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Omega speckles – a novel class of nuclear speckles containing hnRNPs associated with noncoding hsr-omega RNA in *Drosophila*

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SUMMARY

Fluorescence RNA:RNA in situ hybridization studies in various larval and adult cell types of *Drosophila melanogaster* showed that the noncoding hsr-omega nuclear (hsrω-n) transcripts were present in the form of many small speckles. These speckles, which we name 'omega speckles', were distributed in the interchromatin space in close proximity to the chromatin. The only chromosomal site where hsrω-n transcripts localized was the 93D locus or the *hsrω* gene itself. The number of nucleoplasmic speckles varied in different cell types. Heat shock, which inhibits general chromosomal transcription, caused the individual speckles to coalesce into larger but fewer clusters. In extreme cases, only a single large cluster of hsrω-n transcripts localizing to the *hsrω* locus was seen in each nucleus. In situ immunocytochemical staining using antibodies against heterogenous nuclear RNA binding proteins (hnRNPs) like HRB87F, Hrp40, Hrb57A and S5 revealed that, in all cell types, all the hnRNPs gave a diffuse staining of chromatin areas and in addition, were present as large numbers of speckles. Colocalization studies revealed an absolute colocalization of the hnRNPs and the omega speckles. Heat shock caused all the hnRNPs to cluster together exactly, following the hsrω-n transcripts. Immunoprecipitation studies using the hnRNP antibodies further demonstrated a physical association of hnRNPs and hsrω transcripts. The omega speckles are distinct from interchromatin granules since nuclear speckles containing serine/arginine-rich SR-proteins like SC35 and SRp55 did not colocalize with the omega speckles. The speckled distribution of hnRNPs was completely disrupted in *hsrω* nullosomics. We conclude 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.

Key words: 93D, Heat shock, Amide, ICG, SR protein, *Drosophila melanogaster*, HnRNP

INTRODUCTION

Recent studies have revealed a dynamic structural organization of the eukaryotic nucleus in relation to processing of nascent transcripts (Spector, 1993; Carter, 1994; de Jong et al., 1996; Lamond and Earnshaw, 1998). Increasing biochemical information on components of the nuclear RNA processing machinery and application of improved molecular cell biological approaches have permitted delineation and microscopic visualization of different substructures/domains in nuclei (Fakan et al., 1986; Carter et al., 1993; Moen et al., 1995). Chromosomes are organized in domains with defined territories (Schardin et al., 1985; Lamond and Earnshaw, 1998; Verschure et al., 1999). Centres of transcription and RNA processing factors are well organized in specified nuclear compartments in relation to each other and to the chromosome territories. An important structural feature of nuclear compartments is the distribution of various RNPs in the form of granules/speckles. Clusters of interchromatin granules (ICGs) form a well known class. A typical mammalian cell contains 25-50 clusters of interchromatin granules each measuring 0.5-1.8 μm in diameter (Spector et al., 1991; Huang and Spector, 1992; Spector, 1993). Each cluster is composed of granules measuring 20-25 nm in diameter. These are believed to be the storage centres for various snRNAs, snRNPs, serine/arginine-rich proteins (SRps) and RNA polymerase II, all of which are required at different levels in relation to the dynamic physiological status of the cell (Fu and Maniatis, 1990; Thiry, 1993; de Jong et al., 1996; Misteli and Spector, 1997; Singer and Green, 1997). ICG clusters do not contain the heterogenous nuclear RNA-binding proteins (hnRNPs; Fakan, 1994). ICGs are distributed throughout the interchromatin domain/space (ICD) in proximity to the chromosomal territory and are linked together by thin fibrils (Spector, 1993; Thiry, 1993; de Jong et al., 1996). The ICGs are also suggested to contain high molecular weight unidentified nuclear poly(A)$^+$ transcripts, which are distinct from nascent pre-mRNAs (Thiry, 1993; Huang et al., 1994). Another nuclear domain, the perichromatin fibrils (PCFs), are distributed throughout the nucleus at the chromosome territory. PCFs are heavily labeled

with [^{3}H]uridine (Spector, 1993) and contain all snRNPs, non-snRNPs and hnRNPs. These suggest that the PCFs are the sites of transcription and pre-mRNA processing (Fakan et al., 1984; Fakan, 1994). Other than these, several other nuclear domains, such as coiled bodies (Lamond and Carmo-Fonesca, 1993; Sleeman and Lamond, 1999), promyelocytic leukemia protein (PML) nuclear bodies (Doucas and Evans, 1996; Hodges et al., 1998), nuclear dots (ND; Ascoli and Maul, 1991) of specialized functions have also been reported in the eukaryotic nucleus.

hnRNPs are a family of about 25-30 RNA binding proteins, 34-120 kDa in size, and are generally associated with nascent RNA polymerase II transcripts (Dreyfuss et al., 1993). The core hnRNPs are distributed diffusely throughout the nucleus in association with chromatin. These proteins are involved in an array of functions extending from packaging of pre-mRNAs to post-transcriptional processing, including alternative splicing (Mayeda and Krainer, 1992; Krecic and Swanson, 1999).

At least 10 major hnRNPs, structurally and functionally homologous to their mammalian counterparts, have been identified in *Drosophila melanogaster* (Haynes et al., 1991; Matunis et al., 1992a,b; Buchenau et al., 1997; Reim et al., 1999; Hovemann et al., 2000). Earlier immunocytochemical studies on spreads of larval salivary gland polytene chromosomes of *D. melanogaster* using antibodies against several hnRNPs and some other related RNA binding proteins showed that these proteins bind specifically to the actively transcribing regions, but after heat shock at 37°C, these proteins disappear from most of the chromosomal locations and concentrate only on the heat shock-induced 93D puff (Dangli and Bautz, 1983; Dangli et al., 1983; Hovemann et al., 1991; Samuels et al., 1994; Zu et al., 1998; Lakhotia et al., 1999).

The 93D heat shock or the *hsr-omega* (*hsrω*) locus of *D. melanogaster* produces several noncoding transcripts (Lakhotia et al., 1999), which are developmentally expressed in most cell types (Bendena et al., 1991; Mutsuddi and Lakhotia, 1995). Levels of the different hsrω transcripts are further elevated by heat shock and amide treatments (Bendena et al., 1989; Lakhotia and Sharma, 1995). We recently reported that in Malpighian tubule and testis cyst cells of *D. melanogaster* larvae and adults, respectively, the large hsrω nuclear transcripts (hsrω-n) are seen as speckles which, after heat shock, coalesce to form bigger clusters (Lakhotia et al., 1999). After about 40 minutes of heat shock, all the hsrω nuclear transcripts get clustered at the *hsrω* locus itself. It was also shown that HRB87F, an hnRNP A1 homolog in *Drosophila*, colocalized with these hsrω RNA speckles in unstressed cyst cells (Lakhotia et al., 1999).

In the present paper, we extend these preliminary observations and show that hsrω-n RNA speckles are present in all the larval and adult cell types and they are dynamically associated with several different *Drosophila* hnRNPs but not SRps. We name these hsrω-n RNA- and hnRNP-containing speckles as omega speckles. We further show that in the absence of hsrω transcripts, the hnRNPs do not form nucleoplasmic speckles. We also show that the hnRNP complexes immunoprecipitated by antibody against Hrp40 contain hsrω transcripts. We believe that the hsrω-n transcripts play an important role in the assembly of hnRNP-containing omega speckles and that this novel class of speckles helps regulate the availability and trafficking of hnRNPs and related RNA processing factors in the nucleus.

MATERIALS AND METHODS

Fly strains and genotypes

Wild-type (Oregon R$^+$) and *Df(3R)^{eGP4}/TM6B* and *Df(3R)GC14/TM6B* stocks of *D. melanogaster*, reared on standard cornmeal-agar-yeast food at 22±1°C, were used. *Df(3R)^{eGP4}* and *Df(3R)GC14* are small deletions in the third chromosome which, when in trans-heterozygous combination, delete only the 93D6-7 band on both homologs (Mohler and Pardue, 1984). For experimental purposes, the *Df(3R)^{eGP4}/TM6B* and *Df(3R)GC14/TM6B* flies were crossed to obtain *Df(3R)^{eGP4}/Df(3R)GC14* progeny (*hsrω*-nullosomic). Most (approx. 80%) of the *hsrω*-nullosomic embryos die but a few larvae and adults survive (Mohler and Pardue, 1984). These rare non-tubby *hsrω*-nullosomic survivors, which could be differentiated from the *Df(3R)^{eGP4}/TM6B* or *Df(3R)GC14/TM6B* tubby sibs (see Lindsley and Zimm, 1992), were used for experiments involving *hsrω*-nullosomics.

Antibodies, clones and RNA probes

Mouse monoclonal antibodies against HRB87F (P11), Hrb57A (Q18), S5 and SRp55 (B52) were generous gifts from Dr H. Saumweber (Saumweber et al., 1980). Mouse monoclonal antibodies against Hrp40 (8G6) and SC35 were kindly provided by Dr G. Dreyfuss (Matunis et al., 1992a) and Dr J. Gall (Fu and Maniatis, 1990), respectively. FITC-conjugated anti-mouse secondary antibody was from Sigma Chemicals, USA.

Digoxigenin (dig)-labeled antisense riboprobe, generated from the *pDRM30* clone, was used for hybridizations to cellular RNA. The *pDRM30* riboprobe specifically detects the hsrω-n RNA (Lakhotia and Sharma, 1995).

Squash preparation of polytene chromosomes from larval salivary glands

Squash preparations of salivary gland polytene chromosomes of third instar wild-type and *hsrω*-nullosomic larvae were prepared following the protocol of Dangli and Bautz (1983) and processed for immunostaining (see below).

Partial squash preparations

Partially squashed tissue preparations were obtained essentially following the procedure of Bendena et al. (1991), with some modifications. Imaginal discs, brain, salivary glands and Malpighian tubules from wild-type or *hsrω*-nullosomic third instar larvae and testes from 3-4 day old adult males were dissected in Poels' salt solution (PSS; Lakhotia and Tapadia, 1998). For heat shock (HS), the dissected tissues were incubated in PSS at 37°C for 40 minutes. Tissues were fixed for 1 minute in 3.7% paraformaldehyde and transferred to 45% acetic acid for 5 minutes. All the larval tissues were lightly squashed under a coverslip on a poly-L-lysine-coated slide. For squashes of adult testes, 3-4 testes were transferred to a poly-L-lysine-coated slide in a drop of 45% acetic acid and were cut into three pieces (proximal, middle and distal) with the help of a needle. The three pieces were arranged one after the other on a slide and pressed gently under a coverslip. After freezing the slides in liquid nitrogen, coverslips were flipped off with a sharp blade and the slides were dehydrated in 95% ethanol (3×10 minutes each). The slides were used immediately for in situ hybridization and/or immunostaining.

Fluorescence RNA-RNA in situ hybridization (FRISH)

Tissue squashes

The slides were rehydrated in phosphate-buffered saline (PBS, 80 mM

Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.2), 3×5 minutes each, treated with 0.1% diethyl pyrocarbonate (DEPC) in PBS for 3 minutes, washed in PBS and dehydrated through ethanol grades and air dried. The slides were hybridized overnight at 50°C with 30-50 ng of dig-labeled antisense RNA probe (*pDRM30*) in 10 μl of the hybridization solution (50% deionized formamide, 5× SSC, 100 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, 50 μg/ml heparin, 0.1% Tween 20). After hybridization, the slides were washed twice in 2× SSC for 10 minutes each followed by 1× SSC at 50°C thrice for 20 minutes each. The slides were further washed for 5 minutes in Buffer-I (100 mM Tris, pH 7.5, 150 mM NaCl) and blocked in Buffer-II (0.1% of Boehringer's blocking reagent no. 11 in Buffer-I) for 1 hour at room temperature (RT). Slides were incubated in a 1:20 dilution of rhodamine-conjugated anti-dig antibody in Buffer-II for 1 hour at RT in a moist dark chamber. The slides were washed thrice for 20 minutes each in Buffer-I. After counterstaining the nuclear DNA either with propidium iodide (PI, 1 μg/ml) or diaminophenylindole (DAPI, 1 μg/ml), the slides were mounted in Vectashield (Vector Labs, USA).

Whole organs

Larvae and flies were heat shocked at 37°C for 40 minutes and the desired tissues were quickly dissected out in PSS. For control samples, the desired tissues were dissected out in PSS directly from unstressed larvae and flies. In certain experiments, the heat-shocked larvae or flies were allowed to recover at RT for 2 hours before dissection. Whole-organ in situ hybridization was carried out as described by Lehmann and Tautz (1994), with slight modifications as follows. The paraformaldehyde-fixed tissues were treated with 0.1% DEPC for 3 minutes and washed in PBT (PBS, 0.1% Tween 20), 3× 5 minutes each and treated further as described by Lehmann and Tautz (1994).

Tissues were prehybridized for 2 hours (50% deionized formamide, 5× SSC, 100 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, 50 μg/ml heparin, 0.1% Tween 20) at 58°C and were hybridized in the same solution using dig-labeled *pDRM30* riboprobe (30-50 ng) at 58°C for 14-16 hours. The posthybridization washes were carried out as described by Lehmann and Tautz (1994). The detection was carried out as described for the squash preparations.

Immunostaining

Polytene chromosome squashes

Squash preparations of polytene chromosomes were processed for immunostaining using primary antibodies against HRB87F (1:5), Hrp40 (1:50) and Hrb57A (1:5), as described by Dangli and Bautz (1983). The signal was detected using an FITC-conjugated secondary antibody (1:50) and the preparations were counterstained with DAPI or PI.

Tissue squashes

Partial squash preparations were rehydrated twice for 5 minutes each in PBS. After 1 hour in blocking solution (PBS, 10% foetal calf serum, 0.1% Triton X-100, 0.1% sodium deoxycholate, 0.02% thiomersol) at RT, the preparations were incubated with antibodies against HRB87F (1:5) or Hrb57A (1:5) for 2 hours at room temperature in a moist chamber. Slides were washed three times with PBS for 20 minutes each, incubated with FITC-conjugated secondary antibody, washed in PBS (3×20 minutes each), counterstained with DAPI or PI and mounted in Vectashield.

Combined FRISH and immunostaining

After processing for FRISH as described above, the squash preparations and the intact tissues were processed for immunostaining. All the treatments were carried out in the dark to prevent quenching of the rhodamine-tagged RNA signal. The slides were washed thrice in PBS, treated with 1% Triton X-100 for 20 minutes at RT and finally washed in PBST (PBS containing 0.1% Triton X-100, 0.1% bovine serum albumin, BSA), 3× 10 minutes each. Preparations were blocked in the blocking solution for 2 hours and were incubated with any one of the following primary antibodies: HRB87F, Hrb57A, S5, B52 (all diluted in 1:5), SC35 (1:20) and Hrp40 (1:50), for 12-14 hours at 4°C. After incubation, slides were washed in PBST three times for 20 minutes each and incubated with FITC-conjugated secondary antibody (anti-mouse) for 2 hours at room temperature. After further washing in PBST, three times for 20 minutes each, they were counterstained either with PI or with DAPI and mounted in Vectashield.

Fluorescence microscopy

All the FRISH and/or immunostained preparations were observed under an E800 Nikon fluorescence microscope using a 60× (1.40 NA, plan Apo) or 100× (1.30 NA, Plan Fluor) oil objective and appropriate excitation and barrier filter combinations, and photographed with Fuji 200 ASA film. Finally the images were scanned and assembled using Adobe Photoshop 5.0.

Immunoprecipitation

A single larva was opened and turned inside out in PBS. All the internal tissues were suspended in 100 μl IP buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$ containing 0.5% (v/v) Triton X-100) and homogenized on ice with 20-25 strokes of a Dounce homogenizer. The extract was layered over a 30% sucrose cushion (30% sucrose w/v in IP buffer) and centrifuged at 5000 ***g*** for 15 minutes at 4°C. The top layer of whole cell lysate was incubated with 10 μl of anti-Hrp40 (8G6) or anti-HRB87F (P11) mAb for 2 hours at 4°C followed by incubation with 50 μl packed protein-A agarose beads (Bangalore Genei, India) for 2 hours at 4°C with gentle agitation. The beads were pelleted, washed five times each with 1 ml of IP buffer and the bound material was eluted from the beads by the addition of 50 μl SDS-PAGE sample buffer (without β-mercaptoethanol) for western blotting or 300 μl TEL buffer (200 mM Tris-HCl, pH 7.4, 25 mM EDTA, 100 mM LiCl) containing 1% SDS for RT-PCR.

SDS-PAGE and western blotting

Immediately after addition of 50 μl SDS-PAGE sample buffer (without β-mercaptoethanol) to the beads, samples were boiled for 10 minutes and centrifuged at 5000 ***g*** for 2 minutes at room temperature. Proteins in the supernatant were fractionated by SDS-PAGE (12.5% nonreducing polyacrylamide gel) and blotted on Immobilon-P membrane (Millipore Corp., USA). The blot was probed simultaneously with anti-HRB87F (1:100) and anti-Hrb57A (1:100) mAb and later with anti-Hrp40 (1:250) mAb. After antibody decoration, immunoblots were developed using a horseradish peroxidase-labeled anti-mouse antibody in conjunction with the ECL detection system (Amersham Pharmacia Biotech UK).

RT-PCR and Southern hybridization

After adding TEL buffer to the beads, the RNA was extracted from the supernatant twice with phenol/chloroform/isoamyl alcohol after digestion with proteinase K (350 μg/ml) for 15 minutes at 37°C and precipitated overnight with 3 volumes of cold ethanol at –20°C. The pellet was washed in 70% ethanol and dissolved in 10 μl DEPC-treated water. The samples were incubated for 20 minutes at 37°C with RNase-free DNase I. First-strand cDNA was synthesized using Superscript™ II RNase H⁻ reverse transcriptase (Life Technologies, USA) with *hsrω* specific reverse primer (P2: 5′-ATGATAGTAA-GCGACAGAAGGC-3′, see Fig. 7a for details). Half (10 μl) of the reaction mixture was used for PCR amplification using 0.5 μM of 93D specific primers (reverse primer, P2 and forward primer, P1: 5′-GGAAACAAAGAAACCATACGC-3′, see Fig. 7a). For PCR, 5 minutes at 94°C was followed by 35 cycles of 1 minute at 94°C, 1.10 minutes at 62°C, 1 minute at 72°C and final extension for 5 minutes at 72°C. The RT-PCR products were electrophoresed, blotted and Southern hybridized with dig-labeled *pJG10* DNA probe as described earlier (Lakhotia and Tapadia, 1998).

RESULTS

hsrω nuclear transcripts are present as nucleoplasmic speckles in all cell types

We used digoxigenin-labeled anti-sense riboprobe corresponding to the repeat region of the *hsrω* gene (Lakhotia and Sharma, 1995) to hybridize in situ with the large (>10 kb) hsrω-nuclear (hsrω-n) transcripts in intact (Fig. 1h-n) or partially squashed (Fig. 1a-g,o-t) tissues from normally grown or heat-shocked larvae and adult flies. The hybridization was detected using a rhodamine-conjugated anti-dig antibody. In all the cell types examined, the largest hsrω-n transcripts were seen as small speckles in the nucleoplasm and at one single site on chromatin. The RNA:RNA hybridization signal on the single chromosomal site was always the largest. Observations on the RNA:RNA in situ hybridization in salivary gland polytene chromosome spreads (Lakhotia and Sharma, 1995; our other unpublished observations) also showed that the only chromosomal site where the hsrω-n transcripts were present was the 93D locus. Therefore, in other cell types also, the chromosomal site of hybridization of the riboprobe was identified as the 93D locus. In addition to their location at the site of transcription (the 93D locus), the hsrω-n transcripts were also present in the nucleoplasm of all untreated larval and adult diploid (Fig. 1a-g) and polytene (Fig. 1h-t) cell types as variable numbers of discrete small speckles. We name these novel speckles formed by the hsrω nuclear transcripts as 'omega speckles'. The signal at the 93D chromosomal site included a diffuse staining in the core region surrounded by speckles similar to those seen free in the nucleoplasm. The number of omega speckles in a nucleus varied in a cell-type-specific pattern. The small cells in larval brain and imaginal discs showed about 6-8 speckles/nucleus (Fig. 1a,c). On the other hand, as reported earlier (Lakhotia et al., 1999), the somatic cyst cells of adult testis showed nearly 100-120 omega speckles in each nucleus (Fig. 1e). The large polytene nuclei in larval salivary glands (Fig. 1h) showed the maximum number (>1000/nucleus) of omega speckles while the polytene cells in larval gastric caecum (not shown) and mid gut (Fig. 1k) had very few speckles (approx. 4-5/nucleus). The hind gut polytene nuclei, on the other hand, showed nearly 40 omega speckles per nucleus (Fig. 1m). The larval Malpighian tubule showed about 80-100 speckles/nucleus (Fig. 1o). A careful examination of DAPI-stained partly squashed polytene nuclei of larval Malpighian tubules and certain somatic cells in adult testis revealed that except for the one cluster of hsrω speckles that was on chromatin (the 93D site), all the other hsrω speckles were present in very close proximity to the chromatin, i.e. in the perichromatin space (see Fig. 1o,p,s,t).

It is well known that the *hsrω* is strongly induced by heat shock at 37°C (for a review, see Lakhotia et al., 1999). We earlier reported (Lakhotia et al., 1999) that following heat shock, the nucleoplasmic omega speckles in larval Malpighian tubules and

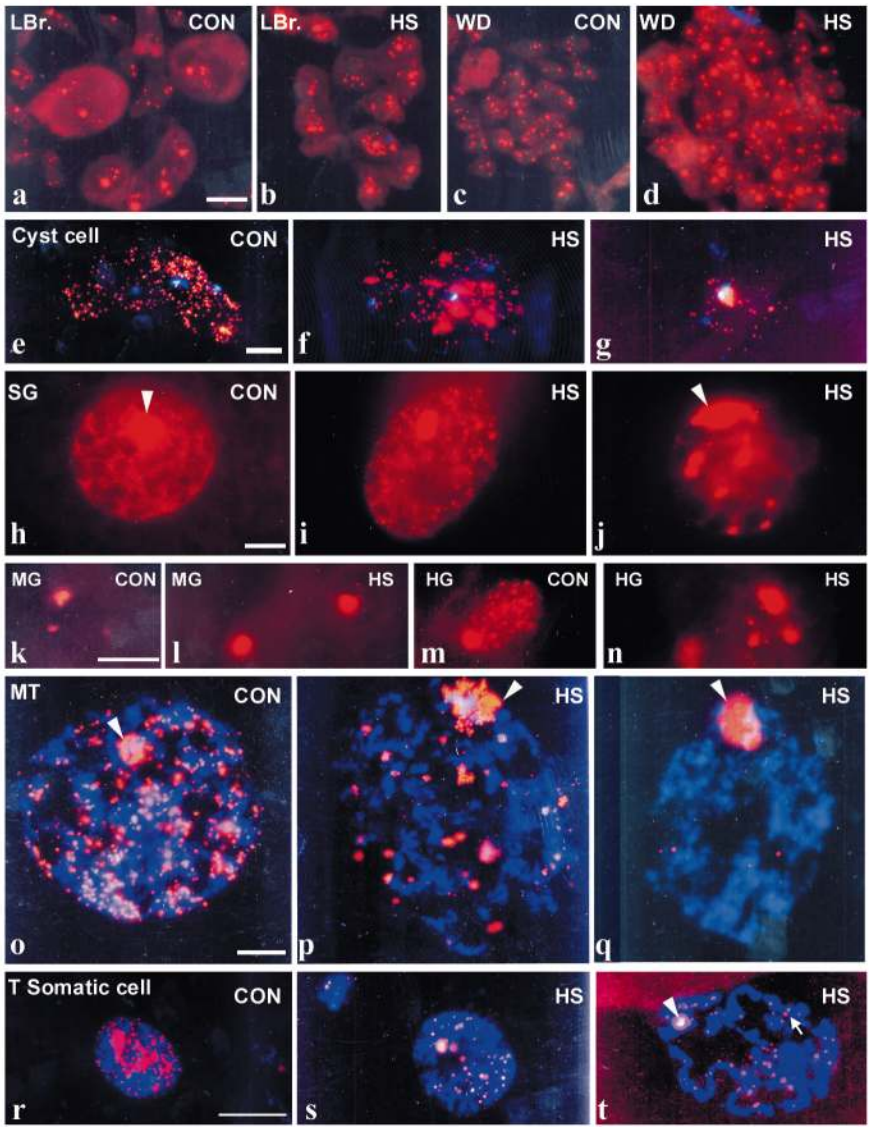

Fig. 1. Localization of hsrω-n RNA by fluorescence (red) in situ hybridization using dig-labeled *pDRM30* riboprobe in unstressed (a,c,e,h,k,m,o,r; CON) or heat-shocked (b,d,f,g,i,j,l,n,p,q,s,t; HS) cells of larval brain (a,b; LBr), wing imaginal disks (c,d; WD), adult testis cyst cells (e-g; Cyst cell), larval salivary glands (h-j; SG), mid gut (k,l; MG), hind gut (m,n; HG), Malpighian tubule (o-q; MT) and adult testis (somatic) polytene cells (r-t; T Somatic cell). Except in a-d and h-n, DNA was counterstained with DAPI (blue fluorescence). In unstressed cells (a,c,e,h,k,m,o,r), in addition to a variable number of nucleoplasmic omega speckles, a large signal is seen in each nucleus (marked by an arrowhead in h and o) on the 93D6-7 site. Heat shock (b,d,f,g,i,j,l,n,p,q,s,t) results in aggregation of speckles into larger clusters and on the 93D site; in many heat-shocked nuclei, the hybridization is restricted essentially to the 93D site (g,q and t). The polytene nuclei in p,q and t, were partially squashed to reveal the proximity of omega speckles with chromatin. Arrowheads (j,p,q,t), 93D locus; arrow (t), omega speckle clusters. Bars (apply to a row), 10 μm.

the adult testes cyst cells aggregated to form larger clusters and that after 40 minutes at 37°C, hybridization to the hsrω-n transcripts in squash preparations of wild-type polytene chromosomes was exclusively at the 93D6-7 site (Lakhotia and Sharma, 1995; our other unpublished data). We have now seen that in partly squashed preparations of heat-shocked salivary glands, where the nuclear envelope was not disrupted, in addition to the large hybridization signal at the 93D chromosome site, there were a few additional clusters of hybridization present in the nucleoplasm (Fig. 1j). In all the larval and adult cell types examined in the present study, the omega speckles formed larger aggregates after heat shock in a time-dependent manner, together with a drastic reduction in the number of free speckles in nucleoplasm (Fig. 1b,d,f,g,i,j,l,n,p,q,s,t). In all types of heat-shocked cells, among the fewer but larger hybridization signals, one was on chromatin and this invariably showed the strongest hybridization signal (Fig. 1g,i,j,l,n,p,q,s,t). In cells with only one site of very strong hybridization signal, it was always on chromatin (Fig. 1q). By analogy with salivary gland polytene cells, we believe that this was the 93D6-7 chromosomal site. In partly squashed polytene cell types also, the heat-shock-induced nucleoplasmic clusters of hsrω transcripts were always in close proximity to chromatin (Fig. 1p,t).

Localisation of hnRNPs

In unstressed cells, hnRNPs are associated with chromatin and with omega speckles; after heat shock, hnRNPs are mostly restricted to the clusters of omega speckles. In our initial study (Lakhotia et al., 1999), we reported that in larval Malpighian tubule and adult testis cyst cells, the HRB87F was localized in the omega speckles in addition to the chromatin. We have now examined the distribution of HRB87F and three other hnRNPs (Hrb57A, Hrp40 and S5) in many other unstressed and heat-shocked cell types of *D. melanogaster*.

HRB87F, a *D. melanogaster* homologue of the mammalian hnRNP A1 (Hovemann et al., 1991), showed localization in two compartments in all unstressed cells. One was in the form

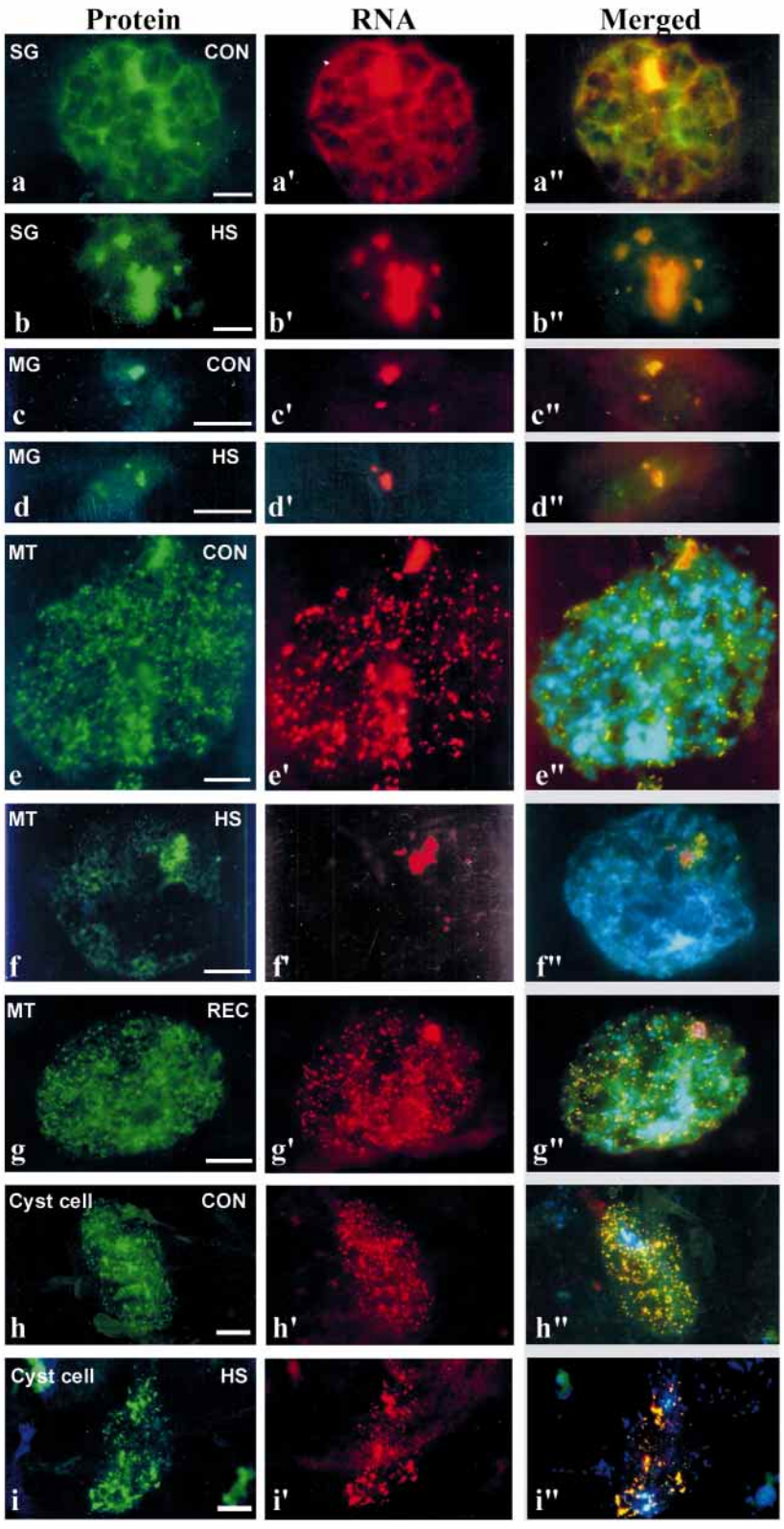

Fig. 2. Combined immunostaining with HRB87F antibody (a-i, green fluorescence; Protein) and in situ hybridization of *pDRM30* riboprobe to RNA (a′-i′, red fluorescence; RNA) in unstressed (a,c,e,h; CON) or heat-shocked (b,d,f,I; HS) larval salivary gland (a,b; SG), mid gut (c,d; MG) and malpighian tubule (e,f; MT) polytene cells and cyst cells from adult testis (h,i). The nucleus in row g is from Malpighian tubules that were allowed to recover (REC) for 2 hours at RT from a 40 minute heat shock. The nuclei in rows e-i were counterstained with DAPI to show chromatin (blue). (a″-i″; Merged) The corresponding nuclei when viewed through a triple band-pass filter to demonstrate colocalization of HRB87F and hsrω-n RNA in the omega speckles (orange in a″-i″) and at the 93D chromosome region. Note that all the hsrω-n RNA-containing speckles also contain HRB87F (orange fluorescence in a″-i″). In heat-shocked cells (b,d,f,i), very little HRB87F is seen on chromatin. Instead, the clusters of omega speckles in nucleoplasm as well as at the 93D site show strong HRB87F immunostaining. Bars (apply to a row), 10 μm.

of diffuse staining on chromatin throughout the nucleus (Fig. 2a,c,e,g,h). The second site of location of HRB87F was the omega speckles (Fig. 2a′,c′,e′,g′,h′). The colocalization of the hsrω transcripts and HRB87F in omega speckles was complete since no speckles were seen in any of the unstressed cell types that contained only one of these two (Fig. 2a″,c″,e″,g″,h″).

After heat shock, the diffused staining of chromatin with HRB87F was extremely weak in all the cell types (Fig. 2b,d,f,i); the HRB87F was now mostly localized at the larger clusters of omega speckles (see Fig. 2b′,b″,d′,d″,f′,f″,i′,i″). In nuclei, where all the hsrω-n transcripts had aggregated at the 93D chromosomal site following heat shock, most of the HRB87F also localized to this site only (Fig. 2f,f′,f″). When heat-shocked (40 minutes at 37°C) tissues were allowed to recover for 2 hours at room temperature (22±1°C), the free nucleoplasmic pattern of omega speckles and chromatin bound and omega speckles associated distribution of HRB87F was restored (Fig. 2g,g′,g″).

The in situ distribution patterns of two other hnRNPs, viz. Hrb57A, an hnRNP K homologue (Buchenau et al., 1997; Hovemann et al., 2000) and Hrp40, an hnRNP D homologue (Matunis et al., 1992a,b) in control and heat-shocked cells in different larval and adult tissues were identical to those of HRB87F (Fig. 3). In all control cells, both these proteins showed a diffuse distribution on chromatin and a speckled pattern that was coincident with the omega speckles. In heat-shocked cells, the diffuse staining of chromatin was minimized while the larger omega clusters were strongly decorated with antibodies for Hrb57A as well as Hrp40 (Fig. 3).

Another RNA binding protein, recently identified as an hnRNP M homologue, is recognized by the S5 antiserum (H. Saumweber, personal communication). The S5 antibody is known to decorate many of the RNA polymerase II transcribing loci in polytene chromosomes and to bind to the 93D and other heat-shock loci after heat shock (Dangli and Bautz, 1983; Risau et al., 1983). Immunostaining with the S5 antibody, in conjunction with FRISH using the hsrω-n riboprobe, in different cell types of *D. melanogaster*, revealed that, like the other hnRNPs, this protein also showed a diffuse chromatin binding and colocalization with omega speckles in control cells (Fig. 3h,h′,h″). After heat shock, all the aggregated omega speckles were positive for S5. However, unlike the other three hnRNPs, a few of the S5 speckles were independent of the omega speckles *(*Fig. 3i,i′,i″).

Omega speckles do not contain SRps

We examined if non-hnRNP splicing factors like SC35 (Fu and Maniatis, 1990) and SRp55 (Champlin et al., 1991; Kraus and Lis, 1994) also colocalize with omega speckles. Our results showed that, in addition to diffused staining on chromatin, the SC35 (Fig. 4a,a′,a″,b,b′,b″) as well as SRp55

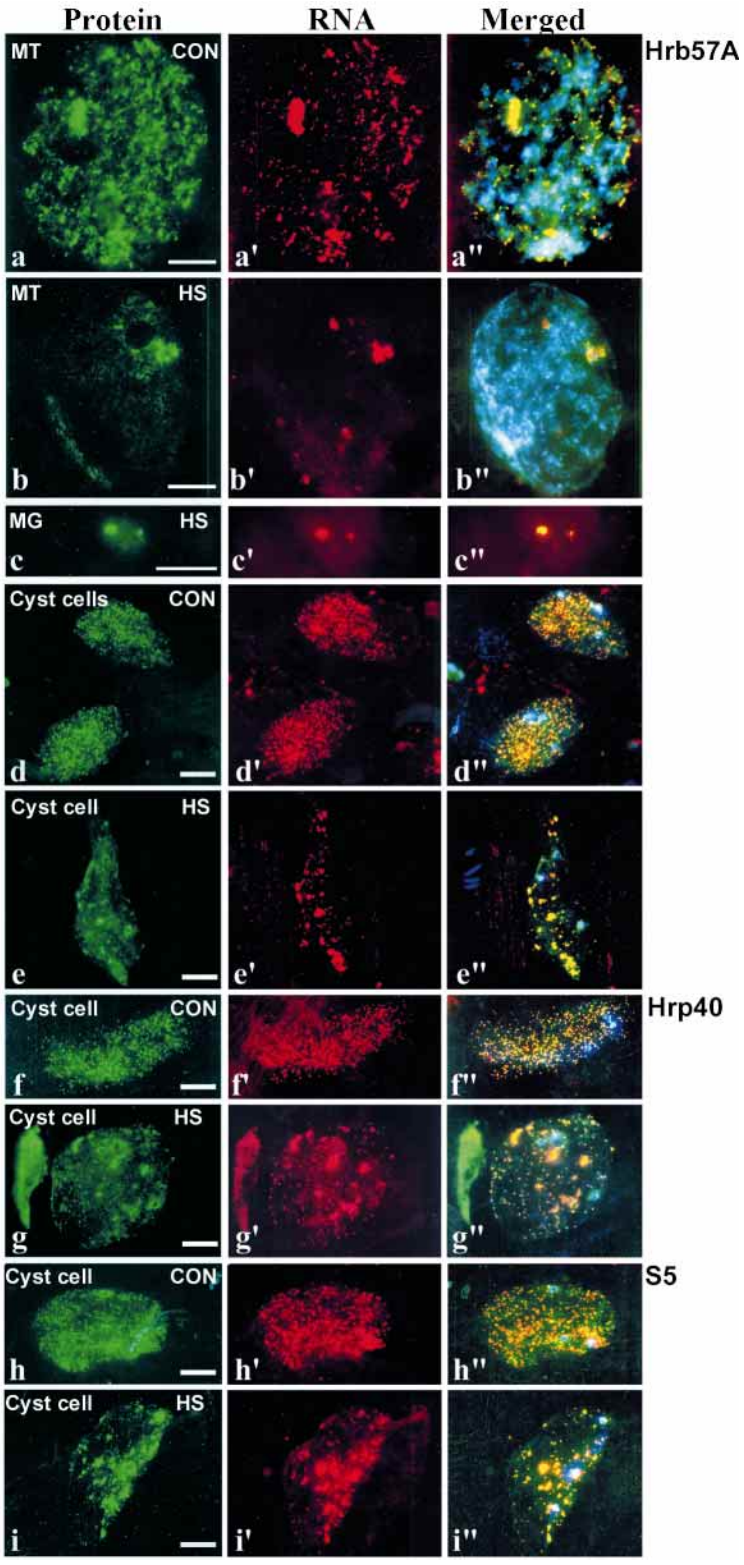

Fig. 3. Combined immunostaining (green fluorescence; Protein) with Hrb57A (a-e), Hrp40 (f,g), and S5 (h,i) antibodies and in situ hybridization (red fluorescence; RNA) of *pDRM30* riboprobe to RNA (a′-i′) in unstressed (a,a′,d,d′,f,f′,h,h′; CON) or heat-shocked (b,b′c,c′,e,e′,g,g′,i,i′; HS) larval Malpighian tubule (a,a′,b,b′; MT) and mid gut (c,c′; MG) polytene cells and adult testis cyst cells (d-i; Cyst cell). (a″-i″; Merged) The combined fluorescence of nuclei in the corresponding rows; except for the nucleus in row c, all were counterstained with DAPI to show chromatin (blue in Merged column). Note the complete correspondence of speckles of Hrb57A (a-e) and Hrp40 (f,g) with the omega speckles/clusters. Bars (apply to a row), 10 μm.

(Fig. 4c,c′,c″) formed nucleoplasmic speckles but none of them were associated with the omega speckles, neither in control nor in heat-shocked cells. It is also known that SRp55 does not bind with the 93D locus in polytene chromosomes from control or heat-shocked salivary glands (Champlin et al., 1991; our unpublished results).

hsrω transcripts are essential for establishing the speckled distribution of hnRNPs

In order to understand the role of hsrω transcripts in the speckled distribution of hnRNPs, we carried out immunostaining for the various hnRNPs in tissues from the few surviving *Df(3R)e^{GP4}/Df(3R)GC14 hsrω*-nullosomic trans-heterozygous larvae and flies. It was remarkable that no hnRNP speckles were seen in control or heat-shocked cells of *hsrω*-nullosomic larvae or flies. As seen in the representative examples of *hsrω*-nullosomic control larval Malpighian tubule (Fig. 5a,a′) or adult testis cyst cells (Fig. 5c,c′), immunostained for HRB87F (Fig. 5a,a′,c,c′) and counterstained with DAPI, instead of speckles, a strong diffuse and more-or-less homogenous staining of the entire nucleus was seen. Unlike in wild type, heat shock to the nullosomic tissues did not cause any clustering of the hnRNPs; rather the HRB87F (Fig. 5b,b′,d,d′) as well as the Hrb57A (Fig. 5e,e′) continued to remain more or less uniformly distributed through the nucleoplasm. DAPI staining of Malpighian tubule polytene nuclei suggested that compared to wild type, fewer of the chromatin areas were divested of the hnRNPs after heat shock in *hsrω*-nullosomic cells (for example, compare Fig. 5a′ and 5b′). Another interesting observation in the nullosomic cyst cells was the presence of three DAPI-bright chromatin areas, all of which showed stronger hnRNP binding in control cells (Fig. 5c,c′); after heat shock, however, only one of the three DAPI-

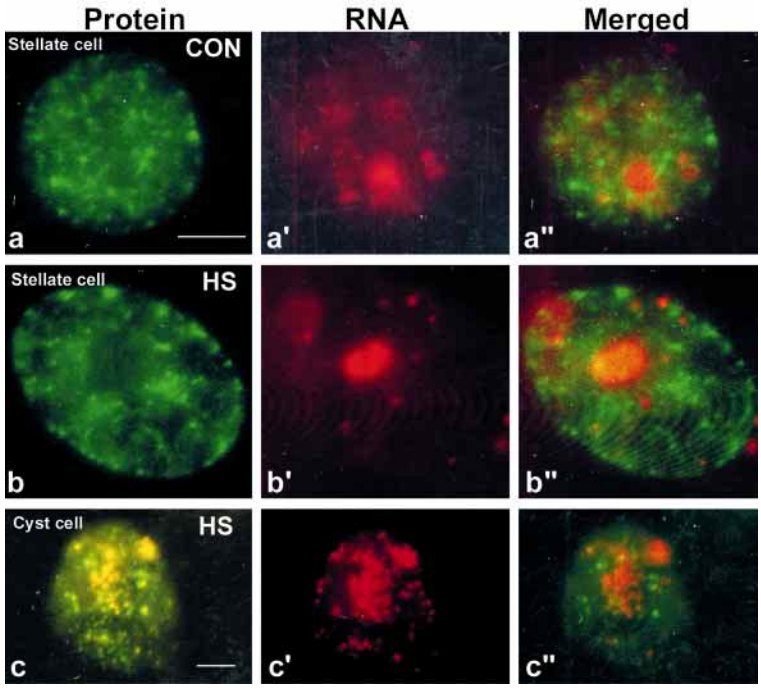

Fig. 4. Combined immunostaining (green fluorescence; Protein) with SC35 (a-b) and SRp55 (c) antibodies and in situ hybridization (red fluorscence; RNA) of *pDRM30* riboprobe to RNA (a′-c′) in unstressed (a,a′; CON) or heat-shocked (b,b′, c,c′; HS) Stellate cells (a-b) of larval Malpighian tubules or Cyst cells (c) of adult testis. (a″-c″; Merged) The combined fluorescence. Bars, 10 μm (rows a,b), 5 μm (row c).

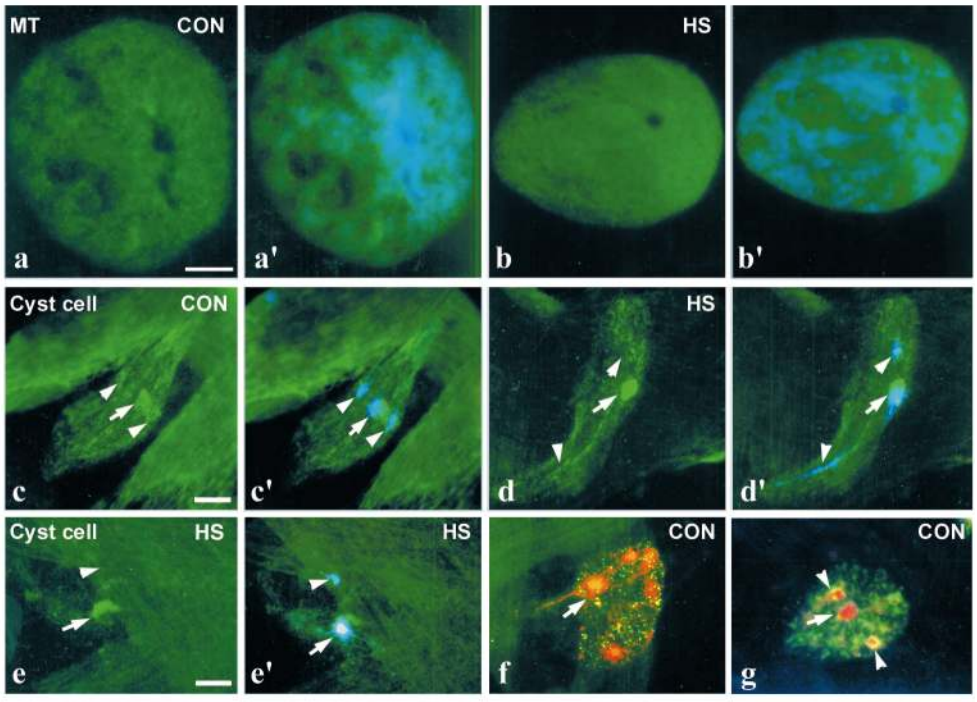

Fig. 5. Immunolocalization of HRB87F (a,a′,b,b′,c,c′,d,d′) and Hrb57A (e,e′) in unstressed (a,c; CON) or heat-shocked (b,d,e; HS) larval Malpighian tubule polytene cells (a,b; MT) and in adult testis cyst cells (c-e; Cyst cell) from *Df(3R)e^{Gp4}/Df(3R)GC14 hsrω*-nullosomics. a′-e′ images show combined FITC (green) and DAPI (blue, DNA) fluorescence. Note the complete absence of any speckles or clusters of HRB87F (a-d) or Hrb57A (e) in the *hsrω*-nullosomic cells. The cell in f shows in situ localization of hsrω-n RNA (green) in an unstressed wild-type testis cyst cell. The unstressed cyst cell in g is immunostained (green) for HRB87F. The chromatin in both the cases is counterstained with PI (red). The arrowheads in c,c′d,d′,e,e′,g, point to DAPI bright (c′-e′) or PI bright (g) chromocentres, which are surrounded with HRB87F or Hrb57A in unstressed but not in heat-shocked cells. The arrow in these figures and in f points to the larger DAPI (or PI) bright block, which is surrounded by HRB87F or Hrb57A after heat shock but not by hsrω-n transcripts (f). Bars (apply to a row), 10 μm.

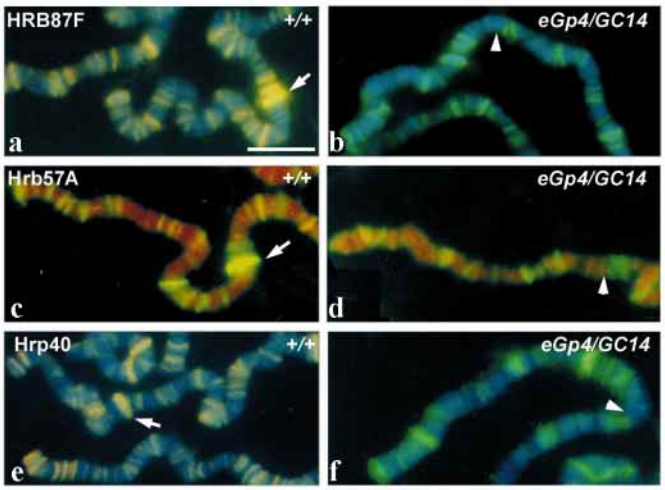

Fig. 6. Immunostaining of unstressed salivary gland polytene chromosomes to show localization (green/yellow fluorescence) of HRB87F (a,b), Hrb57A (c,d) and Hrp40 (e,f) on a segment of the right arm of chromosome 3 in wild type (a,c,e; +/+) and *Df(3R)e*^{Gp4}*/Df(3R)GC14 hsrω*-nullosomic (b,d,f; *eGp4/GC14*) larvae. The DNA was counterstained with DAPI (blue fluorescence; a,b,e,f) or with PI (red fluorescence; c,d). The arrow in a,c,e points to the 93D puff site while the arrowhead in b,d and f points to the deleted 93D6-7 band in *hsrω*-nullosomics. Bar, 10 μm.

bright areas was much more strongly decorated by both the antibodies (Fig. 5d,d′,e,e′). In view of the intense DAPI fluorescence, we believe that these regions with strong hnRNP binding most likely correspond to heterochromatic chromocentres. hnRNP (HRB87F, Hrb57A, Hrp40 and S5) immunostaining in wild-type cyst cells showed that two out of the three DAPI-bright chromocentres were associated with these proteins (see Fig. 5g) but not with the *hsrω* transcripts (Fig. 5f). Immunostaining of larval salivary gland polytene chromosome spreads also confirmed that the chromocenter displayed strong staining for these hnRNPs but not for *hsrω* transcripts (not shown).

A comparison of immunostaining of polytene chromosome squashes of wild type and *hsrω*-nullosomic larval salivary glands did not show any detectable difference in the binding of HRB87F (Fig. 6a,b), Hrb57A (Fig. 6c,d) and Hrp40 (Fig. 6e,f) with specific bands, except that as expected there was no strong binding in the 93D region in the *hsrω*-nullosomic chromosomes.

hsrω transcripts are present in immunoprecipitated hnRNP complexes

We used antibody to Hrp40 or HRB87F to immunoprecipitate hnRNP complexes from unstressed and heat-shocked wild-type larval homogenates. Primers that can distinguish between the spliced 1.2 kb smaller transcript and the unspliced larger transcript (see Fig. 7a) were used for RT-PCR with the Hrp40 immunoprecipitate to see if hsrω transcripts were present in the complex. As shown in Fig. 7b, the Hrp40 immunoprecipitated complexes from control as well as heat-shocked larvae produced two amplicons (1.2 kb and 0.55 kb, respectively), expected from the presence of hsrω-n and hsrω-c transcripts.

The HRB87F antibody immunoprecipitated complex was fractionated by SDS-PAGE, western-blotted and the blot challenged first with Hrb57A and HRB87F antibodies and then with Hrp40 antibody. As shown in Fig. 7c, Hrb57A as well as Hrp40 were coprecipitated with HRB87F. Using antibody to Hrp40 also, we found HRB87F to be coprecipitated (not shown). Silver-staining of the SDS-PAGE fractionated immunoprecipitates revealed the presence of many polypeptide bands (not shown); these are being characterized.

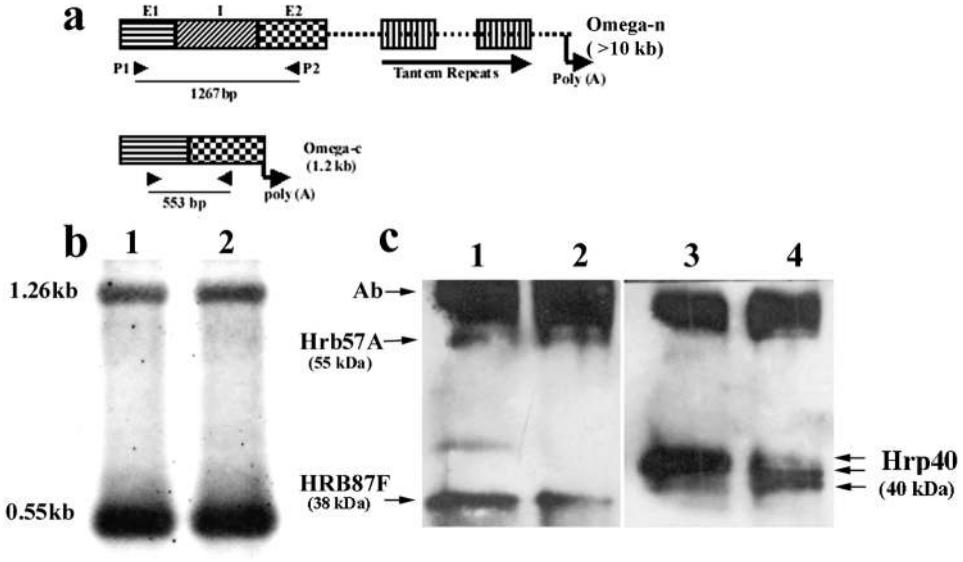

Fig. 7. (a) Diagram of the hsrω-n and hsrω-c transcripts showing the locations of two exons (E1 and E2), the single intron (I) and the tandem repeats in the *hsrω*-n RNA (for details, see Lakhotia et al., 1999), the locations of the two primers (P1 and P2, see text) and the sizes of expected amplicons following RT-PCR using the two primers. (b) Products of RT-PCR, visualized by Southern hybridization with the dig-labelled *pJG10* probe (representing E1 and E2 of the *hsrω* gene), of Hrp40-immunoprecipitated complexes from unstressed (lane 1) or heat-shocked (lane 2) larvae; sizes of the amplicons are noted on the left margin. (c) Western-blotting detection of different hnRNPs in HRB87F immunoprecipitated complexes from unstressed (lanes 1 and 3) or heat-shocked (lanes 2 and 4) larvae; the blot was first simultaneously challenged with Hrb57A and HRB87F (lanes 1 and 2) and the same blot, after stripping the membrane of the two primary antibodies, was challenged with Hrp40 antibody. The heavy band (Ab) at the top in all lanes corresponds to the heavy chain of the primary antibody used for immunoprecipitation. As already reported (Matunis et al., 1992a), Hrp40 produced multiple bands in immunoblots (lanes 3 and 4).

DISCUSSION

The 93D or *hsrω* gene of *D. melanogaster* is an intriguing gene, which is developmentally expressed in almost all cell types, is one of the major heat-inducible genes and is selectively induced by a variety of amides (for a recent review, see Lakhotia et al., 1999). In view of its noncoding transcripts, its functions have remained uncertain, but the very high lethality associated with its nullosomy is indicative of vital functions. Lakhotia et al. (1999) suggested that an important function of this gene's transcripts appears to be related to the fact that a variety of hnRNPs and certain other nuclear RNA-processing proteins, like Sxl, specifically bind with the 93D puff site in squash preparations of heat-shocked salivary glands of *Drosophila* larvae (Dangli and Bautz, 1983; Dangli et al., 1983; Hovemann et al., 1991; Samuels et al., 1994; Zu et al., 1998; Lakhotia et al., 1999). Our initial observations (Lakhotia et al., 1999) on the in situ distribution of hsrω-n transcripts in unstressed intact nuclei of larval Malpighian tubules and cyst cells of adult testes showed these noncoding transcripts to be uniquely present in the form of numerous nucleoplasmic speckles to which the HRB87F also colocalized. To our knowledge, hsrω-n RNA is the only known large nucleus-limited cellular poly(A)$^+$ RNA to display a speckled distribution. On the basis of these observations, we (Lakhotia et al., 1999) proposed that the hsrω-n RNA or omega speckles are distinct from the SRp-containing ICGs and that the hsrω-n RNA functions as a 'sink' for the hnRNPs and related RNA-processing factors. The present results show that the omega speckles containing hsrω-n RNA and hnRNPs are universal in *Drosophila* cells and that the hsrω-n transcripts are essential for the speckled distribution of hnRNPs.

The hsrω-n transcripts exist as speckles in nuclei of all cell types of *Drosophila* with a dynamic cell-type-specific distribution

Our in situ localization of hsrω-n transcripts in intact cells from a variety of larval and adult tissues revealed that the hsrω-n transcripts were present in all the cell types on the *hsrω* gene locus and as many free nucleoplasmic speckles in close proximity to, but not on, chromatin. A speckled distribution of the hsrω-n transcripts was also noted in all embryonic cells from blastoderm stage onwards (T. K. Rajendra, unpublished). In agreement with earlier reports (Bendena et al., 1991; Mutsuddi and Lakhotia, 1995) on varying expression of *hsrω* in cell-type- and developmental-stage-specific manners, the number of the omega speckles varied in cell types, reflecting their differential needs. Buchenau et al. (1997), using short oligonucleotide probes for in situ localization of hsrω transcripts in polyploid amnioserosa cell nuclei in embryos, suggested that the observed multiple hybridization signals represent the asynapsed multiple sites of transcription of the *hsrω* gene. As in other cell types, in the embryonic serosa cells we also found many omega speckles (not shown). We believe that the multiple sites of nuclear hybridization noted by Buchenau et al. (1997) actually represented the many omega speckles rather than transcriptionally active multiple *hsrω* gene loci. Our results clearly showed that in all cell types, only one (or at most two, if the homologs were asynapsed) site of hybridization to hsrω-n RNA was actually on the chromatin and by comparison with data on polytene chromosomes (Lakhotia and Sharma, 1995; Lakhotia et al., 1999), this site in every nucleus was the *hsrω* or the 93D locus itself. Other than this one site, all the other sites of hybridizations were not on chromatin but, as clearly seen in partially squashed polytene nuclei from larval Malpighian tubules and adult testis somatic cells (see Fig. 1p,t), were in the perichromatin space in close proximity to chromosome territory. We believe that the omega speckles seen in our studies are the same as the large RNP particles of approx. 300 nm diameter and containing smaller granules of approx. 30 nm diameter reported on the *hsrω* locus and free in nucleoplasm in earlier electron microscopic studies (Derksen et al., 1973; Dangli et al., 1983).

The hnRNPs that are not bound to chromatin colocalize with hsrω-n RNA in the omega speckles

A series of studies (Saumweber et al., 1980; Kabisch and Bautz, 1983; Samuels et al., 1994; Lakhotia et al., 1999; Hovemann et al., 2000) using immunostaining of squashed polytene chromosomes of *D. melanogaster* with antibodies against a variety of hnRNPs and other RNA-processing factors revealed binding of these proteins to different puffs and other transcriptionally active sites. In nonpolytene interphase nuclei, the hnRNPs are diffusely distributed over chromatin (Jones et al., 1980; Spector, 1993; our present results). Our earlier results (Lakhotia et al., 1999) and the present results of immunostaining of intact cells from different tissues with antibodies for several of the hnRNPs revealed that, in addition, all of them were also present in extrachromosomal domains as speckles. The absence of free speckles of hnRNPs in squash preparations of polytene chromosomes is presumably due to the breakdown of the nuclear envelope and consequent loss of the extrachromosomal hnRNPs during processing. It is most significant that all these hnRNP-containing speckles in intact nuclei were the same as the hsrω-n RNA-containing omega speckles.

Since one of the hsrω transcripts (hsrω-pre-c) is spliced to produce the 1.2 kb cytoplasmic hsrω-c RNA (Lakhotia et al., 1999), it is possible that the omega speckles are actually sites where the hsrω-pre-c transcripts are spliced and packaged for transport to cytoplasm. This, however, is unlikely, because firstly, like many other transcripts (Beyer and Osheim, 1988; Lamond and Earnshaw, 1998), hsrω-pre-c RNA is also spliced and processed at the site of transcription (Lakhotia and Sharma, 1995), and secondly, the nuclear hsrω-n transcripts present in the omega speckles are not the precursors of the hsrω-pre-c (Hogan et al., 1994) and hence do not need to assemble the splicing/processing factors. The presence of hsrω-n RNA at the 93D site as hnRNP-free smear in the central area (Figs 2e″,f″,3a″,b″) surrounded by hnRNP-bound speckles suggests that fresh hsrω-n transcripts quickly associate with the hnRNPs to form the omega speckles.

The presence of hnRNP containing omega speckles in *Drosophila* cells (Lakhotia et al., 1999 and the present results) is significant, since most reports of the nuclear speckles relate to the ICG clusters, which contain the snRNPs, non-snRNP splicing factors (SR proteins) but not hnRNPs (Fakan et al., 1984; Spector, 1993). Since the hnRNP- and hsrω-n RNA-containing omega speckles do not contain the SRps like SC35 and SRp55, while the ICGs do not contain hnRNPs, it is clear that the omega speckles are distinct from the ICGs. We are aware of only two recent studies on comparable hnRNP

speckles in mammalian cells. Weighhardt et al. (1999) reported speckles in mammalian cell nuclei containing an hnRNP A1 associated protein (HAP). Jagatheesan et al. (1999) also found nuclear speckles in HeLa cells when immunostained with the antibody against Hrb57A of *Drosophila*. Weighhardt et al. (1999) suggested that the HAP-containing speckles in mammalian cells have some structural RNA, which may help to assemble the speckles. The ICGs in mammalian cells have also been reported to contain non-coding poly(A)$^+$ RNAs, which may have a structural role (de Jong et al., 1996). Our results show that the hsrω-n RNA is one such candidate RNA species that plays a structural role in the assembly of speckles containing hnRNPs etc. It is likely that analogous species of RNAs may help assemble the ICGs, the HAP-containing speckles etc.

Reorganization of omega speckles after heat shock

The 93D or the *hsrω* locus is one of the most strongly induced heat shock genes in all cell types of *D. melanogaster* (Mutsuddi and Lakhotia, 1995; Lakhotia and Sharma, 1995). Heat shock is known to inhibit general chromosomal transcription, splicing and nucleocytoplasmic transport of RNAs (Yost and Lindquist, 1986; Bond, 1988). As noted earlier, several studies on localization of hnRNPs in squashed preparations of salivary gland polytene chromosomes established that many hnRNPs congregate at the heat-shock-induced 93D puff. Our preliminary report on localization of hsrω-n RNA and HRB87F in intact larval Malpighian tubule polytene cells and in the non-polytene cyst cells of adult testes (Lakhotia et al., 1999), and the present more extensive observations on a variety of larval and adult cell types, have revealed a very interesting correlated relocalization of the hsrω-n transcripts and the hnRNPs following heat shock. As in polytene chromosomes, in the other cell types the chromatin-bound hnRNPs disappeared with increasing duration of heat shock. Concomitantly, the nucleoplasmic omega speckles containing hnRNPs and the hsrω-n RNA congregated into bigger clusters, all of which ultimately localized more or less exclusively on the *hsrω* locus on the chromosome. Weighardt et al. (1999) have shown that in HeLa cells, the HAP also gets reorganized from a fine speckled distribution in unstressed cells to a few large nuclear granules after heat shock. Two other mammalian hnRNPs, hnRNP M and 2H9, have also been reported (Gattoni et al., 1996; Mahe et al., 1997) to dissociate from the ribonucleoprotein complexes upon heat shock and associate with the nuclear matrix. Since the poly(A)$^+$ RNA in ICGs are also suggested to be matrix bound (He et al., 1990; Huang et al., 1994), it is likely that the hsrω-n transcripts comprising the omega speckles are also in some way associated with nuclear matrix, and the sequestration of the hnRNPs by the omega speckles may be related to the dynamics of the nuclear matrix in relation to nuclear physiology. This will be an interesting aspect for further studies.

It is significant that the clusters or aggregates of omega speckles in heat-shocked cells were not randomly distributed in the nucleoplasm. As seen in partially squashed polytene cells, the largest cluster was on the 93D site while the others were mostly in close vicinity of chromosomes (perichromatin space). It will be interesting to examine if the perichromatin sites that the heat shock-induced clusters of omega speckles associate with are specific.

S5 antiserum recognizes the hnRNP M homologue in *Drosophila* (H. Saumweber, personal communication). This protein colocalized with omega speckles in unstressed cells. However, after heat shock, a few S5 speckles were outside the clusters of omega speckles. Other results (Dangli and Bautz, 1983; our unpublished results) showed that in heat-shocked salivary gland polytene chromosomes, the S5 antiserum also decorated, in addition to the 93D puff, certain other chromosomal sites. Therefore, it is possible that in heat-shocked cells, the protein recognized by S5 antiserum has some other functions, independent of the clusters of omega speckles.

hsrω-n RNA has an essential role in the formation of the omega speckles

Our finding that the various hnRNPs in unstressed and stressed cells from *hsrω*-nullosomic individuals do not assemble in speckles is very significant. While the binding of these hnRNPs to chromosomal sites did not appear to be affected (Fig. 6), their speckled distribution in nucleoplasm was completely absent in all *hsrω*-nullosomic cells. Furthermore, unlike in wild-type cells, heat shock did not alter the distribution of hnRNPs in nucleoplasm in the nullosomic cells. We therefore conclude that the hsrω-n RNA is primarily responsible for the assembly of various hnRNPs in omega speckles in the nucleoplasm of unstressed cells and as clusters of omega speckles in heat-shocked cells. A role of the hsrω transcripts in the assembly of hnRNP complexes is also evident from the fact that the immunoprecipitated complexes from unstressed or heat-shocked tissues using the antibody against Hrp40 contained hsrω transcripts. The primers chosen for RT-PCR were designed to distinguish between the spliced 1.2 kb hsrω-c and the larger unspliced hsrω-n nuclear transcripts (see Fig. 7a). The amplification of a 1.26 kb long amplicon in the RT-PCR reactions is in agreement with the results of in situ hybridization with the hsrω-n RNA specific riboprobe. Amplification of 0.55 kb amplicon in RT-PCR reactions suggests that the 1.2 kb hsrω-c RNA species is also present in the hnRNP complexes immunoprecipitated by the Hrp40 antibody. The presence of hsrω-c RNA in the immunoprecipitated complex may be because some of these hnRNPs are associated either with its transport to cytoplasm or with its processing from the hsrω-pre-c RNA. The presence of other hnRNPs like Hrp40 and Hrb57A in the immunoprecipitates resulting from the HRB87F antibody shows that the different hnRNPs exist as fairly stable complexes. This is further supported by our unpublished observations that HRB87F is also coprecipitated with Hrp40 when anti-Hrp40 antibody is used.

It is known that Hrp40 remains bound to some RNA in cells (Matunis et al., 1992a). It has also been recently reported that the glycine-rich domain (GRD) of HRB87F directs this protein to the 93D transcription site while the RNA binding domain II (RBD-II) of HRB87F is essential for its tight binding to the 93D or *hsrω* locus (Zu et al., 1998). It is also known that the hnRNPs (Hrp38, Hrb57A) and other RNA processing factors like Sxl, that bind exclusively to 93D locus after heat shock, contain the GRD domain (Samuels et al., 1994; Buchenau et al., 1997; Zu et al., 1998). On the basis of these observations and our present results, we suggest that the nuclear hnRNPs that are not productively engaged in RNA processing in the perichromatin fibril domain (i.e. other than the chromatin

bound fraction) are directed to the hsrω-n RNA because of RNA-protein and protein-protein interactions. Since Hrp40 is reported to directly bind with RNA (Matunis et al., 1992a), this may be one of the proteins that directly contacts the hsrω-n RNA and this in turn may help the other hnRNPs to assemble at the omega speckles. This is being examined.

Unlike in wild type, in *hsrω*-nullosomic cells, hnRNPs were seen to associate with some of the heterochromatic regions, especially after heat shock. As noted above, association of the hnRNPs in omega speckles seems to be related to certain sequence motifs present in the repeat region of the hsrω-n RNA (Zu et al., 1998). It appears that when the hsrω-n transcripts are not available, the hnRNPs may get associated to some extent with heterochromatin, due to the presence of certain motifs in the repetitive sequences. In this context, it may be noted that the heterochromatin is known to act as a storage site for some transcription factors like GAGA-binding protein during mitosis when this protein is removed from chromatin (Csink and Henikoff, 1998). In the spermatocytes of *Drosophila*, which do not contain hsrω transcripts, some of the hnRNPs like hnRNP M (S5) and Hrb57A (Q18) are associated with the repeat-rich transcribing Y-chromosome loops (Bonaccorsi et al., 1988; our unpublished observations).

Biological significance of omega speckles

Our results clearly establish the omega speckles as new members of the various nuclear domains in relation to the processing of the nuclear transcripts. Fig. 8 summarises our current view of the dynamics of hnRNPs and their assembly into omega speckles (also see Lakhotia et al., 1999). A polytene nucleus is depicted in this model since polyteny permits better spatial resolution of what happens in other interphase nuclei. In an unstressed nucleus, certain proportions of the various hnRNPs are productively associated with transcriptionally active gene loci. Such actively engaged hnRNPs are not associated with the hsrω-n RNA and show binding with specific chromosome regions in polytene nuclei, but remain as diffuse staining of chromatin in non-polytene interphase nuclei (Fig. 8a). The remainder of the nuclear hnRNPs and related proteins (like Sxl), which at any given time are not productively engaged in RNA processing etc, bind with the hsrω-n RNA to form the omega speckles in the perichromatin space for storage. The closeness of nucleoplasmic omega speckles to the perichromatin fibrils presumably allows a quick exchange of hnRNPs between the active chromatin sites and the omega speckle storage sites. The observed variations in the numbers of omega speckles in different cell types reflects the variable amounts of hnRNPs that need to be sequestered at storage sites in relation to nuclear activity. One of the factors that may regulate transcription of the *hsrω* gene can be the pool of hnRNPs that need to be sequestered. The rapid turnover of hsrω-n transcripts in unstressed cells (Bendena et al., 1989) perhaps relates to the dynamic equilibrium between hnRNPs actively associated with splicing substrates and those sequestered in the omega speckles. It is possible that as the hnRNPs are withdrawn from the omega speckles, the freed hsrω-n RNA is degraded; alternatively, degradation of the hsrω-n transcripts may provide the mechanism for release of hnRNPs. The reduced transcription and RNA processing under conditions of heat shock causes increasing amounts of hnRNPs to disengage from chromatin. The released hnRNPs are soaked by the hsrω-n transcripts, whose level is enhanced due to increased transcription and stability following heat shock (Bendena et al., 1989; Hogan et al., 1994; Lakhotia and Sharma, 1995). We think that clustering of omega speckles in heat-shocked cells (Fig. 8b) ensures a more tight binding of the hnRNPs and other related proteins like Sxl. The clustering of omega speckles may, firstly, more effectively prevent the hnRNPs in getting involved in promiscuous RNA processing under nonpermissive heat shock conditions and secondly, may also provide some chaperoning functions against thermal damage (Lakhotia et al., 1999). The culmination of this sequestering of hnRNPs by hsrω-n transcripts under heat shock conditions is the

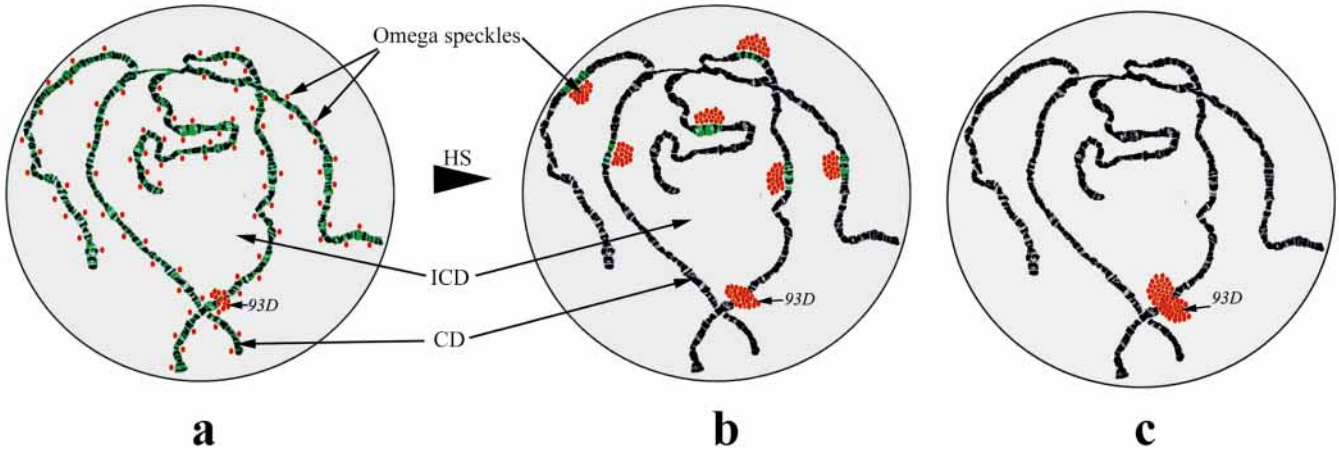

Fig. 8. Dynamics of hsrω-n transcripts and the different hnRNPs in a polytene nucleus under unstressed (a) and heat-shocked (b-c) conditions. The location of hnRNPs on active chromosomal sites is in green while the omega speckles containing hsrω-n RNA and the hnRNPs are shown in orange. The omega speckles in unstressed cells (a) are present at the 93D site on the chromosome and in close proximity to chromosomal domains (perichromatin space). Soon after heat shock (b), the hnRNPs begin to disappear from chromosomal sites and the omega speckles aggregate to form larger and fewer clusters, still close to active and/or potentially active sites and at the 93D site. After a heat shock for 40 minutes or so (c), nearly all the hnRNPs are localized at the 93D site as a massive cluster of omega speckles. CD, chromosome domain; ICD, interchromosome domain; HS, heat shock.

congregation of all the omega speckles into a massive cluster at the 93D site itself (Fig. 8c). This may represent a still higher order storage and protective state. As the cells recover from stress and resume chromosomal transcription and RNA processing, the free nucleoplasmic omega speckles reappear and the chromatin-bound hnRNPs also become detectable in 1-2 hours of recovery from heat shock. A prime role of hsrω-n transcripts in the dynamic redistribution of hnRNPs is also suggested by our observation that the chromatin binding of hnRNPs during recovery from heat shock is considerably delayed in *hsrω*-nullosomics (unpublished observations). A major cause for the high (approx. 80%) embryonic lethality of the *hsrω*-nullosomics (Mohler and Pardue, 1984; Lakhotia et al., 1999) appears to be the absence of omega speckles in their nuclei.

Besides storage, the sequestering of hnRNPs and related RNA-processing proteins by the omega speckles may serve the following additional functions. (1) By increasing the local concentration of hnRNPs and providing preassembled partial complexes near the sites of transcription, omega speckles may facilitate efficient processing of nascent transcripts. (2) Since the relative ratio of hnRNPs and SR proteins can affect alternative splicing (Mayeda and Krainer, 1992), the organization of hnRNPs and SRps in distinct domains of omega speckles and ICGs, respectively, may provide a mechanism for differential mobilization of these proteins for alternative RNA processing in relation to cellular needs.

Titration of RNA processing factors, including SRps and hnRNPs, by specific noncoding nuclear RNAs produced late after adenovirus-2 infection or trinucleotide repeat disorders, affects processing of other pre-mRNAs (Himmelspach et al., 1995; Timchenko, 1999). The hsrω-n RNA seems to carry such functions in normal and stressed cells. We believe that the equivalent of omega speckles must be present in other eukaryotic nuclei as well. Our finding that immunoprecipitation of hnRNP complexes allowing the recovery of hsrω transcripts could provide a strategy for the identification of noncoding transcripts that we suspect are present in various RNP speckles in mammalian cells.

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