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Effects of tethering HP1 to euchromatic regions of the *Drosophila* genome

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SUMMARY

Heterochromatin protein 1 (HP1) is a conserved non-histone chromosomal protein enriched in heterochromatin. On *Drosophila* polytene chromosomes, HP1 localizes to centric and telomeric regions, along the fourth chromosome, and to specific sites within euchromatin. HP1 associates with centric regions through an interaction with methylated lysine nine of histone H3, a modification generated by the histone methyltransferase SU(VAR)3-9. This association correlates with a closed chromatin configuration and silencing of euchromatic genes positioned near heterochromatin. To determine whether HP1 is sufficient to nucleate the formation of silent chromatin at non-centric locations, HP1 was tethered to sites within euchromatic regions of *Drosophila* chromosomes. At 25 out of 26 sites tested, tethered HP1 caused silencing of a nearby reporter gene. The site that did not support silencing was upstream of an active gene, suggesting that the local chromatin environment did not support the formation of silent chromatin. Silencing correlated with the formation of ectopic fibers between the site of tethered HP1 and other chromosomal sites, some containing HP1. The ability of HP1 to bring distant chromosomal sites into proximity with each other suggests a mechanism for chromatin packaging. Silencing was not dependent on SU(VAR)3-9 dosage, suggesting a bypass of the requirement for histone methylation.

Key words: Gene silencing, *Drosophila*, Heterchromatin protein 1 (HP1)

INTRODUCTION

Proper control of gene expression is established by interactions among cis-regulatory elements and trans-acting factors. One mechanism to regulate these interactions is the packaging of genomic DNA into chromatin. The fundamental packaging unit is the nucleosome, consisting of approximately 146 bp of double-stranded DNA wrapped around a histone octamer (Wolffe, 2001). The majority of the genome in eukaryotes is packaged into nucleosomes and further subdivided into regions of condensed heterochromatin and dispersed euchromatin. Heterochromatic regions are located near centromeres and telomeres that contain repetitive DNA sequences with relatively low gene densities. These regions are often packaged into regular arrays of nucleosomes consisting of hypoacetylated histones (Jenuwein and Allis, 2001; Richards and Elgin, 2002). By contrast, euchromatic regions contain the majority of genes and are packaged into nucleosomes that can exhibit irregular spacing. The histones in transcriptionally active euchromatic regions are typically acetylated. In addition to having distinct histone tail modifications, heterochromatic and euchromatic regions associate with distinct non-histone chromosomal proteins.

One protein that exhibits a localization bias towards heterochromatic regions is Heterochromatin protein 1 (HP1) (Eissenberg and Elgin, 2000). On *Drosophila* polytene chromosomes, HP1 localizes to centric regions, telomeric regions and in a banded pattern along the small fourth chromosome. In addition to these heterochromatic locations, HP1 is observed at approximately 200 sites throughout the euchromatic arms (James et al., 1989). HP1 contains two domains, the N-terminal chromo domain and the C-terminal chromo shadow domain, conserved from yeast to humans (Eissenberg and Elgin, 2000). Structural studies show that these two domains form hydrophobic pockets that are the sites of protein-protein interactions (Ball et al., 1997; Brasher et al., 2000; Cowieson et al., 2000). Mutations within these domains affect chromosome segregation and gene silencing (Fanti et al., 1998b; Wang et al., 2000).

One mechanism by which HP1 associates with chromosomes is through an interaction of the chromo domain with methylated lysine nine of histone H3 (H3 K9 methylation), a modification generated by the histone methyltansferase SU(VAR)3-9 (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Lachner et al., 2001; Nielsen et al., 2002). This interaction is consistent with the histone code hypothesis that states histone tail modifications serve as specific recognition markers for chromosomal proteins (Jenuwein and Allis, 2001). Experimental data support an interaction of HP1 with methylated K9 of H3 for centric localization. For example, *Drosophila* homozygous *Su(var)3-9* mutant flies show reduced levels of HP1 in centric regions (Schotta et al., 2002). By contrast, other experimental data suggest that HP1 uses alternative mechanisms for localization

at non-centric locations (Li et al., 2002). On *Drosophila* polytene chromosomes, methylated K9 of H3 and HP1 do not exhibit complete co-localization within euchromatin (Cowell et al., 2002; Li et al., 2002). Furthermore, a mutation in the chromo domain that abolishes an interaction with methylated K9 of H3 does not eliminate association at telomeres (Fanti et al., 1998b; Jacobs and Khorasanizadeh, 2002). Direct interactions of HP1 with unmodified histones and non-histone chromosomal proteins have been proposed as alternate mechanisms of HP1 association (Nielsen et al., 2001; Zhao et al., 2000).

To understand the relationship between HP1 and gene expression better we have developed a system to tether HP1 upstream of a reporter transgene inserted at sites within euchromatic domains of the *Drosophila melanogaster* genome. We determined that tethered HP1 is sufficient to nucleate the formation of silent chromatin at some, but not all sites tested within euchromatin. Silencing of the downstream reporter gene correlated with formation of ectopic fibers that frequently connected the tethered site to other sites, some of which contain HP1. This finding suggests that HP1 can partner with itself to bring distant chromosome sites into close proximity. Such a mechanism might be used for chromatin packaging, regulating gene expression or nuclear organization. Silencing of the reporter gene was independent of SU(VAR)3-9 dosage, suggesting that HP1 functions downstream of SU(VAR)3-9 in the silencing pathway.

MATERIALS AND METHODS

Tethering system

The tethering system has two components, a transgene expressing a *lac* repressor DNA binding domain (*lacI* BD) fused to a protein of interest, and a reporter transgene containing repetitive binding sites for the *lacI* BD (*lac* repeats) upstream of a reporter gene (Robinett et al., 1996). *lacI* fusion genes were cloned downstream of the *hsp70* promoter in the *Drosophila melanogaster* P-element vector pCaSpeR-hs-act containing mini-*white* as a transformation marker (thummel.genetics.utah.edu/vector%20map%20htmls/pcasper-hs-act.html). The resulting constructs were used to generate transformed stocks (Rubin and Spradling, 1982) carrying a single copy of a *lacI* BD fusion gene; these stocks will be referred to as expressor stocks. Expressor stocks were generated that contained a transgene encoding the *lacI* BD fused to full-length HP1 (Eissenberg et al., 1990). When these stocks were heat shocked for 45 minutes and allowed to recover for 3 hours, the *lacI*-HP1 fusion protein was present at levels that were 0.9 to 2.3 times that of endogenous HP1 (data not shown). Expressor stocks containing a transgene encoding green fluorescent protein (GFP) fused to *lacI* BD were used as a control in the gene silencing assay.

Stocks with transgenes containing 256, 32 and four copies of *lac* repeats cloned 500 bp upstream of a mini-*white* reporter gene were generated by standard transformation procedures (Rubin and Spradling, 1982); these stocks will be referred to as reporter stocks. Expression of the mini-*white* gene, required for eye pigmentation, was used in the silencing assays. Additional reporter stocks with 15-256 copies of *lac* repeats were generated by P-element mobilization (Robertson et al., 1988) using reporter stock 157.1, containing a transgene with 256 copies of *lac* repeats, as a starting stock. During the process of mobilization, copies of *lac* repeats were lost, generating stocks with smaller numbers of repeats.

In order to score silencing of mini-*white* in reporter stocks, the mini-*white* transformation marker in the expressor stocks needed to be removed. Imprecise P-element excision (Harrison and Perrimon, 1993) was performed on the expressor stocks to recover flies in which the *white*$^{+}$ transformation marker was deleted but the *lacI* fusion gene remained intact. The resulting *white*$^{-}$ expressor stocks were crossed to reporter stocks, heat shocked daily, and silencing of the mini-*white* reporter was scored by visual inspection of the adult eye.

Rescue of lethality

To determine whether the *lacI*-HP1 fusion retained functions of HP1, we tested *lacI*-HP1 for the ability to rescue the lethal phenotype of HP1 mutants. Mutations in the gene encoding HP1 are designated as alleles of *Su(var)2-5* (Eissenberg et al., 1990; Fanti et al., 1998b). Females with the genotype *lacI-HP1*; *Su(var)2-5*^{04}*/CyO, GFP* were crossed to males of the genotype *Su(var)2-5*^{02}*/CyO, GFP. CyO* is a balancer chromosome carrying the *Cy* mutation that generates a curly wing; the GFP transgene allows for scoring of the larvae containing this marker under a fluorescent dissecting microscope (FlyBase). Crosses were heat shocked at 37°C for 45 minutes daily. Rescue of lethality was indicated by the presence of non-GFP, straight winged adult progeny, representing the genotype *lacI-HP1*; *Su(var)2-5*^{04}*/Su(var)2-5*^{02}. Approximately 100 rescued adult progeny (the expected ratio for complete rescue) were obtained from five independent crosses.

Immunostaining of polytene chromosomes

To examine the chromosomal localization of the *lacI*-HP1 fusion protein with respect to the location of endogenous HP1 and H3 K9 methylation, third instar larvae were heat-shocked at 37°C for 45 minutes and allowed to recover at room temperature for 2 hours. Salivary glands were dissected, fixed and squashed as described (Platero et al., 1995). To detect HP1, the monoclonal antibody C1A9 (gift of Dr S. C. R. Elgin) was used. To detect the *lacI* fusion proteins, a rabbit polyclonal antibody to *lacI* (Stratagene) was used. To detect methylated histone H3, a rabbit polyclonal antibody to H3 K9 di-methyl (gift of Dr C. David Allis) and a rabbit polyclonal antibody to H3 K9 tri-methyl (gift from P. Singh) were used. To detect SU(VAR)3-9EGFP, a polyclonal antibody to GFP (Molecular Probes) was used. FITC-conjugated (Sigma) and Cy5-conjugated (Jackson ImmunoResearch Laboratories) secondary antibodies were used for detection. Images were photographed using a DMLB fluorescence microscope (Leica) and a Spot RT slider digital camera (Spot Diagnostic Instruments).

Determination of P-element insertion sites

Inverse PCR was used to determine the P-element insertion sites in reporter stocks according to published methods (Cryderman et al., 1998). A BLAST search was performed with the sequences obtained to determine the location of the P-element within the *Drosophila* genome.

RESULTS

Tethered HP1 nucleates the formation of silent chromatin at most, but not all, sites within euchromatic regions

HP1 localization at centric regions depends on SU(VAR)3-9 and correlates with gene silencing and a closed chromatin configuration (Schotta et al., 2002; Wallrath and Elgin, 1995). By comparison, relatively little is known about the mechanism of localization and function of HP1 within euchromatic regions of the genome. To address this issue, we targeted HP1 to sites within euchromatin by fusing HP1 to a heterologous DNA-binding domain. This allowed us to determine whether HP1 is sufficient to nucleate the formation of silent chromatin in the

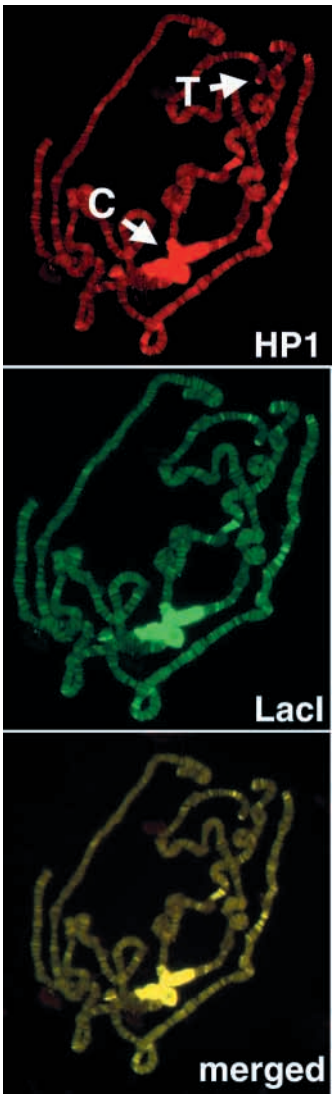

Fig. 1. *lacI*-HP1 fusion protein co-localizes with endogenous HP1. Polytene chromosomes from expressor stock LH_2, expressing *lacI*-HP1, were squashed and stained with antibodies to HP1 (red) and antibodies to *lacI* (green). The chromocenter (C) and telomeres (T) are indicated. Complete co-localization is observed as yellow in the merged image.

absence of the methyl mark laid down by the histone methyltransferase SU(VAR)3-9 and in the absence of repetitive DNA sequences typically found in centric heterochromatin. Full-length HP1 was fused in frame to the *lacI* BD (Robinett et al., 1996). The resulting fusion gene was expressed under control of an *hsp70* promoter. To determine whether fusion of the *lacI* BD to HP1 altered properties of HP1, the localization pattern of the fusion protein was examined on salivary gland polytene chromosomes. Antibodies to the *lacI* BD showed strong staining at the chromocenter, along the fourth chromosome, and at specific euchromatic locations (Fig. 1). This pattern is consistent with wild type HP1 and suggests that features required for localization were not disrupted by the fusion of the *lacI* BD. We wondered whether this complete co-localization might be due to associations between endogenous HP1 and *lacI*-HP1 through their chromo shadow domains, which have been shown to dimerize (Cowieson et al., 2000). To test this possibility, the localization pattern of *lacI*-HP1 was examined in larvae lacking endogenous HP1. Mutations in the gene encoding HP1, alleles of *Su(var)2-5*, are homozygous lethal. Mutant larvae survive until the late third instar larval stage, presumably owing to maternally contributed HP1 (Fanti et al., 1998b; Lu et al., 2000). Polytene chromosomes from late third instar larvae carrying two different mutant alleles of the gene encoding HP1, $Su(var)2\text{-}5^{05}$ and $Su(var)2\text{-}5^{04}$, were stained for *lacI*-HP1. The staining pattern in the mutant background showed that the *lacI*-HP1 protein retained a wild-type pattern, even in the absence of endogenous HP1 (data not shown).

As a second assay of function, *lacI*-HP1 was tested for the ability to rescue the lethality of an HP1 mutant. A genetic cross was performed to test whether flies carrying two different mutant alleles of *Su(var)2-5* could be rescued by expressing the *lacI-HP1* transgene (see Materials and Methods). When the *lacI-HP1* transgene was expressed by heat shock once a day for the first 5 days of development, the expected ratio of adult flies lacking a wild type *Su(var)2-5* gene was produced. By contrast, flies raised under non-heat shock conditions did not give rise to rescued adult progeny. These results indicate that *lacI*-HP1 possessed the ability to complement the lethality of the HP1 mutant.

Given that the *lacI*-HP1 fusion protein was functional, we tested for effects on gene silencing. Flies expressing the fusion protein were crossed to flies carrying a reporter transgene with 256 to four copies of *lac* repeats located 500 bp upstream of a mini-*white* reporter gene. For 25 out of 26 reporter stocks, tethered HP1 caused silencing of mini-*white*, as indicated by a reduction in the eye pigmentation observed in the adult progeny (Fig. 2; Table 1). In the single case where silencing was not observed, stock J3.2, the eye phenotype was red in the presence or absence of tethered HP1 (Fig. 2, middle row). Taken together, the results indicate that tethered HP1 is sufficient to nucleate the formation of silent chromatin at the majority of sites tested.

What influences the ability of HP1 to nucleate silent chromatin? Likely factors include features of the local genomic environment such as gene density and transcriptional activity. In order to determine the genomic location of mini-*white* with respect to nearest genes, inverse PCR was performed and the sequences adjacent to the P-element ends were determined. For 13 out of the 25 lines that exhibited silencing of mini-*white*, the P-element was inserted at distances greater than 10 kb away from a known or annotated gene (Fig. 2; Table 1). Thus, HP1 is sufficient to nucleate the formation of silent chromatin at many sites throughout the genome where transcriptional activity is predicted to be low.

In contrast to the lines where the nearest gene was greater than 10 kb away, 13 lines (12 that showed silencing) had the P-element inserted at distances less than 10 kb from a gene. We then examined the relationship between the silencing phenotype of mini-*white*, the distance to the transcription start site of the nearest gene, and the expression pattern of the nearest gene. For 11 out of the stocks in which silencing was observed, the nearest gene was not expressed or expressed only at low levels, during the time of development when *white* was expressed (FlyBase and http://genome.med.yale.edu/Lifecycle). Two exceptions were stock S9.2 in which the P-element was inserted 48 bp downstream of the *agros* gene and stock J3.2 in which the P-element was inserted 62 bp upstream of the *Atpα* gene (Feng et al., 1997; Freeman et al., 1992). Both of these genes are expressed in the developing eye at a similar time in development as the *white* gene (Sawamoto et

Table 1. Summary of silencing assays

lacI repeat line	*lacI* repeat location	Copies of *lacI* repeats	Eye color phenotype without *lacI*-HP1	Eye color phenotype with *lacI*-HP1	P-element insertion site
157.1	1E4	256	Orange	White	None
179.1	86D	256	Orange	White	8.63 kb downstream of *Sodh-2*
157.4.112	61F3	256	Orange	White	17 bp upstream of *BtbII*,
P6.4	88E12-F1	256	Yellow	White	1.69 kb downstream of *ea*
P20.21	99A4-5	256	Orange	White	None
P8.6	96B1	256	Orange	White	25 bp upstream of BcDNA:GH12663
P11.3	96C1-2	256	Yellow	White	None
P19.9	63C5	256	Orange	White	None
P2.5	65D4-5	256	Orange	White	6 bp upstream of *sgl*
P23.9	87D9-11	256	*Yellow*	*White*	4 bp downstream of CtBP
J11.1	42C2-3	256	Yellow	White	444 bp upstream of *l(2) 01289*
J3.2	93A7-B1	256	Red	Red	62 bp upstream of *Atpα*
F3.1.1	54B17-C1	256	Orange	White	None
107.1	40B-D	256	Orange	White	None
P25.22	89B2-B3	256	Orange	White	3.45 kb upstream of *He189B*
J8.1	89B16-17	~58	Orange	White	6.5 kb upstream of *tara*
S4.5	73A1-73A2	~58	Dark orange	Yellow	None
S11.7	61D1	~55	Dark orange	Yellow	None
S9.2	73A2-73A4	~46	Red	Yellow	48 bp downstream of *argos*
S15.14	1E4	~33	Orange	Yellow	None
CN2	50C20-22	32	Orange	Light orange	3.84 kb upstream of *Aats-phe*
CN10	63C5	32	Dark orange	Yellow	None
P9.4	85A7-9	~20	Dark orange	Yellow	None
J2.3	86E13-15	~15	Orange	Light orange	None
6.4P5	77B6-9	4	Dark orange	Orange	166 bp upstream of *trb1*
64.AB	67A8-B1	4	Orange	Light orange	None

Nearby genes listed are only those in which the transcription start site, or a predicted transcription start site, resides within 10 kb of the P-element insert according to FlyBase. 'None' indicates absence of genes within 10 kb of the P-element insert site. 'Upstream' and 'downstream' denote the location of the P-element with respect to the gene. '~' indicates lines in which the number of *lac* repeats were estimated based on the size of the *lac* repeat-containing fragment.

al., 1998; Yasuhara et al., 2000). Interestingly, only stock S9.2 showed silencing, while stock J3.2 has a red eye phenotype in the absence or presence of *lacI*-HP1 (Table 1). The failure of HP1 to nucleate silent chromatin in stock J3.2 suggests that chromatin modifications associated at the promoter region of an active gene might block *lacI*-HP1 association. Alternatively, they might allow association of *lacI*-HP1 but prevent the formation or stabilization of a silencing complex. To investigate these possibilities, polytene chromosome staining experiments were performed following expression of *lacI*-HP1 in stock J3.2. The results indicated that *lacI*-HP1 was bound at the location of the *Atpα* gene (Fig. 2). Thus, association of HP1 to a site might be insufficient to nucleate the formation or stabilization of silent chromatin in the promoter region of an active gene.

In contrast to stock J3.2, silencing might be permitted in stock S9.2 because the P-element was inserted within the coding region of a gene. Chromatin modifications that antagonize silencing might be associated with active promoters and not coding regions. Alternatively, a P-element insertion within a gene might disrupt transcription of that gene, resulting in the absence of modifications associated with gene activity.

Tethered HP1 causes ectopic associations

In addition to silencing, a second effect of tethered HP1 was observed when examining polytene chromosomes that were fixed, squashed, and stained with antibodies to *lacI*-HP1. There was an obvious appearance of ectopic fibers that frequently connected the tethered site to other nearby sites that contain HP1 (Fig. 3A,B). This phenomenon was observed in the four stocks (157.1, 157.4.112, P2.5 and 179.1) that were examined. Both intra- and inter-chromosomal associations were observed within a given stock, although intra-chromosomal associations were more frequent. In some cases, chromosomal contacts occurred between the tethered site and a site that did not show visible staining for HP1 (Fig. 3C), suggesting other proteins might participate in ectopic associations. The fibers are likely to be composed of protein because they disappear with acid treatment and do not stain with dyes used to visualize DNA. For a given stock, ~50% of the nuclei showed HP1-dependent ectopic associations. This frequency became nearly 100% upon increasing SU(VAR)3-9 dosage through introduction of a heat shock driven *Su(var)3-9-EGFP* transgene. Under conditions of daily heat shock treatments, presumably tethered HP1 recruits SU(VAR)3-9-EGFP and methylation of nearby histone H3 occurs (Fig. 4C). Importantly, ectopic associations were not observed when *lacI*-HP1 was not expressed or when the control GFP-*lacI* was expressed (data not shown). Interestingly, the associations did not appear in chromosome preparations of J3.2, the stock that did not show silencing with tethered HP1, implying that these associations correlated with the process of silencing.

Silencing is not dependent on SU(VAR)3-9 dosage

One model proposed for the spreading of silent chromatin is that HP1 recruits SU(VAR)3-9, which methylates histone H3, resulting in additional association of HP1 along the chromosome (Bannister et al., 2001). This model predicts that

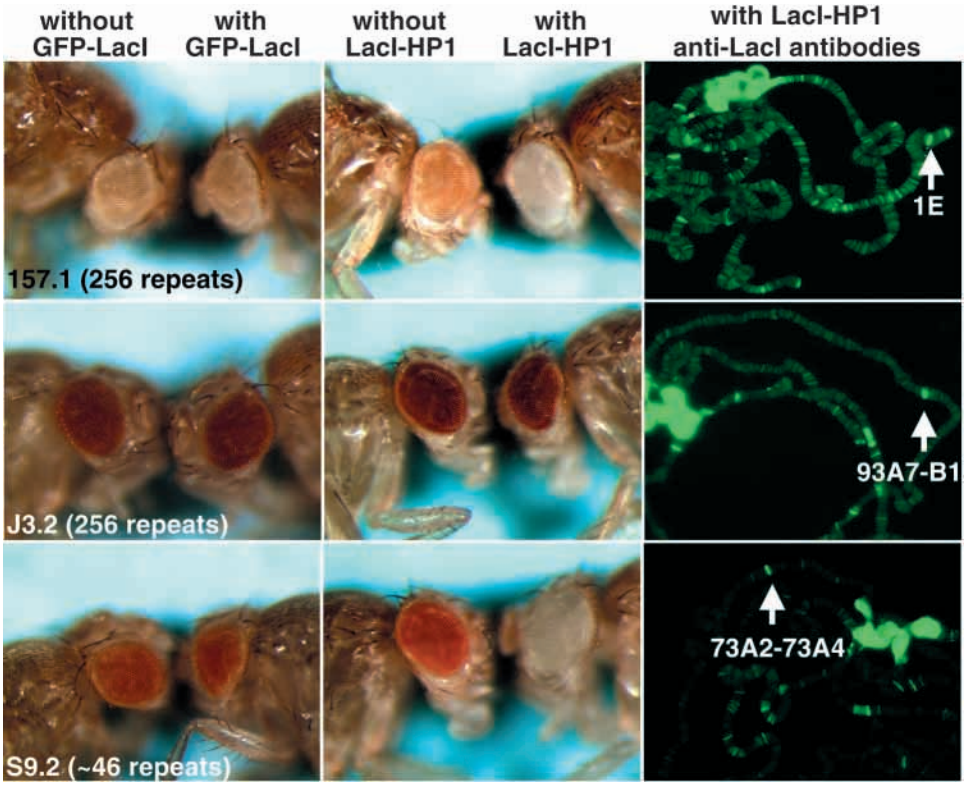

Fig. 2. Tethered HP1 causes silencing at some, but not all, genomic sites. The left column shows the eye phenotype observed for stocks 157.1, J3.2 and S9.2 with and without tethered GFP-*lacI*. The middle column shows the eye phenotype with and without tethered *lacI*-HP1. The right column shows localization of the *lacI*-HP1 fusion protein on polytene chromosomes. The arrow denotes the location of *lac* repeats. Polytene chromosomes were stained with antibodies against *lacI* (green).

SU(VAR)3-9 should be recruited to the site of tethered HP1 leading to increased H3 K9 methylation. Antibodies specific for *Drosophila* SU(VAR)3-9 are currently not available, therefore, we examined polytene chromosomes for H3 K9 methylation as a mark of SU(VAR)3-9 activity. Polytene chromosomes from three reporter stocks carrying transgenes with 256 copies (stocks 157.1, 157.4.112 and P2.5) of *lac* repeats were simultaneously stained with antibodies against HP1 and either H3 K9 di-methylation or H3 K9 tri-methylation (Cowieson et al., 2002). We first examined the chromosomal localization of HP1 and H3 K9 methylation in the host injection stock used to generate the reporter stocks. No staining was observed for either antibody at the cytological positions 1E4, 61F3 and 65D4-5, corresponding to the site of insertion of the reporter transgene in stocks 157.1, 157.4.112 and P2.5. It is well known that HP1 localizes to repetitive sequences within the genome, including tandem arrays of transgenes (Fanti et al., 1998a). Therefore, it was not surprising to detect a low level of HP1 over the 256 copies of *lac* repeats (Fig. 4A) inserted at sites that normally showed no HP1. Staining was not observed for transgenes with smaller copies of *lac* repeats (data not shown). In conjunction with this low level of HP1 staining, a low level of H3 K9 methylation was also observed over transgenes with 256 copies of *lac* repeats (Fig. 4A). This low level of staining prompted us to test whether mutations in the gene encoding HP1 affected expression of the mini-*white* reporter gene in the absence of tethered HP1. All eleven stocks tested show no effect in a *Su(var)2-5*^{04} mutant background (data not shown), suggesting that the variation in the eye color of the reporter stocks in the absence of tethered HP1 was likely to be due to typical euchromatic position effects. In contrast to this low level of staining, intense staining over the *lacI* repeats was observed with antibodies to HP1 upon expression of the *lacI*-HP1 fusion protein (Fig. 4B). Interestingly, a corresponding increase in H3 K9 methylated staining was not observed (Fig. 4B), suggesting that SU(VAR)3-9 was not recruited to the tethered site.

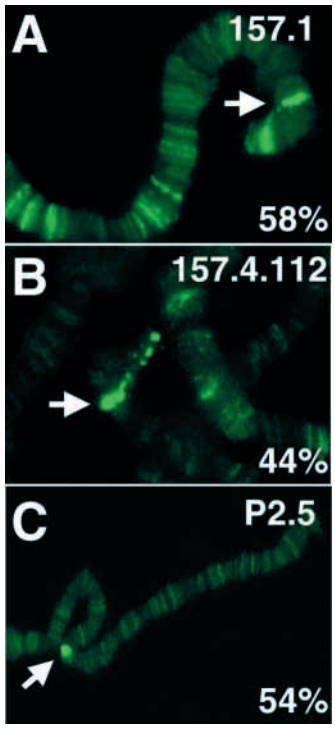

Fig. 3. Tethered *lacI*-HP1 causes ectopic associations**.** Third instar larvae of stocks 157.1, 157.4.112 and P2.5 expressing *lacI*-HP1 were stained with antibodies to *lacI* (green). Arrows indicate the locations of *lac* repeats. The percent of nuclei showing ectopic associations, out of 60 nuclei scored for each cytological position, are shown at the bottom of each panel. Associations were observed between the tethered site and other HP1-containing sites (A,B), but were also observed at sites not enriched with HP1 (C).

Several possible explanations could account for the lack of enhanced H3 K9 methyl staining at the tethered site. One explanation is the fusion of the *lacI* BD to HP1 allows for a bypass of the histone methylation requirement. This suggests HP1 functions downstream of SU(VAR)3-9 in silencing. A second explanation is that the fusion of the *lacI* BD to HP1 disrupts the ability of HP1 to interact with SU(VAR)3-9, an interaction shown by two-hybrid analysis and co-immunoprecipitation experiments (Schotta et al., 2002). We tested this possibility by adding an *hsp70* driven *Su(var)3-9-EGFP* transgene into the system. Under conditions of daily heat shock treatments SU(VAR)3-9-EGFP was recruited to the tethered site, implying an interaction with *lacI*-HP1 can occur in vivo (Fig. 4C). A third explanation for the lack H3 K9 methylation is that the methyl mark is masked under the fixation conditions. This seems unlikely as co-localization of the H3 K9 methylation and HP1 was observed within the chromocenter and at discrete euchromatic sites (Cowieson et al., 2000; Li et al., 2002). After considering these possibilities, the cytological data are most consistent with the idea that SU(VAR)3-9 activity is not required for silencing by tethered HP1.

The lack of SU(VAR)3-9 recruitment predicts that silencing due to tethered HP1 should be independent of *Su(var)3-9* gene dosage. We genetically tested this by examining the ability of tethered HP1 to silence in a *Su(var)3-9* mutant background. In general, gene silencing by heterochromatin is sensitive to both the dosage of HP1 and SU(VAR)3-9 (Wallrath, 1998). As examples, stocks containing a *white*$^{+}$ gene juxtaposed to centric heterochromatin or inserted within fourth chromosome heterochromatin are dominantly suppressed by *Su(var)3-9*^{06}, a null mutation (Fig. 4D) (Schotta et al., 2002). Three reporter stocks (157.1, 157.4.112 and 6.4P5) expressing *lacI*-HP1 that were either heterozygous or homozygous for *Su(var)3-9*^{06} showed no suppression of silencing (Fig. 4D; data not shown). Furthermore, a reduction in the intensity of HP1 staining on polytene chromosomes at the site of the *lacI* repeats was not observed (data not shown). Taking the cytological and genetic data together, HP1 appears to function downstream of SU(VAR)3-9 in the silencing pathway in *Drosophila*.

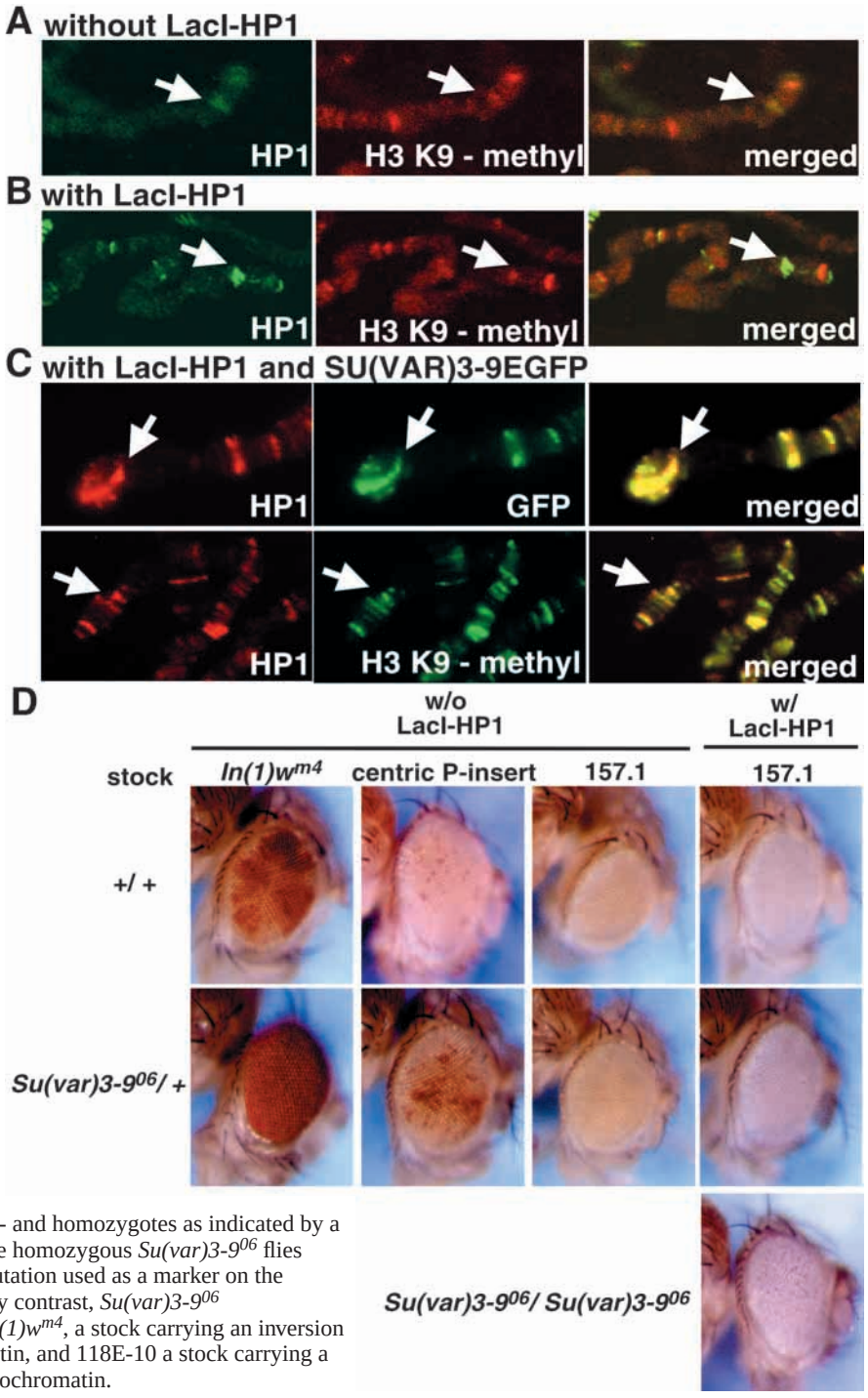

Fig. 4. HP1 functions downstream of SU(VAR)3-9. (A) The *lac* repeats show a low level of HP1 and H3 K9 methylation in the absence of *lacI*-HP1. Polytene chromosomes from stock 157.1 containing a reporter transgene with 256 copies of *lac* repeats were stained with antibodies to HP1 (green) and antibodies to H3 K9 di-methyl (red). Arrows indicate the locations of *lac* repeats. (B) Tethered HP1 does not intensify H3 K9 methylation. Strong staining is observed for the HP1 antibody, but not the H3 K9 di-methyl antibody, upon expression of *lacI*-HP1. (C) Expression of a *Su(var)3-9-EGFP* transgene leads to recruitment of SU(VAR)3-9-EGFP (upper row) and increased H3 K9 methylation (bottom row). (D) Silencing by tethered HP1 (stock 157.1) was unaffected in *Su(var)3-9*^{06} hetero- and homozygotes as indicated by a white eye phenotype (middle and bottom rows). The homozygous *Su(var)3-9*^{06} flies show a rough eye phenotype due to the *roughest* mutation used as a marker on the *Su(var)3-9*^{06}-bearing chromosome (bottom row). By contrast, *Su(var)3-9*^{06} heterozygotes show suppression of *white* gene in *In(1)w*^{m4}, a stock carrying an inversion placing the *white* gene next to centric heterochromatin, and 118E-10 a stock carrying a *hsp70-white* P-element inserted within centric heterochromatin.

DISCUSSION

In the present study, we have shown that tethered HP1 is sufficient to nucleate the formation of silent chromatin at most, but not all, genomic sites tested within euchromatin. This finding implies that simple satellite sequences and middle repetitious elements frequently located within heterochromatin are not required for the formation of silent chromatin. In the exceptional case (stock J3.2) in which silencing did not occur, the *lac* repeats were within a gene that is normally active in the same tissue and at the same developmental stage as the reporter gene. We hypothesize that features of the local chromatin structure associated with active chromatin, such as histone phosphorylation or acetylation, might play a role in preventing the establishment and/or spreading of silent chromatin even though HP1 is present.

Overall, our results using tethered *Drosophila* HP1 are consistent with those using tethered mammalian HP1 proteins. Both human and mouse HP1 proteins have been tethered to a small number of binding sites immediately upstream of a reporter gene on transient transfected plasmids, resulting in gene repression (Lehming et al., 1998; Seeler et al., 1998; van der Vlag et al., 2000). Our studies reported here extend these findings demonstrating that *Drosophila* HP1 is sufficient to nucleate the formation of silent chromatin on a native chromatin template.

When we compare silencing with *lacI*-HP1 to a study using a *Drosophila* Gal4-HP1 tethering system (Seum et al., 2001), some interesting differences emerge. In the Gal4-HP1 studies, tethered HP1 silenced a *white* reporter gene at only one of six genomic locations tested. The authors concluded that HP1 was necessary, but not sufficient, to form silent chromatin. In their study, the one site that supported silencing was surrounded by middle repetitious sequences thought to promote the formation of silent chromatin. One explanation for the different results obtained with the two HP1 fusion proteins is that the Gal4-HP1 fusion protein possessed limited capabilities, silencing only in a genomic context already favoring the formation of silent chromatin. In support of this hypothesis, the Gal4-HP1 fusion did not rescue the lethality of an HP1 mutant as did *lacI*-HP1 in our study. A second explanation for the different results obtained with the two different HP1 fusion proteins is that the five locations unable to support silencing by Gal4-HP1 were within active regions of the genome, similar to the reporter gene insertion of stock J3.2 described here. However, it is difficult to know whether this is the case as the genomic locations of the sites that did not support silencing by Gal4-HP1 were not reported.

Gene silencing by heterochromatin frequently is observed by a phenomena known as position effect variegation (PEV) (Weiler and Wakimoto, 1995). PEV is the cell-by-cell variation in expression of a gene that is brought into juxtaposition with heterochromatin through a chromosomal rearrangement or transposition event. In the study reported here, the reporter stocks selected for analysis were those that did not have the P-element inserted near heterochromatin, or ones in which the insert was within repetitive DNA sequences typically found within heterochromatin. In all cases, silencing by tethered *lacI*-HP1 was observed as a uniform, non-variegated, reduction in eye pigmentation. By contrast, the single example of silencing by tethered Gal4-HP1 was in a stock in which the reporter gene was inserted near repetitive elements. Taking this data together, it is tempting to speculate that the PEV phenotype might be related to the presence of repetitive DNA sequences. Such sequences might play a role in the 'on/off' decision of a promoter by 'locking in' a particular conformation state.

Previously, we have shown that gene silencing due to an association of HP1 correlates with a 'closed' chromatin structure and regular nucleosome arrays (Sun et al., 2001; Wallrath and Elgin, 1995). One possible mechanism to explain the formation of a closed chromatin configuration is through HP1-HP1 interactions on adjacent or nearby nucleosomes. Such interactions might render DNA sequences inaccessible to transcription factors and/or prevent nucleosome sliding induced by chromatin remodeling machines (Vignali et al., 2000). HP1-HP1 interactions at more distant chromosome locations might facilitate chromatin folding, leading to a closed chromatin structure. In addition, HP1-HP1 interactions might facilitate looping of regulatory elements (both enhancers and silencers) to promoter regions. This mechanism could explain how HP1 has different effects on distinct genes depending on their chromatin context. Finally, HP1-HP1 interactions might indirectly regulate gene expression by directing the localization and arrangement of chromosomes within the nucleus. This idea is supported by the discovery that HP1 interacts with the lamin B receptor at the nuclear membrane (Ye et al., 1997). Nuclear organization is thought to play a pivotal role in gene regulation (Hediger and Gasser, 2002).

The results from the *lacI*-HP1 tethering studies suggest that in the majority of cases silent chromatin 'spreads' at least 500 bp downstream of the *lac* repeats, hindering the promoter activity of the reporter gene. In preliminary studies, we have extended these studies showing that tethered HP1 can silence strong heat shock promoters up to distances of 5 kb from the *lac* repeats (J.R.D. and L.L.W., unpublished). Based on a current model for heterochromatin spreading (Bannister et al., 2001), tethered HP1 would be predicted to recruit SU(VAR)3-9, which would in turn methylate adjacent histone tails, serving as additional substrates for HP1 association. Both our cytological and genetic data do not support this model for spreading at distances of 500 bp to 5 kb from the tethered site in a wild-type genetic background. Only upon increased *Su(var)3-9* gene dosage do we observe recruitment of the SU(VAR)3-9-EGFP and subsequent enhancement of H3 K9 methylation. These results imply that under normal circumstances, SU(VAR)3-9 is a limiting component for silent chromatin formation. Therefore, we favor alternative models for spreading that include the recruitment of histone deacetylases and/or additional proteins that propagate the silent state along the chromosome. Our tethering system, in conjunction with the powerful technique of chromatin immunoprecipitaton (Orlando et al., 1997), will allow us to identify the histone modifications and protein components of silent chromatin extending from the *lac* repeats and determine the mechanism of silent chromatin spreading in greater molecular detail.

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