Heterochromatin protein 1 (HP1) is associated with induced gene expression in *Drosophila* euchromatin

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**Introduction**

Heterochromatin protein 1 (HP1)* is a chromosomal protein first defined in *Drosophila melanogaster* by its association with the heterochromatin and by mutations that suppressed the silencing effect of heterochromatin in position effect variegation (James and Elgin, 1986; James et al., 1989; Eissenberg et al., 1990). HP1 is also a telomere-capping protein whose function is necessary for chromosome stability (Fanti et al., 1998). It has since been shown by molecular studies to be a highly conserved protein (Singh et al., 1991) enriched in heterochromatin and telomeres, and involved in gene silencing in other eukaryotes, including *Schizosaccharomyces pombe* and mammals (Jones et al., 2000; for review see Wang et al., 2000). HP1 has two prominent structural motifs, the chromo domain (Paro and Hogness, 1991) and chromoshadow domain (Aasland and Stewart, 1995), which are thought to be important for chromatin binding and protein interactions, respectively. Although different sets of data have shown that HP1 may associate to other several different proteins (Pak et al., 1997; Nielsen et al., 1999; Zhao et al., 2000; Nielsen, Oulad-Abdelghani et al., 2001; Nielsen, Schneider et al., 2001), until recently, we lacked precise molecular models to explain how these motifs might function to recognize chromatin, mediate protein–protein interactions, and induce heterochromatization and gene silencing. Recent studies have identified specific HP1 interacting histone methyltransferases enzymes, respectively, called SUV39H1 and Clr4 (Rea et al., 2000; Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Nielsen, Oulad-Abdelghani et al., 2001; Nielsen, Schneider et al., 2001) in mammals and yeast. These proteins are homologues to the *Drosophila* PEV modifier SU(VAR)3-9 protein (Tschiersch et al., 1994), which also displays methyltransferase activity (Schotta et al., 2002). This finding has suggested a model in which the interactions among a histone methyltransferases enzyme, the methylation of histone H3 and HP1 are proposed as the underlying basis for heterochromatin formation and epigenetic gene silencing. According to the model, the SU(VAR)3-9 enzymes methylate the histone H3 at lysine 9 creating selective binding sites for itself and the chromo domain of HP1. This three-component complex is proposed to form a specialized higher order chromatin state that defines heterochromatin and represses gene activity.

Recently, using antibodies that specifically recognize components of the complex, we compared the patterns of HP1 and histone H3 methylated at lysine 9 on *Drosophila* salivary gland chromosomes and found that these proteins are located at specific euchromatic sites, as well as in the heterochromatin, in patterns that only partially overlap (Cowell et al., 2002; Abdelghani et al., 2001; Nielsen, Schneider et al., 2001). Because the puffs are the cytological phenotype of intense gene activity, we did a detailed analysis of the heat shock–induced expression of the HSP70 encoding gene in larvae with different doses of HP1 and found that HP1 is positively involved in *Hsp70* gene activity. These data significantly broaden the current views of the roles of HP1 in vivo by demonstrating that this protein has multifunctional roles.

**Key words:** HP1; heterochromatin; HSP70; *Drosophila* euchromatin; developmental and heat shock–induced puffs on polytene chromosomes.
Fanti et al., 2003). These observations suggest that these proteins can be independently involved in different euchromatic domains.

To analyze the functional meaning of the unique association of HP1 to euchromatic sites, we mapped such sites and examined the relationship of the localization pattern with gene expression. Strikingly, we observed that HP1 is associated with induced developmental and heat shock puffs. A detailed analysis of the heat shock–induced expression of the Hsp70 encoding gene in larvae lacking or overproducing HP1 has shown that HP1 is positively involved in Hsp70 gene activity. These data significantly broaden the current views of the roles of HP1 in vivo by demonstrating that this protein has multiple functional roles in different chromosomal contexts.

**Results**

**HP1 binds multiple euchromatic regions and is associated with active loci**

Immunostaining of larval salivary gland chromosomes with an HP1 antibody revealed an enrichment of HP1 on the chromocenter, the fourth chromosome, and telomeres as already observed (James et al., 1989; Fanti et al., 1998; Fig. 1). In addition, the antibody also detected numerous sites along the euchromatic arms whose mapping is reported elsewhere (Fanti et al., 2003). Inspection of the specific loci to which HP1 binds revealed a striking result. Among the numerous euchromatic binding sites, we observed a localization of the protein to loci that form developmentally regulated chromosome puffs. We realized that, although not discussed, examples of puff staining with the HP1 antibody are evident also in a previous work by James et al. (1989). It is well-known that the puffs on polytene chromosomes of *Drosophila* and other Diptera are regions of high rates of RNA synthesis representing the visible expression of an intense gene activity at the chromosomal level. In salivary glands of third instar larvae, ~10 prominent puffs are stably visible. During the late third instar larval and prepupal stages, the release of the hormone ecdysone into the hemolymph induces a sequence of puffing activity that involves ~130 loci. Many of these loci have been mapped, and their characterization has shown that each puff has a specific temporal pattern of activity (Ashburner, 1972). As shown in Fig. 2 (A and B), three prominent ecdysone-induced puffs are clearly decorated by the HP1 antibody. This association is particularly suggestive of an involvement of HP1 in induced gene activity. To examine whether this result could be generalized, we asked if HP1 might also be found on other types of puffs such as those formed by transgenes under the control of heterologous promoters or heat shock–inducible puffs. We took advantage of the FLFW-1 transgenic *Drosophila* line characterized by Cavalli and Paro (1998, 1999). This strain contains the yeast transcriptional activator GAL-4 expressed under the control of the Hsp70 promoter and a GAL-4 activable UAS sequence that drives a lac-Z reporter gene. The reporter gene is flanked by Fab-7 and the mini-white gene. Previous characterization of the FLFW-1 insert by Cavalli and Paro (1998, 1999) showed that upon GAL-4 induction, the UAS-lac-Z gene forms a puff at the 61C9 region of the left arm of the third chromosome. As shown in Fig. 2 (C and D), we observed that, after induction of Gal-4, HP1 strongly accumulates on the puffed FLFW-1 insert.

**HP1 is recruited to heat shock–induced loci and is positively related to their expression**

To examine whether HP1 might accumulate at heat shock–induced puffs, we focused our assays on the best characterized heat shock–inducible puffs located at the 87A, 87C, 93D, and 95D regions on the right arm of the third chromosome. The 87A and 87C regions contain two and three genes, respectively, all coding the HSP70 protein isoforms (Leigh Brown and Ish-Horowicz, 1981), whereas the 95D region contains the gene encoding the HSP68 protein (for review see Pauli et al., 1992). The 93D region contains a noncoding gene whose activity produces untranslated transcripts (for review see Lakhotia and Sharma, 1996). We did not detect a significant presence of HP1 on these loci when larvae were raised under standard laboratory conditions (25°C). However, when larvae were treated for 30 min at 37°C, and their salivary glands immediately processed for immunostaining, we detected a strong association of HP1 with the heat shock loci. As shown in Fig. 2 E, along the right arm of the third chromosome there are two prominent heat shock puffs at 87A and 87C, and one at 93D and 95D. All of these sites show intense antibody staining, with signals for HP1 dispersed throughout the entire puffs. Strictly
speaking, the detection of HP1 in the heat shock puffs could be due either to the exposure of masked epitopes as the loci expand into puffs, or to the recruitment of new proteins upon induction. The latter possibility was suggested by the observation that the HP1 accumulation on heat shock–induced puffs was accompanied by a strong reduction of HP1 staining at nearly all euchromatic sites (Fig. 2 F). To further distinguish between the recruitment versus the epitope exposure possibilities, we used a transgene that increases the level of HP1 at the time of heat shock induction. This transgene, P[(neo)HSHP1.83C] places an HP1 cDNA under the control of the Hsp70 promoter (Eissenberg and Hartnett, 1993). With high temperatures, high levels of HP1 are expressed from the transgene, coincident with heat shock puff formation. Under these conditions, we observed an even stronger accumulation of HP1 on the heat shock puffs than observed in nontransgenic larvae and again a reduction of other euchromatic signals (unpublished data). Most significantly, we also performed heat shock experiments in HP1 mutant larvae carrying the heat shock–inducible P[(neo)HSHP1.83C] transgene. As shown in Fig. 2 G, the HP1 immunostaining does not reveal strong staining on polytene chromosomes from untreated larvae. However, in mutant polytene chromosomes, HP1 accumulation on heat shock–induced puffs is evident after heat shock induction, the puffs are already visible and show an abundant HP1 accumulation (Fig. 2 H). Together, the results demonstrate that HP1 is rapidly recruited to the heat shock–induced puffs likely due to the remobilization of this protein from its euchromatic sites. This interpretation is also consistent with observations described below that verify that the heat shock treatment used in our experiments does not affect the amount of HP1 in larvae (see Fig. 4 H).

The rapid accumulation of HP1 on the induced puffs is temporally coincident with the accumulation of the heat shock factor (HSF), the protein that is essential for heat shock gene activation. To test whether HP1 recruitment depends on the presence of HSF at the puffs, we used a strain homozygous for the temperature-sensitive hsf4 mutation (Jedlicka et al., 1997). We observed that after heat shock treatment of this strain, HP1 is not recruited to heat shock loci and does not appear removed from the other euchromatic regions (unpublished data). To test whether HSF directly recruits HP1 to the induced heat shock loci, we used a strain of flies carrying a transgene with a polymer of native HSF binding sites (HSE; Shopland and Lis, 1996). Previous studies showed that HSF is strongly recruited to the HSE sites in this construct (Shopland and Lis, 1996). The comparison of the immunostainings with HSF and HP1 antibodies on polytene chromosomes of heat shock–treated transgenic larvae showed that the two proteins colocalize at all the induced puffs (unpublished data). However, HP1 is not present on two sites corresponding to the transgenic HSE arrays (Fig. 3 B) even though HSF is clearly strongly bound to those sites (Fig. 3 A). These observations indicate that the presence of HSF is not sufficient to recruit HP1 to chromosomal sites.
The immunostaining assay does not allow us to assess whether HP1 is actually associated with the Hsp70 gene itself. To address this issue, we performed a formaldehyde cross-linked chromatin immunoprecipitation (X-ChIP) assay (Orlando et al., 1997) using the C1A9 anti-HP1 antibody. We designed three nonoverlapped primer pairs that amplify two 400–500-bp fragments of the Hsp70 gene promoter, including the TATA, HSE, and GAGA elements, and one corresponding to a portion of an exon (Fig. 3 C). We used these primers to amplify the DNA immunoprecipitated with C1A9 from chromatin of SL-2 cultured cells (Strahl-Bolsinger et al., 1997). As reported in Fig. 3 D, this assay showed that HP1 is not detected on the untreated heat shock gene. However, after heat shock induction, the protein is enriched in the Hsp70 coding region. We did not see enrichment of HP1 in the promoter region. These results support the conclusion that HP1 is associated with the Hsp70 gene itself after heat shock induction, specifically in the coding sequences.

The association of HP1 with induction of the heat shock puffs, raised the possibility that HP1 might be required for the heat shock response in Drosophila. To investigate this possibility, we asked whether larvae lacking HP1 exhibited a normal heat shock response. Previous studies showed that larvae transheterozygous for the HP1 Su(var)2-504 and Su(var)2-505 mutations survive until the late third instar stage and do not produce detectable HP1 in their salivary glands (Fanti et al., 1998). The immunostaining of the polytenic chromosomes of these mutant larvae after heat shock treatment showed that the absence of HP1 does not perturb the formation of puffs and the accumulation of high levels of the heat shock transcription factors HSF and hyperphosphorylated Pol II (Fig. 4, A–D). This was also confirmed by the evidence that mutant larvae are able to respond to heat shock by producing the HSP70 protein (Fig. 4, E–F). However, as shown in Fig. 4, we observed that in mutant and P[(neo)/HSHP1.83C] transgenic larvae, the level of Hsp70 transcript (Fig. 4 G) and protein (Fig. 4 H) 3 h after heat shock treatment was, respectively, significantly lower and higher than in wild-type larvae. Intriguingly, 7 h after heat shock treatment, all the genotypes showed a notably, but different, reduction of the transcripts. The mutant and the transgenic larvae, respectively, showed a higher and a lower level of transcript compared with wild-type larvae (Fig. 4 G). This difference is probably due to the different timing in the regression of transcription and it may reflect the dosage dependence, self-regulatory transcriptional control, of the gene. It has been shown that the transcription of heat shock loci is self-regulated depending on the critical quantity of their encoded proteins (DiDomenico et al., 1982).

**HP1 may bind the transcripts of active loci**

The chromatin IP studies indicated an enrichment of HP1 in the coding region of Hsp70, but did not indicate whether HP1 binding is dependent on the presence of RNA. To test this point, we treated polytenic chromosomes of untreated and heat-shocked wild-type larvae with RNase, and fixed the chromosomes followed by immunostaining with anti-HP1 antibody. Fig. 5 A shows the results of the RNase treatment on HP1 staining in nonheat-shocked polytenes. We observed differences in the effects of the RNase treatment depending on the chromosomal region. A loss of HP1 immunosignals was evident at many euchromatic sites with a pattern very similar, if not identical, to the immunopattern observed after heat shock in RNase untreated chromosomes (Fig. 2 F). However, the RNase treatment did not affect the immunofluorescence on the chromocenter, telomeres, and the 31 region. In addition, we observed that RNase treatment results in the removal of HP1 at the heat shock–induced puffs (Fig. 5 B). We also found that HP1 is not recruited to the heat shock puffs when they are induced by sodium salicylate (unpublished data). This substance is known to induce heat shock puff formation without RNA transcription (Winegardener et al., 1996) and, therefore, our observation strongly suggests that HP1 recruitment to the puffs depends on the presence of RNA transcripts.

**Figure 3. Mapping of HP1 binding sites in the Hsp70 gene after heat shock treatment.** A and B show a segment of a polytene chromosome from heat shock–treated larvae. These chromosomes contain an array of HSF binding sites contained on a transposon inserted in the 30A region (arrows). The immunostaining with antibodies against (A) HSF and (B) HP1 shows a strong accumulation of HSF at the site of the transposon insertion, but no significant HP1 immunosignal is visible. The arrowheads in A and B indicate the euchromatin 31 region. We show this region as a control for the immunostaining of HP1. The 31 region is, in fact, stained by the HP1 antibody (B), but not by the HSF antibody (A). (C and D) X-ChIP assay. The location of the DNA fragments amplified by the three primer pairs (Hsp70 promoter 1, Hsp70 promoter 2, and Hsp70 coding region) is shown in C. The first two fragments correspond to the promoter regions that include the TATA, HSE, and GAGA elements. The third fragment corresponds to a portion of the Hsp70 coding region. (D) PCR analysis of immunopurified DNA from SL-2 chromatin (SL-2-ChIP). The amplification products of each primer pair using genomic DNA (g), anti-HP1 immunoprecipitation (+) and mock immunoprecipitation (−) from heat shock–treated (HS) and untreated (noHS) SL-2 cells are shown.
Finally, we asked if the chromo domain of HP1 is involved in its interaction with transcripts, using the Su(var)2-5^{02} strain, which carries a mutation that disrupts the known function of the chromo domain (Platero et al., 1995). To this end, Su(var)2-5^{02}/Su(var)2-5^{05} mutant larvae were either untreated or heat shocked, and their polytene chromosomes showed smaller and larger amounts of Hsp70 transcripts, respectively, compared with levels in wild-type larvae. At 7 h, an inverse situation is present. Compared with the controls, mutant and transgenic larvae show larger and smaller amounts of Hsp70 transcripts, respectively. (H) Western blot analysis of HSP70 and HP1 proteins in untreated and heat shock–treated wild-type larvae (×/H11001), HP1 mutant larvae (×/H11002), and wild-type larvae carrying the heat shock–inducible Su(var)2-5 transgene (×/H11001/H11001). As expected, in untreated larvae, HSP70 is absent, whereas 3 h after the heat shock treatment, in mutant and transgenic larvae, the protein is less and more abundant than in wild-type. The differential abundance of HSP70 is clearly correlated to the absence and the overexpression of HP1. Note that in wild-type larvae, the quantity of HP1 is not affected by the heat shock treatment. The rp49 transcripts and the α-tubulin protein were used as a control.
somes were stained with anti-HP1 antibody. As shown in Fig. 5 C, we observed on chromosomes of untreated mutant larvae that the majority of euchromatic immunosignals were absent giving an immunopattern very similar to those observed in (Fig. 2 F) wild-type heat shocked chromosomes or (Fig. 5 A) RNase-treated chromosomes. After heat shock, we did not detect HP1 on the induced puffs (Fig. 5 D). These results suggest that the accumulation of HP1 on the active loci depends on the presence of transcripts.

Discussion

The HP1 protein in Drosophila and its counterparts in S. pombe, mammals, and other organisms have been extensively studied. In nearly all of these studies, the focus has been on the role of HP1 in inducing heterochromatin formation and gene repression. Recent studies have also noted a euchromatic localization of HP1 (Fanti et al., 2003), and a role in silencing in euchromatin has also been suggested (Hwang et al., 2001; Li et al., 2002). One apparent exception seems to be represented by genes that are embedded into Drosophila heterochromatin. Previous studies have shown that the activity of the heterochromatic light and rolled genes, is reduced by HP1 mutations (Hearn et al., 1991; Clegg et al., 1998; Lu et al., 2000; for review see Wakimoto, 1998). These data have suggested that the transcription of heterochromatic genes is mediated by the heterochromatin whose formation is HP1 dependent. As a result, the HP1 involvement in both gene silencing and heterochromatic gene activation seems to correspond to the two sides of the same medal, namely the role of such protein in forming heterochromatic domains.

In contrast to the most commonly cited role of HP1 in heterochromatin formation, the present data show a clear association of HP1 with induced gene expression in euchromatin. We have shown that association is true whether the induction occurs as a result of the developmental stage (as with the edcsyne regulated puffs), a heat shock–induced response, or induced ectopic expression (as with the GAL4/UAS transgene). In addition, the recruitment of HP1 to transgenic, developmental, and heat shock–induced puffs suggests that the association of HP1 with gene expression depends on the induction per se and not on a specific type of induction, specific promoter, or specific transcript.

Our analyses of gene expression have failed to detect a difference in heat shock–induced puffs between individuals with or without a functional HP1 gene. Although, the puff formation is not visibly affected, a quantitative Northern analysis reveals that genotypes with different doses of the HP1-encoding gene differ in the amount of Hsp70 transcripts. We found that, during the first hours after heat shock, the amount of Hsp70 transcripts in mutant larvae lacking HP1 and in transgenic larvae carrying four doses of the HP1-encoding gene is, respectively, lower and higher compared with the transcript level in wild-type larvae, thus, showing that HP1 affects heat shock RNA, either its expression or stability. The results of the X-ChIP assay show that, after heat shock induction, HP1 accumulates on the coding regions and not on the promoter region. This is consistent with a role of this protein on transcription rates, transcript elongation, transcript processing, or transcript stability rather than a role in gene induction. This role seems to be corroborated by our observations suggesting that HP1 accumulation depends on the presence of Hsp70 transcripts and by the integrity of its chromo domain. Because it has been shown that the chromo domain could be a module of interaction with RNA (Akhtar et al., 2000), we propose that HP1 may directly bind the Hsp70 transcripts. However, whatever the mechanism, it is clear that these results suggest a new role for HP1 in its association with induced, actively transcribed genes in euchromatin, and predict also its biochemical association with factors compatible with gene expression. Given that the physiological and heat shock–induced genes show accumulation of this protein, the network of interacting proteins may include mediators of the induction itself, such as hormone receptors and HSF. An interesting point in this regard, is that the accumulation of HP1 on heat shock–induced puffs seems coincident with its removal from many other sites including the developmental puffs. This opens the possibility that HP1 could be involved, at least in part, in the well-known extensive silencing of the genome after heat shock.

We believe the positive versus negative effects of HP1 are determined by its interacting proteins. Whether the positive and negative effects will map to the same interacting protein domains of HP1 will be interesting to determine. The activator and repressor activities require distinct protein domains for different DNA–protein, RNA–protein, or protein–protein interactions. HP1 has different domains that shares with other PEV modifier proteins or transcriptional regulators that should confer to it the necessary structural flexibility required for multiple functional roles. Further studies will tell us if our observation in Drosophila represents just an exception or instead represents, as we suspect, the first evidence that HP1 has multiple separate, nonoverlapping functions acting as either positive or negative transcriptional regulator also in euchromatin, depending on chromosomal context. To this regard, we anticipate that we observed also in other Drosophila species an HP1 association with active loci (unpublished data).

Materials and methods

Drosophila strains

The Ore-R stock used here has been kept in our laboratory for many years. The Su(var)203 strain was obtained from B. Wakimoto (University of Washington, Seattle, WA). The Su(var)2-5th and Su(var)2-21st strains were provided by G. Reuter (Institute of Genetics, Martin Luther University, Halle, Germany). The P(neo)1/H5HP1.83C stock was provided by J. Eisenberg (St. Louis University Medical Center, St. Louis, MO). All mutations were balanced with the TSTL (2;3) translocation carrying the larval dominant marker Tb, so that, in all the combinations, the mutant larvae could be recognized by the Tb phenotype. The hs71 mutant line was provided by C. Wu (NHI, National Cancer Institute, Bethesda, MD). The transgenic line containing a polymere of native HSF-binding site was provided by J. Lis (Cornell University, Ithaca, NY). The FLFW-1 strain was provided by G. Cavalli (Institut de Genetique Humaine, UPR 1142–CNR5, Montpellier, France). Cultures were maintained at 24°C on standard cornmeal-sucrose-yeast-agar medium. Heat shock experiments were performed according to Eisenberg and Hartnett (1993).

Immunofluorescence assays

Immunofluorescence analyses of polytene chromosomes were performed according to James et al. (1989). In brief, salivary glands from heat-
shocked and control larvae were rapidly dissected in Cohen and Gotchell medium G containing 0.5% Nonidet P-40 and incubated in a formaldehyde fixative solution. The same protocol was applied for immunostaining of whole salivary glands.

For RNAse treatments, approximately half of the glands was dissected in medium G and incubated in TBS (10 mM Tris-HCl, pH 7.15, and 150 mM NaCl). The other half was incubated in TBS plus 50 μg/ml RNase (Roche) for 45 min at room temperature. The glands were transferred to TBS/0.05% Tween 20 for 5 min and fixed in formaldehydefixative solution (James et al., 1989) for 25 min. The preparations were incubated with anti-H1C1A9 antibody (1:50), monoclonal mouse H14 (lgM) antibody (1:50) to the phosphorylated CTD of RNA Pol II (Covance), and rat anti-HSF antibody to the phosphorylated histone H2A (Covance) for 2 h followed by incubation with goat anti-rat IgG [Jackson ImmunoResearch Laboratories] in various pairwise combinations) for 1 h at room temperature in a humid chamber. The slides were washed three times in TBS at 4°C, stained with 4,6-diamidino-2-phenylindole (DAPI) at 0.01 μg/ml, and mounted in antifading medium. Chromosome preparations were analyzed using a computer-controlled Eclipse epifluorescence microscope (model E1000, Nikon) equipped with a CCD camera (CoolSnap). The fluorescent signals, recorded separately as grayscale digital images, were pseudocolored and merged using the Adobe Photoshop program.

Northern and Western blot analyses
RNA samples were isolated using the RNA extraction kit (QAGEN) according to manufacturer’s instruction. Store sample and ImageQuant software (Amersham Biosciences) were used for Northern blot hybridization signals detection and quantitation.

To perform Western blots, larvae were homogenized in SDS gel-loading buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 10 mM dithiothreitol, and 0.1% bromophenol blue) in the presence of protease inhibitors (10 μM benzamidine HCl, 1 mM PMSF, 1 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A) and heated at 95°C for 4 min. Insolubles were pelleted by centrifugation before electrophoresis. Proteins fractionated by 10% SDS-PAGE were electroblotted onto Immobilon-P polyvinylidifluoride membranes (Millipore) in a buffer containing 10 mM 3-cyclohexylamino-1-propanesulfonic acid (Sigma-Aldrich), pH 11, and 20% methanol, in a semi-dry transfer apparatus (Amersham Biosciences).

The blot was blocked with 0.2% I Block (Tropix) for AP detection or 5% blocking reagent (Amersham Pharmacia) for ECL in PBS (58 mM Na2HPO4, 17 mM NaH2PO4, 95 mM NaCl/0.1% Tween 20 (PBST). After blocking, proteins were probed with antibody against H1C (1:500), α-tubulin (1:3,000), and HPST0 (1:3,000), and detected with a 1:5,000 dilution of goat anti-mouse conjugated to alkaline phosphatase (HP1 and α-tubulin) or protein A HRP linked (HPST0). The AP detection kit and Enhanced Chemiluminescence kit were purchased from Tropix and Amersham Biosciences, respectively.

In vivo formaldehyde fixation and chromatin IP (X-ChIP)
Tissue cultured cells, from D. melanogaster cell line SL-2, were grown in serum-free medium. Cell densities were maintained between 3 × 106 and 8 × 106 cells/ml. The heat shock was performed at 36.5°C for 1 h. After heat shock, cells rested for 30 min at room temperature.

Cross-linked chromatin, prepared from SL-2 cultured cells, and immunoprecipitation were performed as described previously (Orlando et al., 1997). The precipitated DNA was redissolved in a suitable volume of TE buffer (10 mM Tris buffer, pH 8, and 1 mM EDTA) and stored at 4°C or used directly for PCR (Strahl-Bolsinger et al., 1997). PCR reactions were performed in 50-μl volumes using 2–3 μl of the template of the immunoprecipitated material or 200 ng of total genomic DNA from SL-2 culture cells, by using Taq polymerase and reaction buffer (GIBCO BRL). PCR scheme was performed as follows: 94°C for 3 min, once; 94°C for 1 min, 55°C for 1 min, 72°C for 45 s, 30 times; and 94°C for 1 min, 55°C for 1 min, 72°C for 7 min, once.

For each primer pair, the optimal magnesium concentration (1–2 mM MgCl2) was determined, and the annealing temperature and number of cycles were adjusted until no signal was detected for the mock-immunoprecipitated DNA, but the amplification on the genomic DNA was not altered. Signals obtained with the antibody-immunoprecipitated DNA under these conditions were considered significant. The amplified DNA was separated on 1.5% agarose gel and visualized with ethidium bromide.

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