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

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**Introduction**

Heterochromatin protein 1 (HP1) is a conserved non-histone chromosomal protein, which is involved in heterochromatin formation and gene silencing in many organisms. In addition, it has been shown that HP1 is also involved in telomere capping in *Drosophila*. Here, we show a novel striking feature of this protein demonstrating its involvement in the activation of several euchromatic genes in *Drosophila*. By immunostaining experiments using an HP1 antibody, we found that HP1 is associated with developmental and heat shock–induced puffs on polytene chromosomes. 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

**Abbreviations used in this paper:** HP1, heterochromatin protein 1; HSE, heat shock factor binding sites; HSF, heat shock factor; X-ChIP, formaldehyde cross-linked chromatin immunoprecipitation.

**Article**

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The Journal of Cell Biology, Volume 161, Number 4, May 26, 2003 707–714
http://www.jcb.org/cgi/doi/10.1083/jcb.200303012
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 ecdyson 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 ec dysone-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.
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 from untreated larvae. However, in mutant polytenues fixed just 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 polynucleot 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 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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{Su(var)2-5\textsuperscript{83C}} and \textit{Su(var)2-5\textsuperscript{85}} 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 polytene 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 \textit{P\bigl(\textit{neo}\\bigr)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 \textit{Hsp70}, but did not indicate whether HP1 binding is dependent on the presence of RNA. To test this point, we treated polytene 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 polytene. 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 (Winegar-den et al., 1996) and, therefore, our observation strongly suggests that HP1 recruitment to the puffs depends on the presence of RNA transcripts.

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**Figure 3.** Mapping of HP1 binding sites in the \textit{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 euchromat 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)2-502 strain, which carries a mutation that disrupts the known function of the chromo domain (Platero et al., 1995). To this end, Su(var)2-502/Su(var)2-505 mutant larvae were either untreated or heat shocked, and their polytene chromosomes were analyzed.

Figure 4. HP1 mutations do not affect the formation of puffs and HSF and POLII binding after heat shock treatment. (A) HSF and (B) POLII binding heat shock-induced puffs in polytene chromosomes of wild-type larvae. C and D show that in HP1 mutant larvae, the heat shock treatment induces puffs along with (C) HSF and (D) POLII binding. E and F show an immunostaining with HSP70 antibodies of whole salivary glands from untreated (E) and heat shock–treated (F) HP1 mutant larvae. Note the absence of any immunosignal in (E) untreated nuclei and the accumulation of the HSP70 in (F) heat shock–treated nuclei. (G) Northern blot analysis of Hsp70 transcripts in different times after heat shock induction in wild-type larvae (+), HP1 mutant larvae (−), and wild-type larvae carrying a heat shock–inducible Su(var)2-5 transgene (+ +). Hsp70 transcripts are not detectable in wild-type and HP1 mutant untreated larvae. 3 h after heat shock induction, HP1 mutants and transgenic larvae show 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, respectively, 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.

Figure 5. The RNase treatment of wild-type polytene chromosomes and the Su(var)2-502 HP1 mutation affects the euchromatic HP1 binding with similar immunopatterns observed. (A) HP1 immunostaining of RNase-treated polytene chromosomes from salivary glands of untreated wild-type larvae. Many of euchromatic HP1 immunosignals apparent in the controls are very faint or not visible at all in the RNase-treated case. Interestingly, the physiological puffs also lack HP1 immunosignals. The few immunosignals still visible, such as the signal on the 60B region (arrow) of the second chromosome and on the 14A and 14C regions of the X chromosome (arrows), are the same present in the polytene chromosomes of wild-type larvae after the heat shock treatment reported in Fig. 2 (F and G). Note that the immunostaining on the telomeres (asterisks), the chromocenter, the fourth chromosome, and the 31 region does not appear to be affected (large arrowheads). (B) A higher magnification showing HP1 immunostaining of RNase-treated polytene chromosomes from salivary glands of heat-shocked wild-type larvae. The heat shock–induced puffs in 87A and 87C do not show immunostaining (arrows). The arrowhead points to one of the few residual immunosignals. (C) HP1 immunopattern on polytene chromosomes from Su(var)2-502/ Su(var)2-502 mutant larvae. The chromocenter, the telomeres, and the 31 region (arrowheads) do not seem to be strongly affected, whereas the euchromatic immunopattern appears identical to that observed after RNase treatment showing only the same few immunosignals on 60B, 14A, and 14C (arrows). (D) A higher magnification view of an HP1-immunostained polytene chromosomes from salivary glands of heat-shocked Su(var)2-502/Su(var)2-502 mutant larvae. The heat shock–induced puffs at 87A and 87C are not immunostained (arrows). Note that in this case the same residual immunosignal, visible in B, is also present (arrowhead).
some 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 (Fantin 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 heterochromat remodeling seems to correspond to the two sides of the same medal, namely the role of such protein in forming heterochromat 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 edysone 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 PEF 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)205 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(neck1HSHP1.83C) stock was provided by J. Eisenberg (St. Louis University Medical Center, St. Louis, MO). All mutations were balanced with the TSTL (2;3;1) translocation carrying the larval dominant marker Tb so that, in all the combinations, the mutant larvae could be recognized by the Tb phenotype. The hs701 mutant line was provided by C. Wu (NHL, National Cancer Institute, Bethesda, MO). 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-CNRS, 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 Gohette 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 A (Roche) for 45 min at room temperature. The glands were transferred to TBS/0.05% Tween 20 for 5 min and fixed in formaldehyde fixative solution (James et al., 1989) for 25 min. The preparations were incubated with anti-H1P1 C1A9 antibody (1:50), monoclonal mouse H14 (lgM antibody) (1:50) to the phosphorylated CTD of RNA Pol II (Covance), and rat anti-HSF antibody (1:1000), both in PBS with 0.1% Tween 20 (PBST). After blocking, proteins were probed with antibody against HP1 (1:500), α-tubulin (1:3,000), and HPSP70 (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 (HPSP70). The AP detection kit and Enhanced Chemiluminescence kit were purchased from Tropix and Amersham Biosciences, respectively.

**References**


