had roles in nuclear activities, the cytoplasmic hsrω-c transcript was related to translational activities. Because only the first four amino acids of the short but translatable ORF-ω in the cytoplasmic transcript (Fini *et al.* 1989) are strongly conserved, it appeared that it was the act of translation of ORF-ω, rather than its product, that was important to the cell (Bendena *et al.* 1989a). Accordingly, while reviewing the 93D puff story at the first conference of the Asia-Pacific Organization of Cell Biology (APOCB) at Shanghai in 1991, I suggested that the 1.2 kb hsrω-c transcript monitors the cells' translational activities. In a later more detailed review, we (Lakhotia and Sharma 1996) suggested that the hsrω locus 'has important house-keeping functions in transport and turnover of some transcripts and in monitoring the "health" of the translational machinery of the cell'.

Using quantitative RNA:RNA *in situ* hybridization with squash preparations of polytene chromosomes, we (Lakhotia and Sharma 1995) examined relative levels of the different hsrω transcripts *in situ* at the 93D puff following various treatments. Heat shock enhanced levels of all the three transcripts, viz., hsrω-n, hsrω-pre-c and hsrω-c at the 93D puff site in a coordinated manner; BM treatment caused a significant increase in the levels of hsrω-n and hsrω-pre-c, whereas colchicine resulted in increased levels of the hsrω-n and hsrω-c RNA species at the 93D site. This study also suggested that the hsrω-pre-c RNA is spliced at the site of synthesis with the spliced-out 'free' intron (hsr-omega-fi) accumulating at the puff site in a treatment specific manner. It was also found that in certain conditions under which the 93D puff was not induced in polytene cells and showed little ^{3}H-uridine incorporation, the amount of hsrω transcripts present at the 93D region of polytene chromosomes remained nearly as high as when it was typically induced (Lakhotia and Sharma 1995). This suggests additional regulation at the level of turnover of these transcripts.

8. Complexity of regulation of developmental and induced expression of *hsrω*

Although the 93D puff has been known mostly for its unique inducible properties, this locus also forms a developmental puff in salivary glands of wild-type late third instar larvae (Ashburner 1967; Mukherjee and Lakhotia 1979). Northern analyses of total cellular RNA from unstressed salivary glands/embryos/whole organisms or from cells in culture always showed this gene's different transcripts in all preparations (Garbe *et al.* 1986). The very poor viability of *hsrω*-nulls (*Df(3R)e*^{Gp4}*/Df(3R)GC14*) also suggested this gene to have developmental expression and essential functions. Bendena *et al.* (1991), using a radioactively labelled RNA probe for *in situ* hybridization with hsrω transcripts in histological sections of different tissues from the various developmental stages, showed that the three omega transcripts are produced in almost all tissues in a regulated manner, with ecdysone probably playing a role in the tissue- and stage-specific changes in hsrω transcript profile. Only the preblastoderm embryos and the primary spermatocytes were reported (Bendena *et al.* 1991) to be devoid of hsrω transcripts.

We (Mutsuddi and Lakhotia 1995) re-examined the developmental expression of the *hsrω* gene by whole organ colorimetric RNA:RNA *in situ* hybridization and by following expression of the *lacZ* reporter gene placed downstream of different stretches of the *hsrω* promoter in transgenic lines generated by us. Our observations were generally in agreement with those of Bendena *et al.* (1991) but additionally revealed that the male as well as female gonial cells and oocytes in ovary do not contain hsrω transcripts. Based on these and other results, it was suggested that the variety of non-protein-coding transcripts of the *hsrω* gene have vital 'house-keeping' functions (Mutsuddi and Lakhotia 1995; Lakhotia and Sharma 1996), although the nature of such 'functions' remains elusive.

The β-galactosidase reporter expression studies in germline transformants carrying the *lacZ* reporter under different regions of the *hsrω* promoter (Mutsuddi and Lakhotia 1995) also helped in analysis of the promoter of the *hsrω* locus. The β-galactosidase reporter expression suggested that the region between −346 bp to −844 bp upstream contained major regulatory element/s for most of the developmental expression of this gene although this region was not sufficient for its expression in male and female reproductive systems. It was further shown that heat shock but not the BM or colchicine response elements are present within the proximal 844 bp of the *hsrω* promoter (Lakhotia and Mutsuddi 1996). Based on results of another unique study utilizing small chromosomal deficiencies, Lakhotia and Tapadia (1998) suggested that the amide response element

may be located more than 21 kb upstream of the *hsrω* gene and that intervening sequences may not interfere with action of the putative amide response element.

In a later more detailed study (Lakhotia *et al.* 2001), an enhancer-trap line carrying *P-lacZ* transposon insertion at −130 bp position of the *hsrω* promoter ($hsr\omega^{05241}$) was used to monitor the developmental and induced expression of the *hsrω* gene in a greater variety of embryonic, larval and adult tissues. The developmental, heat-shock- and BM-induced β-galactosidase reporter gene expression in this enhancer-trap line was compared with expression of the *hsrω* gene itself in different tissues through RNA:RNA *in situ* hybridization in the transposon insertion line and in wild type. Further, the two earlier generated transgenic lines (Mutsuddi and Lakhotia 1995) with the β-galactosidase reporter activity driven by different regions of the *hsrω* promoter were also used for comparison. This detailed study of the developmental expression revealed that the embryonic pole cells and the hub cells in testes and ovaries also do not express the *hsrω*, neither normally nor after heat shock. Significantly, in spite of insertion of a big transposon in the promoter, expression of the $hsr\omega^{05241}$allele in the enhancer-trap line, as revealed by *in situ* hybridization to cellular hsrω transcripts, was comparable to that in unstressed and heat-shocked wild-type embryonic, larval and adult somatic tissues examined. The BM inducibility of the 93D puff also appeared to be unaffected by the *P*-transposon insertion in the $hsr\omega^{05241}$ chromosome. On the other hand, expression of the *lacZ* reporter in this enhancer-trap line paralleled the *hsrω* RNA in all diploid cell types, but in the polytene cells, the β-galactosidase reporter activity in unstressed as well as heat-shocked tissues was completely absent (Lakhotia *et al.* 2001).

Small differences in the site of insertion of P-transposon in the *hsrω* gene promoter have been found to result in distinctly different phenotypic consequences. Insertion of the EP (enhancer promoter)-element in the promoter region of *hsrω* gene by itself causes small differences in its developmental expression, which are associated with specific phenotypes in adult flies. The EP-transposon insertion in the *EP93D* and *EP3037* lines is at −130 and −144 bp, respectively (Mallik and Lakhotia 2009a). However, even with this small difference in the insertion sites, the *EP3037* homozygous flies are generally much weaker and less fecund than the *EP93D* homozygotes (Mallik and Lakhotia 2011). Further, when driven by GAL4 drivers, these two EP alleles result in distinctly different phenotypic consequences in certain instances (Mallik and Lakhotia 2009a, b, 2010a, 2011). Apparently, the local chromatin structure/regulatory elements in the *hsrω* promoter affect the EP allele expression.

The recently generated Exelixis and Drosdel collections of chromosomal deletions with molecularly defined breakpoints have provided chromosomes in which defined region of the *hsrω* promoter or only its transcribed region is deleted. Using some of these molecularly defined short deletions, Akanksha in my laboratory has recently (unpublished) found that even the proximal 124 bp of the *hsrω* promoter can drive some developmental expression so that genotypes with only one copy of the *hsrω* gene carrying just the proximal 124 bp promoter region can survive under normal conditions although they are sensitive to heat shock.

Together, these results clearly indicate that the *hsrω* gene's promoter is complex, as may be expected from the diversity of tissues in which this gene is expressed in a regulated manner and the variety of environmental factors that influence its transcriptional activity and the stability/turnover of its different transcripts. Several non-exclusive possibilities about its transcriptional regulation need further studies: (i) most of the developmental and heat-shock-induced regulation is at least partially controlled by the proximal 124 bp of the upstream region, (ii) some of the regulatory sites may be present within the transcribed region and/or (iii) the far upstream regulatory elements can exert their effects across the transposon insertion or other intervening genes.

9. Analysis of *hsrω*'s 'functions' through genetic interaction studies

Identification of functions of a non-coding gene is a difficult task when no point mutation is available and, especially more so, when the base sequence of the homologs changes as rapidly between related species as recorded for the *hsrω* locus. The two small deficiencies, $Df(3R)e^{Gp4}$ and *Df(3R) GC14* (Mohler and Pardue 1982, 1984), spanning the *hsrω* locus provided the only opportunity until recently for a genetic approach to examine functions of this enigmatic gene. When these two deletions are brought in trans, they completely delete both copies of the *hsrω* gene (figure 4). However, since their proximal and distal breakpoints are not molecularly mapped (Mohler and Pardue 1984), the extent of flanking regions that would be absent on both homologs or would be present only on one of the deficiency chromosomes remains uncertain. Nevertheless, in spite of this limitation, these two deficiencies did provide some insights into the functions of the *hsrω* gene. The high early lethality of the $Df(3R)e^{Gp4}$/*Df(3R)GC14* trans-heterozygous 93D-null indicated essential developmental functions of this locus. On the other hand, their increased thermo-sensitivity suggested a role of these transcripts in the heat shock response, notwithstanding the fact that the synthesis of heat shock proteins was not affected in the *hsrω*-null individuals (Mohler and Pardue 1984; Lakhotia 1987, 1989). A role of the *hsrω* gene in thermal adaptation has also been noted in a series of population genetic studies by S. McKechnie's group using its natural indel and other alleles in Australian

populations (McKechnie *et al.* 1998; McColl and McKechnie 1999; Anderson *et al.* 2003; Collinge *et al.* 2008, also see section 11.5 for my laboratory's recent observations on the role of this gene in surviving heat shock).

To investigate the possible functions of these non-coding transcripts, we initially made use of the above-noted two deletions in genetic interaction studies. Following a report by Morcillo *et al.* (1993) that heat shock enhanced binding of the Hsp83 with the 93D puff, we found that *hsp83* mutant alleles dominantly enhanced the high lethality of *Df(3R)* e^{Gp4}*/Df(3R)GC14* trans-heterozygous progeny (Lakhotia and Ray 1996). Likewise *Ras* mutant alleles, which were shown to interact with Hsp83 (Cutforth and Rubin 1994), also dominantly enhanced the *Df(3R)*e^{Gp4}*/Df(3R)GC14* trans-heterozygous phenotype (Ray and Lakhotia 1998). Currently, Mukulika Ray in my laboratory is examining interactions of the Ras pathway and Hsp83 with the hsrω transcripts using the *hsrω-RNAi* transgenic lines recently generated in our laboratory (section 11.2).

In an attempt to generate mutant alleles of the *hsrω* gene through transposon mutagenesis, TK Rajendra mobilized the P-transposon insertion in the $hsr\omega^{05241}$ allele. Several of the lines in which the P-element was completely lost shared a common phenotype that the homozygotes displayed a prolonged larval life with small-sized imaginal discs, brain ganglia and salivary glands, culminating in death as very early pupae. This was initially very exciting as we hoped that we may possibly have isolated some mutant alleles of the *hsrω* that had distinct phenotypes! However, further genetic and molecular analyses revealed that in all these lines, the *hsrω* gene and its promoter were normal, having been fully repaired following excision of the P-transposon from their genome and thus the recessive lethal phenotype appeared to be due to a new mutation in some other gene. Sonali Sengupta (Sengupta 2005) mapped the second site mutation generated during the P-mobilization to a 39 kb interval in the 93E13-94 F1 region on the right arm of chromosome 3 by recombination as well as deletion mapping, and renamed the mutation as *l(3)pl* (*pupal lethal*). Since all the then publicly available mutant alleles of genes predicted to be present in the 39 kb interval complemented this phenotype, the identity of the *l(3)pl* remained enigmatic (Sengupta 2005). Of interest in the context of *hsrω* story, however, was the observation that the omega speckles (section 10) in larval tissues of the $l(3)pl^{10R}$ homozygotes were clustered as seen in wild-type cells after a mild heat shock, and thus we believe that the $l(3)pl^{10}$ allele interacts with *hsrω* transcripts (Sengupta 2005). Current studies by Akanksha in my laboratory suggest that the $l(3)pl^{10R}$ mutation nay affect the *DNA-pol epsilon* (*CG6768*) gene located in this region, but for which no mutant allele is publicly available so far. This is being examined further.

10. Association of different hnRNPs and other proteins with *hsrω* locus during heat shock and the essential role of hsrω-n transcripts in organizing the nucleoplasmic omega speckles

Initial ultrastructural studies revealed unique characteristic large RNP particles in the nucleoplasm and on or in the vicinity of the 2-48B puff of *D. hydei* (Derksen *et al.* 1973; Derksen 1975) and the 93D puff of *D. melanogaster* (Dangli *et al.* 1983). These studies further suggested that the proteinaceous core of these particles was surrounded by RNA (Derksen *et al.* 1973).

A remarkable collection of monoclonal antibodies against chromosomal proteins was generated by Saumweber *et al.* (1980). These antibodies, which continue to be generously provided by Dr H Saumweber to users, have significantly helped in understanding some functions of this non-coding gene in normal development and under conditions of cellular stress. Dangli *et al.* (1983; also *see* Dangli and Bautz 1983) reported that in unstressed polytene cells, the P11 and Q18 antibodies, now known to recognize the Hrb87F and Hrb57A hnRNPs, respectively (Haynes *et al.* 1991; Buchenau *et al.* 1997), decorated many active and potentially active chromosome sites. However, their localization changed dramatically following heat shock so that most of the chromosome-bound antibodies were detectable only at the 93D puff site (Dangli *et al.* 1983). Subsequently, nearly all the tested hnRNPs and several other RNA-binding proteins have been found to show exclusive and/or high presence at the 93D puff in heat-shocked cells (reviewed by Lakhotia *et al.* 1999; Lakhotia 2003; Jolly and Lakhotia 2006).

Antibodies present in sera from certain class of patients suffering from autoimmune ankylosing spondilytis show specific binding with the 93D puff in heat-shocked salivary glands (Lakomek *et al.* 1991). It is possible that these auto-antibodies recognize some nuclear proteins like hnRNPs and thus specifically decorate the heat-shock-induced 93D puff. This interesting issue, although with the potential of becoming a diagnostic tool, has not been followed further.

The significance of an early observation of Spruill *et al.* (1978) that cyclic-GMP is present at many active chromosome sites in control cells but preferentially accumulates at the 93D puff following heat shock also remains unknown. In the context of the accumulation hnRNPs, etc., on the 93D puff in heat-shocked cells, it will be interesting to examine if cyclic-GMP is involved in some modifications of the hnRNPs while they are sequestered at the 93D puff.

An interesting finding of Samuels *et al.* (1994) was that the Sxl protein, one of the key players in sex determination and dosage compensation in *Drosophila* in female cells, moved from its normal presence at many chromosomal sites in unstressed polytene cells and became exclusively

localized at the 93D puff following prolonged heat shock. It remains to be seen if the association of Sxl with the hsrω transcripts in omega speckles and the 93D locus has some bearing on the sex-specific lethality seen under certain conditions of under- or overexpression of the *hsrω* gene (Mallik and Lakhotia 2011).

Dangli *et al.* (1983) as well as Samuels *et al.* (1994) suggested that the 93D puff may serve as a storage site for the RNA processing machinery under stress conditions. However, the significance of such unique association of a variety of proteins at a non-coding site during heat shock essentially remained a strange phenomenon until we examined (Lakhotia *et al.* 1999; Prasanth *et al.* 2000) the distribution of the hsrω transcripts in normal and heat-shocked cells in intact, rather than squashed, cells by fluorescence *in situ* hybridization. These studies revealed that in unstressed cells the large nuclear hsrω-n transcripts are present, besides at the 93D site, as distinct speckles in the nucleoplasm but closer to chromatin regions (figure 6). However, in heat-shocked cells this RNA was exclusively localized in large quantities at the 93D site only. This observation was contrary to that of Hogan *et al*. (1994), who, using the spatially less resolved *in situ* hybridization with radioactive-labelled probe, reported that the nuclear distribution of hsrω-n transcripts in control and heat-shocked Schneider cells was similar.

Fluorescence co-immunostaining and RNA:RNA *in situ* hybridization showed that all the so far tested hnRNPs (Lakhotia *et al.* 1999; Prasanth *et al.* 2000 and other unpublished observations) are present in unstressed and *hsrω*-expressing cells at (i) the active chromosome sites, (ii) the 93D puff site and (iii) in the hsrω-n-RNA-containing speckles. Following heat shock, the hnRNPs disappear from nearly all the chromosome sites, and along with the disappearance of the nucleoplasmic hsrω-n RNA speckles, they accumulate at the 93D site (figure 6). Because (i) the formation of these nucleoplasmic speckles containing the diverse hnRNPs and related proteins is dependent upon the presence of hsrω-n RNA and (ii) these speckles are distinct from the interchromatin granule clusters (Spector *et al.* 1991), we (Prasanth *et al.* 2000) named these novel nuclear structures as omega speckles (figure 6). The primary role of hsrω-n transcripts in organizing the omega speckles has been reconfirmed by the observation that ablation of these transcripts through RNAi results in loss of omega speckles so that the diverse hnRNPs get diffusely dispersed in nucleoplasm while overexpression results in larger clusters of omega speckles (Mallik and Lakhotia 2009a, 2011). Prasanth *et al.* (2000) concluded that the hsrω-n transcripts 'play essential structural and functional roles in organizing and establishing the hnRNP containing omega speckles and thus regulate the trafficking and availability of hnRNPs and other related RNA binding proteins in the cell nucleus'. It is now well known that hnRNPs constitute a family of diverse but conserved RNA-binding proteins with multiple roles in nucleic acid metabolism, including the packaging and processing of different species of nuclear RNAs and their translational regulation (Han *et al.* 2010). The omega speckles thus are expected to influence a diverse array of the nuclear RNA processing activities in view of their association with a variety of hnRNPs.

Recent studies in my laboratory indicate that the omega speckles may also be heterogeneous in their composition in different cell types or even in the same nucleus since some of the omega speckle associated proteins, for example, NonA, PEP and S5, are not present in all the speckles in a given nucleus. In addition, the recent finding that the hsrω-n RNA also exists in spliced and unspliced hsrω-n1 and hsrω-n2 forms and that both may be associated with omega speckles (Mallik and Lakhotia 2011), may provide a further means of functional differentiation of the omega speckles. Curiously, while the omega speckles are very distinct in the

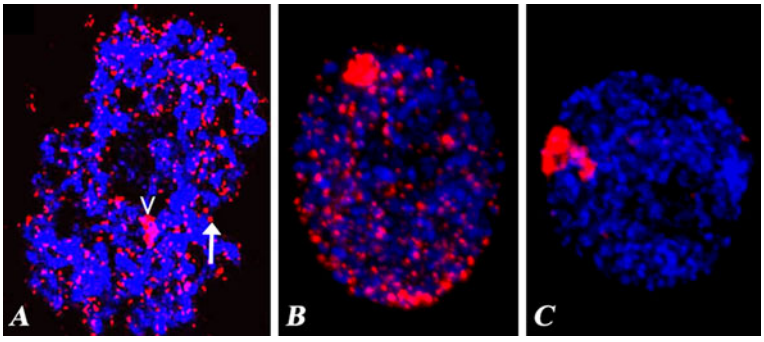

Figure 6. (**A**) Nucleoplasmic omega speckles (red, arrow) seen after *in situ* hybridization of hsrω 280b repeat riboprobe with RNA in Malpighian tubule polytene nucleus (chromatin blue). The arrowhead indicates the *hsrω* locus on the chromosome. Immunostaining of control (**B**) and heat shocked (**C**) Malpighian tubule nuclei with Hrb87F antibody. The Hrb87F (red) follows the same speckled pattern as the hsrω-n to RNA in control cells, but after heat shock, it shows near exclusive localization at the 93D site. DNA, stained with DAPI, is shown in blue in all the images. The image in **A** is from Sengupta (2005), while those in **B** and **C** were provided by Anand K Singh.

larval Malpighian tubule polytene cells, they are less prominent in the more polytenized larval salivary glands due to a more diffuse presence of hnRNPs in the nucleoplasm. The larval salivary glands also appear to have more of the spliced hsrω-n2 form compared to the hsrω-n1; whereas in whole larval or adult, the reverse situation obtains (unpublished observations). The significance of such differences remains to be examined.

Several reports (Jolly and Morimoto 1999; Denegri *et al.* 2001; Jolly *et al.* 2004) on the formation of nuclear stress bodies in human cells following heat shock revealed that the non-coding sat III repetitive sequences, associated with centromeric heterochromatin on chromosome 9, were transcriptionally induced by heat shock and were essential for accumulation of a variety of proteins at the site of transcription in the form of stress bodies. Intrigued by the striking parallels between the behaviour of hsrω transcripts and the human sat III transcripts in heat-shocked cells, I initiated email exchanges with Caroline Jolly, which finally developed into a review wherein we suggested that the hsrω-n and sat III transcripts are functional analogues and work through a common paradigm to dynamically regulate RNA-processing proteins through sequestration (Jolly and Lakhotia 2006).

Zimowska and Paddy (2002) reported that the Tpr protein, normally present at nuclear pores and as granules/speckles associated with the nuclear matrix, accumulates at the 93D puff following heat shock in parallel with proteins like NonA, PEP, etc. This paper, however, does not state if proteins like NonA or PEP associate with the Tpr granules in nucleoplasm although data indicate that the Tpr protein is not present at any of the developmental or other heat shock puffs. Thus, Tpr behaves differently from the other proteins that accumulate at the 93D puff after heat shock. Observations in our laboratory, using the Bx34 antibody against Tpr, obtained from Dr H Saumweber, reveal that, unlike the NonA or PEP or other hnRNPs, the Tpr granules do not colocalize with the omega speckles, although Tpr granules and the omega speckles are on the fibrillar nuclear matrix network that also contains Tpr, Surprisingly, however, contrary to the results of Zimowska and Paddy (2002), we (Anand K Singh and SC Lakhotia, unpublished) do not find any binding of the Bx34 antibody at the 93D puff in heat-shocked cells. These different results need further examination.

Around the time when we were excited about the discovery of omega speckles, we also noted (Rajendra *et al.* 2001) that males homozygous for the $hsr\omega^{05241}$ P-transposon insertion allele were completely sterile and they also displayed overexpression of hsrω-n transcripts in the cyst cells, a pair of which surrounds the bundle of 64 elongating sperm until their individualization. This correlation appeared very attractive and led us to surmise that the larger clusters of omega speckles seen in cyst cells in testes of $hsr\omega^{05241}$ homozygotes sequestered a greater proportion of hnRNPs, etc., and this affected the cyst cell function, which in turn prevented individualization of spermatozoa, resulting in male sterility (Rajendra *et al.* 2001). Subsequent studies in the laboratory, however, revealed that the $hsr\omega^{05241}$ mutation was actually not responsible for the recessive male sterility. It turned out that our earlier study (Rajendra *et al.* 2001) with the $hsr\omega^{05241}$ P-transposon insertion chromosome erroneously failed to detect a second site recessive mutation. Akanksha *et al.* (2008) showed that the male sterility was actually due to a second site recessive mutation rather than to the P-transposon insertion at −130 bp position in the *hsrω* promoter. The second site mutation (ms^{21}) has subsequently been mapped by Roshan Fatima (unpublished) in my laboratory to a novel axonemal dynein intermediate chain gene (*CG7051*) at 61B1 region on left arm of chromosome 3. The cause and consequence of the clustering of omega speckles in cyst cells in dynein intermediate chain mutant background remain unknown.

Although Lakhotia *et al.* (2001) did not notice a difference in the levels of hsrω transcripts in larval tissues of $hsr\omega^{05241}$ homozygotes, either through colorimetric RNA:RNA *in situ* hybridization or the *lacZ* reporter assay, a later study (Sengupta and Lakhotia 2006) using more sensitive fluorescence *in situ* hybridization showed that the omega speckles in eye disc cells of $hsr\omega^{05241}$ homozygous larvae were also clustered and larger, suggesting this allele to be overexpressing. The *hsrω* chromosome used by Sengupta and Lakhotia (2006) still carried the then unsuspected ms^{21} mutation. Subsequently, however, it has been seen that the male fertile $hsr\omega^{05241}$ line also shows slightly enhanced expression of the hsr*ω* transcripts in the larval eye discs and a mild roughening of adult eyes.

Binding of polyADP-ribose (pADPr) to many proteins, mediated by PARP or otherwise, significantly modulates their activities; the removal of pADPr from the ribosylated proteins by poly(ADP-ribose) glycohydrolase (PARG) is also a regulated process. Among the many proteins that undergo such modifications, the ribosylation of hnRNPs is very interesting in the context of *hsrω* activity. Ji and Tulin (2009) have shown that heat shock enhances ribosylation of hnRNPs like Squid and Hrb98DE, so that these proteins lose their affinity for association with RNA at most of the developmentally active loci. PARG activity relieves the released hnRNPs of their pADPr moieties; these de-ribosylated hnRNPs then accumulate at the 93D puff in heat-shocked cells. Ji and Tulin (2009) reported that there is enhanced ribosylation in PARG mutants and, consequently, reduced binding of the two hnRNPs with the heat-shock-induced 93D puff. Further, the compromised PARG activity affected splicing of hsrω and Ddc transcripts, which they

correlated with a reduced binding of proteins like Squid and Hrb87F with RNA in the spliceosome machinery. While this model needs further studies and validation, the observed relationship between PARP and PARG activities and the accumulation of hnRNPs at the *hsrω* locus during heat shock is very interesting. The mechanism and processes underlying the congregation of hsrω-n transcripts and the various proteins at the 93D locus following heat shock remain to be examined. Nuclear matrix components are expected to play important roles in the dynamic nuclear relocation of hnRNPs and hsrω-n transcripts. In this context, our recent finding (Mallik and Lakhotia 2011) that hsrω transcripts interact with nuclear lamins is of interest. Further, as noted in sections 11.4 and 11.5, the *hsrω* activity also relates to ISWI and HP1 proteins. Further studies to explore the interactions between ribosylation, ISWI activity, hnRNPs, HP1, lamins, nuclear matrix, etc., with each other and with hsrω transcripts should provide novel insights into the overall chromatin regulation and nuclear activity.

A number of different speckle compartments are now known in eukaryotic nuclei, all of which contain variety of RNA species together with different RNA-processing and other proteins (Jolly and Lakhotia 2006; Prasanth and Spector 2007). It is likely that all classes of nuclear speckles carry non-coding RNA species. Based on our understanding of the omega speckles, it appears that the non-coding RNA species in these nuclear compartments help them function as dynamic 'storage depots' for a regulated on demand release of different classes of nuclear proteins and other factors involved in functions like organization and modification of chromatin, processing and transport of nuclear (including nucleolar) RNA, etc.

The discovery of omega speckles at the turn of the century was a milestone and made it possible to experimentally address the functional significance of this intriguing non-coding gene. Fortunately, this also happened at a time when non-coding RNAs were becoming increasingly attractive. Although much of the excitement about non-coding RNAs was with reference to the small miRNAs (and their various namesakes), by the beginning of this century, several large non-coding RNAs were also known as valid and functional molecules (Lakhotia 1996), and thus the hsrω transcripts' role in organizing the omega speckles was 'acceptable'. Our paper (Lakhotia *et al.* 1999) in which we raised the possibility that the hsrω transcripts may regulate the dynamics of hnRNPs, etc., was part of a collection of articles on large non-coding RNAs that I edited for *Current Science*. In my editorial (Lakhotia 1999), I argued for a due recognition of the significance of non-coding RNA and stated, 'Among a variety of factors that are already known to affect the higher order chromatin organization and consequently gene expression and "ribotype" of a cell, RNA is one as exemplified by the inactive X-chromosome in female mammals. Since the fine-tuned "ribotype" of a cell results in individual cell phenotype, the "ribotype" actually is subjected to natural selection. Additionally, since the "ribotype" can also generate new components of the genotype through reverse transcription, RNA molecules in a cell remain the prime players'. Since the prejudice against non-coding RNAs as functional molecules essentially stemmed from the popular interpretation of 'central dogma of molecular biology' that biological information that leads to production of proteins only is relevant, I concluded my editorial saying, 'Dogmas are helpful in providing directions for searches in a defined framework but they need continued revisions and modifications so that newer directions are found. Followers of the central dogma of molecular biology need to become less dogmatic since the living world is full of diversity and surprises' (Lakhotia 1999). It may be noted that although the basic point of the 'central dogma' that the flow of information from DNA to protein via RNA is unidirectional remains valid, the common (mis-)interpretation that has generally prevailed is that the genetic information not involved in production of proteins may be 'junk' or 'selfish'. Fortunately, with the large non-coding RNAs making their presence felt, this misconception is losing its ground.

11. Conditional RNAi or overexpression unravels pleiotropic roles of hsrω transcripts

Since classical mutant alleles of the *hsrω* locus have not been available, we (Mallik and Lakhotia 2009a) generated transgenic lines for the GAL4-UAS based (Brand and Perrimon 1993) conditional RNAi, using the 280 bp *hsrω* repeat sequence. We believe that this RNAi construct acts within the nucleus (Mallik and Lakhotia 2011) and thus essentially down-regulates only the hsrω-n transcripts. We also used the EP-transposon (Rorth 1996) for targeted overexpression of hsrω transcripts. These two approaches have provided very exciting insights into the multiple functions that these transcripts perform in a cell during normal development as well as under conditions of cell stress.

11.1 *Developmental effects*

Since the *hsrω* gene is expressed in a regulated manner in somatic cells of all developmental stages, it is expected to perform some functions in all cells. In agreement, overexpression or ablation of the hsrω transcripts in different tissues and developmental stages with a variety of GAL4 drivers revealed that a balanced expression of these non-coding transcripts is critical for survival and normal

development; a change in cellular levels of these transcripts generally had detrimental consequences, with extreme cases resulting in organismal lethality (Mallik and Lakhotia 2011). Since altered levels of the hsrω nuclear transcripts immediately affect the omega speckles, we believe that the developmental effects following the targeted down- or up-regulation of hsrω transcripts disrupt the dynamic homeostasis of RNA-processing proteins in the given tissue, which in turn has cascading effects on downstream events. Interestingly, we also found that in a few cases, ablation of these transcripts suppressed the mutant phenotype resulting from mis-expression of other genes, such as inactivated *apterous* gene or UAS-driven overexpression of lamin C protein (see Mallik and Lakhotia 2011). In a few cases, the hsrω-RNAi or overexpression had sex-specific effects (Mallik and Lakhotia 2010a, 2011); it remains to be seen if such sex-specific effects relate to the earlier noted interactions of Sxl protein with the hsrω transcripts.

In agreement with the abundant presence of the hsrω transcripts in nurse cells (Mutsuddi and Lakhotia 1995; Lakhotia *et al.* 2001), absence or down- or up-regulation of hsrω transcripts in ovarian follicles, due to chromosomal deletion, P-transposon insertion, RNAi or EP allele expression, affects oogenesis (Lakhotia *et al.* 1999; Srikrishna 2008, and other unpublished results). In parallel with the limited transcription of *hsrω* in the male germline, even in the complete absence of the hsrω transcripts, fertile sperms are produced in *Df(3R)e*^{gp4}*/Df(3R)GC14* males (Ray 1997; Lakhotia *et al.* 1999).

The *hsrω*-null condition or global activation of *hsrω-RNAi* transgene with *Act5C-GAL4* driver causes extensive embryonic or larval death but a certain proportion of such individuals regularly survive, and surprisingly, *Act5C-GAL4* and *UAS-hsrω-RNAi* can even be maintained together in a stock (Mallik and Lakhotia 2011). We believe that the successful survival of these flies with global down-regulation of the hsrω transcripts is due to other pathways that can take care of the critical *hsrω* functions, at least to a limited extent. Existence of such 'backup' or alternative pathways appears to be supported by the observation that the continued presence of *Act5C-GAL4* driver and the *UAS-hsrω-RNAi* responder through several generations in a stock reduces, but does not abolish, the lethality compared to that when the driver and the responder are brought together for the first time (Mallik and Lakhotia 2011). This may suggest a rapid selection or activation of the 'backup' pathways through epigenetic or other means. The nature and population dynamics of the possible 'backup' for *hsrω* functions need further study.

Apparently supporting the role of hsrω-n transcripts in sequestering hnRNPs and thus controlling processing of many hnRNAs, Johnson *et al.* (2009) reported that adult flies of certain lines of *D. melanogaster* showed reduced levels of hsrω-n transcript and also displayed higher rates of protein synthesis in adult ovaries. The enhanced rate of protein synthesis was presumed to result from a faster processing of mRNAs by the enhanced availability of hnRNPs consequent to reduced hsrω transcripts in these lines. While this looks attractive, some limitations of the experimental procedures of this study need to be considered. Johnson *et al.* (2009) measured the rate of protein synthesis only in ovaries but the hsrω transcript levels were measured in total RNA from whole flies, rather than from their ovaries only. Thus, a direct correlation between levels of the hsrω transcripts in ovaries with the rate of protein synthesis remains uncertain. Further, their design for measurement of rate of protein synthesis did not eliminate the possibility that unincorporated ^{35}S-methionine also contributed significantly to their estimated 'rate' of protein synthesis. An additional complication arises from the recent finding in our laboratory (Mallik and Lakhotia 2011) that the hsrω-n transcripts can exist in unspliced as well as spliced forms; consequently, the RT-PCR-based estimates of hsrω-c transcripts in earlier studies failed to differentiate between the spliced hsrω-n and hsrω-c transcripts, since the amplicons size in both cases are identical. Therefore, the inferences of Johnson *et al.* (2009) need re-examination.

11.2 *Modulation of induced apoptosis*

Following our observation that eye damage as well as the high frequency of apoptosis in eye discs in organisms carrying two copies of the GMR-GAL4 driver transgene are nearly completely suppressed by hsrω-RNAi, we examined the consequences of down- or up-regulation of hsrω transcripts on apoptosis induced either by directly expressing the proapoptotic Reaper, Grim or Hid proteins or caspases (precursor as well as activated) in the eye and other imaginal discs (Mallik and Lakhotia 2009b). We showed for the first time that down-regulation of hsrω transcripts through RNAi suppressed JNK singalling and stabilized the *Drosophila* inhibitor of apoptosis protein 1 (DIAP1), presumably through its increased association with Hrb57A, which is released following the disappearance of omega speckles (Mallik and Lakhotia 2009a).

11.3 *Modulation of neurodegeneration*

Fernandez-Funez *et al.* (2000) reported the *hsrω*^{05241} allele to be a dominant enhancer of the expanded polyQ toxicity in a fly model of Spinocerebellar Ataxia type 1. Following this report, we examined the interaction in greater detail. In the first study, Sengupta and Lakhotia (2006) confirmed the dominant enhancing effect of the *hsrω*^{05241} allele on 127Q-induced neurodegeneration but found that the *hsrω*^{05241}

allele was actually an overexpressing allele, rather than loss-of-function allele as presumed by Fernandez-Funez *et al.* (2000). This study also showed that the omega speckles do not colocalize with the polyQ nuclear inclusion bodies (IB). However, the hsrω transcripts may affect the polyQ pathogenesis via the association with hnRNPs in omega speckles since a monosomic condition for Hrb87F (also known as Hrp36) was, by itself, also found to dominantly enhance the polyQ phenotype (Sengupta and Lakhotia 2006). The availability of the *hsrω-RNAi* and *EP* lines for down- or up-regulation, respectively, of the hsrω transcripts permitted more detailed mechanistic analyses of the modifying effect of these transcripts on polyQ pathogenesis in several different fly models (Mallik and Lakhotia 2009a, 2010a). Reduction in hsrω-n transcripts through RNAi nearly completely suppressed neurodegeneration following expression of different expanded polyQ proteins, while elevation of these transcripts through EP allele expression aggravated neurodegeneration. Significantly, the down-regulation of hsrω-n transcripts through RNAi was associated with disappearance/reduction of the polyQ IBs (Mallik and Lakhotia 2009a). Levels of the chromatin and transcription modulator CREB-binding protein (CBP) mRNA as well as protein were elevated under this condition (Mallik and Lakhotia 2010a). The elevated levels of free hnRNPs, following hsrω-RNAi, resulted in their enhanced association with CBP. Down-regulation of hsrω transcripts also improved proteasomal activity. The hsrω-RNAi thus interferes with the polyQ pathogenesis pathways at multiple levels (Mallik and Lakhotia 2010b) and thereby facilitates clearance of the polyQ IBs. Together with the above-noted suppression of induced apoptosis (section 11.2), hsrω-RNAi results in near complete suppression of neurodegeneration (Mallik and Lakhotia 2010a, b). Interestingly, unlike the suppression of polyQ damage, hsrω-RNAi had little protective effect on damage in eye discs caused by over-expression of normal or mutated tau protein (Mallik and Lakhotia 2009a).

11.4 *Interaction with ISWI chromatin remodeller*

An email from Dr Davide Corona in October 2007 stating that his laboratory had found *hsrω* to be one of the modifiers of phenotypes resulting from mis-expression of the ISWI-ATP-dependent chromatin remodeller initiated a regular exchange of ideas and reagents (fly stocks, antibodies, DNA clones, etc.) between our labs. This collaboration provided very exciting insights into the interaction between ISWI and hsrω transcripts (Onorati *et al.* 2011). The phenotypes of ISWI-nulls, like poor condensation of the polytene chromosomes, especially the X chromosome in male salivary glands, larval lethality and eye degeneration in mosaic eyes, etc., (Corona *et al.* 2007) are suppressed by hsrω-RNAi. These studies show that ISWI is essential for organization of omega speckles since in ISWI-null cells, the nucleoplasmic hsrω-n transcript and associated hnRNPs are seen as elongated trail-like structures, rather than the typical speckles. Interestingly, while the hsrω-n transcripts and ISWI display limited colocalization, immunoprecipitation with ISWI antibody pulls down the hsrω-n transcripts as well; further, the 280b repeat unit of hsrω-n transcripts can stimulate the ATPase activity of the ISWI *in vitro* (Onorati *et al.* 2011). The mechanistic details of interaction between *hsrω* and ISWI are not yet understood, but the interactions of either of these with PARP/PARG, HP1, nuclear lamins, CBP, etc., appear to have significant roles in this context.

11.5 *Role in recovery from heat shock*

We have also used these RNAi and overexpressing *EP* lines to understand the role of hsrω transcripts in heat shock response. As noted earlier, the *Df(3R)e*^{Gp4}*/Df(3R)GC14* trans-heterozygotes, which are null for the *hsrω* gene, show thermosensitivity in spite of normal induction of synthesis of heat shock proteins. In agreement with these earlier observations, it has been recently observed in our laboratory that, unlike wild-type embryos and larvae, those expressing *hsrω-RNAi* or *EP* during heat shock or those being nullosomic for the *hsrω* gene show nearly 100% death several hours after the thermal stress. To understand this rather enigmatic situation, we (Lakhotia *et al.* 2011, in preparation) have compared the dynamics of hnRNPs, RNA pol II and HP1 after heat shock and during recovery in wild-type salivary glands that have relatively reduced (because of RNAi or *hsrω* mono- or nullo-somy) or elevated (through EP expression) levels of hsrω transcripts during heat shock. The rapid re-distribution of hnRNPs to the 93D puff and of the RNA pol II on the heat-shock-induced loci is comparable in all the cases. However, unlike the rapid return of the hnRNPs and the RNA pol II to different chromosomal regions within 1 hr of recovery from heat shock, glands with reduced or elevated levels of the hsrω transcripts fail to re-localize these proteins to their expected chromosome sites as well as to the omega speckles. In cells with reduced hsrω transcripts, the hnRNPs neither get back to omega speckles nor move efficiently to chromosome sites but get distributed in a rather diffuse manner in the nucleoplasm in addition to some chromosomal sites. The RNA pol II is also seen on fewer developmentally active chromosome sites during recovery in these glands. In the *hsrω* overexpressing glands most of the hnRNPs as well as the RNA pol II continue to remain, even after 1 or 2 hr of recovery, at sites they moved to following the heat shock (Lakhotia *et al.* 2011, in preparation). In glands with down- or up-regulated hsrω transcripts, the hnRNPs showed abnormal association with the 87A and 87C puffs, which may provide an explanation

for the earlier noted (section 6) unequal puffing of these twin puffs when the 93D is not induced during heat shock.

The HP1 protein also shows redistribution to heat shock loci, including the 93D puff, following heat shock (Piacentini *et al.* 2003). Our results (Lakhotia *et al.* 2011, in preparation) show that depletion or overexpression of hsrω transcripts during heat shock affects the redistribution of HP1 protein to pre-stress condition during recovery.

These observations suggest that a balanced level of the hsrω transcripts is essential for a regulated movement of hnRNPs, RNA pol II and other proteins during recovery from heat shock. Consequently, in the absence of normal resumption of cellular activities, the organisms die over a period time, even though they produce the usual set of heat shock proteins during the heat shock period.

In view of the above-noted interactions of CBP, ISWI, lamin C, HP1, etc., with hsrω transcripts, it is likely that components of nuclear matrix and chromatin remodelling complexes are involved in the dynamic movement of hnRNPs, etc., away from the 93D puff during recovery from heat shock. In this context, it is significant that, like the hnRNPs that are associated with the nuclear matrix (Mattern *et al.* 1999; Arao *et al.* 2000), the omega speckles are also mostly distributed along the nuclear matrix and show rapid, but constrained, movements in live cells (Anand K Singh and SC Lakhotia, unpublished; also see supplementary video S1 in Onorati *et al.* 2011).

12. Non-coding transcripts as hubs in cellular networks

The wide variety of apparently unrelated pathways, with which the *hsrω* gene appears to interact, may lead one to suspect that these may be non-specific interactions. However, given the primary role of the hsrω-n transcripts in organizing omega speckles and thus modulating, directly or indirectly, activities of a diverse array of regulatory proteins, such pleiotropy is indeed expected. As discussed above, the *hsrω* locus is also very sensitive to intra- and extra-cellular conditions. Therefore, we believe that the non-coding *hsrω* gene functions as a hub in cellular networks (Arya *et al.* 2007; Mallik and Lakhotia 2010a). Based on the known genetic, cell biological or biochemical interactions of the *hsrω* gene with other pathways, a simplified schematic of the role of hsrω-n transcripts as a potential hub in cellular networks is presented in figure 7. Since each interacting protein has its own network connections, it is likely that the different hsrω transcripts help maintain homeostasis through their influence on the diverse networks; the actual signal transduced to and between the different networks would depend upon the input signals from other intra- and extra-cellular events. This model predicts that an imbalance between these non-coding transcripts and their interacting proteins or other factors will have consequences of varying magnitudes in different cells depending upon the temporal and spatial factors. It also appears likely that for such critical roles, the evolutionary processes would have generated 'backup' systems, mentioned in section 11.1, to provide for at least a limited survival under conditions when the prime transcripts or their activities are compromised.

A significant advantage that a large non-coding RNA has in its role as hub is that it may not only provide platforms for multiple proteins in view of the usually short nucleotide sequences needed at their binding sites, but being single-stranded, the RNA molecules can have a much greater plasticity in their secondary and tertiary structures. The tandem repeats seem to provide additional versatility. The higher-order structures of such large non-coding RNAs may be specifically modulated by binding of one or the other proteins or other conditions. A strong functional conservation in spite of the poor conservation of the *hsrω* base sequence even between different related *Drosophila* species is an indication of the importance of secondary and tertiary structures of the hsrω transcripts rather than the base sequence itself. The functional analogy between the hsrω-n transcripts and human sat III transcripts without any sequence homology is also a pointer in the same direction. A search for functional equivalents of *hsrω* in other organisms will, therefore, have to look for large non-coding RNAs with comparable protein association properties and/or similar higher-order structural features. Improved bioinformatic approaches and knowledge of the non-coding RNomes in different species will hopefully permit identification of the *hsrω* equivalents in increasing number of eukaryotes in the near future.

13. Epilogue

The journey of the 93D locus and its transformation from a 'nice-looking' puff to a mysteriously conserved but apparently non-coding *hsrω* 'gene', to the organizer of omega speckles and, therefore, a potential hub regulating multiple interconnected networks, has indeed been fascinating. As this story progressed, my conviction in a major role played by the enormously large non-coding component of eukaryotic genome in generating the biological complexity has grown (Lakhotia 1987, 1989, 1996, 1999, 2003; Jolly and Lakhotia 2006; Mallik and Lakhotia 2007). My interest in the inactive heterochromatin arose during my doctoral studies primarily because of the chromosomal level differences in the inactive-X in female mammals and the hyperactive-X in male flies (Lakhotia 1970). Karyotyping of some mammals with prominent centromeric heterochromatin (Rao and Lakhotia 1972) and participation in writing a review on heterochromatin (Shah *et al.* 1973) enhanced

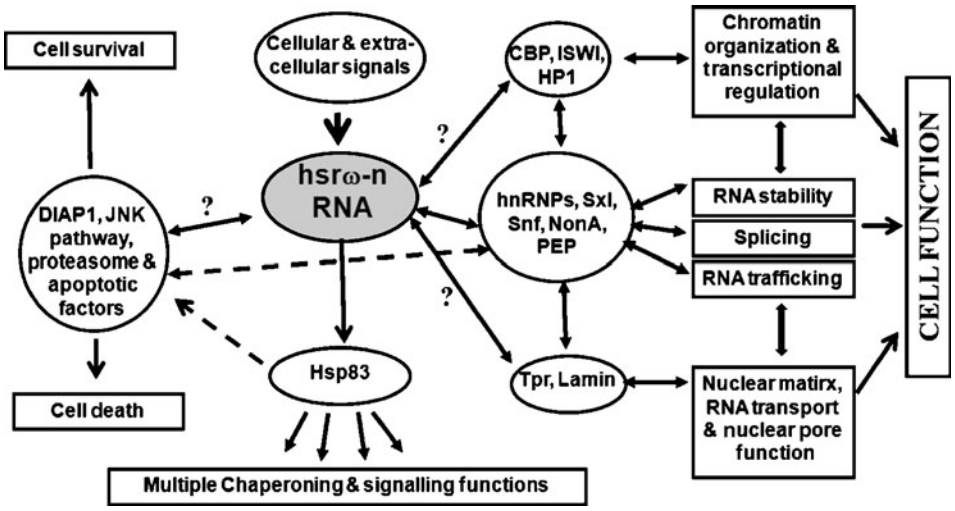

Figure 7. The hsrω-n RNA seems to function as a hub to coordinate several critical cellular networks (shown within individual ovals) that control different activities (noted in rectangles). The hsrω-n RNA is sensitive to a variety of cellular and extra-cellular signals (arrowhead) and relays the signal through its transcripts to multiple networks like (i) chromatin modulators, (ii) factors regulating nuclear RNA stability, processing and transport, (iii) nuclear pore and matrix components, (iv) Hsp83 and/or (v) cell survival or cell death pathways . A direct interaction of the hsrω-n transcripts with other networks is indicated through solid line arrows while indirect affects through other networks are shown with broken line arrows. The interrogation mark close to some solid line arrows indicates that a direct interaction with one or more components of the particular network is yet to be established.

my interest in the enigma of heterochromatin. That heterochromatin has definite functional significance was further strengthened when active transcription in the classical 'gene-deficient' β-heterochromatin was discovered for the first time (Lakhotia and Jacob 1974b). Thus, the increasingly clear evidence that the 93D locus does not code for a typical protein (Lakhotia and Mukherjee 1982; Peters *et al.* 1984; Ryseck *et al.* 1985; Garbe *et al.* 1986) was exciting rather than disappointing. During the 1995 session of the series of annual discussion meetings named 'TRendys', while reviewing the then known large non-coding RNAs, I predicted that they would become 'trendy' in coming years (Lakhotia 1996). It is indeed gratifying that this prediction has come true.

As is natural, many questions that arose during the course of our studies on the 93D puff have remained unanswered or even unaddressed. Several of these have been noted above and would be worth following up. More importantly, while our recent studies have mostly focused on the large nuclear hsrω-n transcripts, significance of the stable spliced-out intron and the 1.2 kb cytoplasmic hsrω-c transcript need more focused attention. The nearly 100% conservation of the intron–exon junction sequences, especially of the junction of the intron and second exon of *hsrω*, in all species of *Drosophila* whose genome sequences are available (Eshita Mutt and SC Lakhotia, unpublished) remains unexplained. This requires in-depth experimental analysis to understand if this conservation has something to do with regulation of the *hsrω* gene and/or with subtle differences in properties and activities of the hsrω-n1 (unspliced) and hsrω-n2 (spliced) transcripts. The mechanistic details of specific interactions of the various hsrω transcripts with the diverse variety of proteins can be more specifically addressed as novel experimental strategies become available.

As the years go by, it is expected that functions of the enormous diversity of large non-coding RNAs, which commonly exist in eukaryotes but currently remain largely unknown or unappreciated, will be understood soon so that the '93D puff' would no more be an exception but would be remembered as one of the 'pioneer' non-coding gene.

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