Mechanisms of HP1-mediated gene silencing in *Drosophila*

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Summary

Heterochromatin Protein 1 (HP1) is a structural component of silent chromatin at telomeres and centromeres. Euchromatic genes repositioned near heterochromatin by chromosomal rearrangements are typically silenced in an HP1-dependent manner. Silencing is thought to involve the spreading of heterochromatin proteins over the rearranged genes. HP1 associates with centric heterochromatin through an interaction with methylated lysine 9 of histone H3, a modification generated by SU(VAR)3-9. The current model for spreading of silent chromatin involves HP1-dependent recruitment of SU(VAR)3-9, resulting in the methylation of adjacent nucleosomes and association of HP1 along the chromatin fiber. To address mechanisms of silent chromatin formation and spreading, HP1 was fused to the DNA-binding domain of the *E. coli* lacI repressor and expressed in *Drosophila melanogaster* stocks carrying heat shock reporter genes positioned 1.9 and 3.7 kb downstream of *lac* operator repeats. Association of *lacI*-HP1 with the repeats resulted in silencing of both reporter genes and correlated with a closed chromatin structure consisting of regularly spaced nucleosomes, similar to that observed in centric heterochromatin. Chromatin immunoprecipitation experiments demonstrated that HP1 spread bi-directionally from the tethering site and associated with the silenced reporter transgenes. To examine mechanisms of spreading, the effects of a mutation in *Su(var)3-9* were investigated. Silencing was minimally affected at 1.9 kb, but eliminated at 3.7 kb, suggesting that HP1-mediated silencing can operate in a SU(VAR)3-9-independent and -dependent manner.

Key words: Chromatin structure, *Drosophila*, Gene silencing, Heterochromatin Protein 1, HP1

Introduction

Within the eukaryotic nucleus, DNA is packaged into two general forms of chromatin, euchromatin and heterochromatin. Euchromatin contains the majority of genes and is typically packaged into irregular nucleosome arrays containing DNase I hypersensitive sites that mark gene regulatory regions (Richards and Elgin, 2002). Nucleosomes within euchromatin are frequently modified by acetylation, which serves to decondense chromatin and attract transcriptional activators (Kurdistani and Grunstein, 2003). By contrast, heterochromatin contains mostly repetitive DNA sequences that are packaged into regular nucleosome arrays (Cryderman et al., 1998; Lohe and Brutlag, 1986; Sun et al., 2001; Wallrath and Elgin, 1995). Heterochromatic nucleosomes typically lack acetylation and frequently possess methylation at lysine 9 of histone H3 (MeK9H3) (Rea et al., 2000). Owing to distinct differences in histone modifications and DNA composition between euchromatin and heterochromatin, specific proteins preferentially localize to these two domains of the genome.

HP1 is a non-histone chromosomal protein enriched in heterochromatin (Eissenberg and Elgin, 2000). On polytene chromosomes of *Drosophila melanogaster*, HP1 associates at ~200 sites along the euchromatic arms, in a banded pattern along the fourth chromosome, near centromeres and near telomeres (Fanti et al., 2003; James et al., 1989). HP1 is an essential component of heterochromatic gene silencing, a phenomenon where active genes repositioned near heterochromatin become silenced (Weiler and Wakimoto, 1995). Heterochromatin-associated proteins are hypothesized to ‘spread’ linearly along the chromosome from the breakpoint and alter the chromatin structure of the relocated sequences (Locke et al., 1988; Tartof et al., 1984; Zuckerkandl, 1974). These sequences adopt a ‘closed’ chromatin structure; nucleosomes are packaged into regular nucleosome arrays that correlate with gene silencing (Cryderman et al., 1998; Cryderman et al., 1999; Sun et al., 2001; Wallrath and Elgin, 1995). There are estimated to be at least 25 genes in *Drosophila* called *Suppressors of variegation* [*Su(var)s*] that modify heterochromatic gene silencing (Weiler and Wakimoto, 1995). Three *Su(var)* gene products, HP1, SU(VAR)3-7 and SU(VAR)3-9, are haplo-insufficient suppressors and triplo-enhancers of heterochromatic gene silencing and are likely to play a central role in the molecular mechanism underlying heterochromatin formation and silencing (Schotta et al., 2003).

HP1 has two conserved protein-protein interaction domains, the chromo domain (CD) at the N terminus and the chromo shadow domain (CSD) at the C terminus (Aasland and Stewart, 1995; Paro and Hogness, 1991). The CD forms a hydrophobic groove that binds to the MeK9H3 modification (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001). A point mutation within the groove disrupts heterochromatic gene silencing (Jacobs et al., 2001; Platero et al., 1995). The CSD homodimerizes (Cowieson et al., 2000) and mediates interactions with a variety of nuclear factors (Li et al., 2002).

Dimerization of the CSD is required for some interactions with nuclear factors that contain a penta-peptide motif (Brasher et al., 2000; Smothers and Henikoff, 2000). The CD and CSD are separated by a hinge region that has been implicated in heterochromatin localization, interactions with histone H1 and non-specific DNA, and chromatin binding (Meehan et al., 2003; Nielsen et al., 2001; Smothers and Henikoff, 2001; Zhao et al., 2000). Together, these observations suggest that HP1 functions as a bridging protein connecting heterochromatic proteins to centric regions.

Of importance is the interaction between the HP1 CSD and SU(VAR)3-9 (Schotta et al., 2002; Schotta et al., 2003; Yamamoto and Sonoda, 2003). The C-terminal SET domain of SU(VAR)3-9 possesses histone methyltransferase activity that generates the MeK9H3 epigenetic mark recognized by the HP1 CD (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Rea et al., 2000). A model to explain heterochromatin spreading has been proposed where HP1 binds to MeK9H3 and recruits SU(VAR)3-9 (Bannister et al., 2001). The methyltransferase activity of SU(VAR)3-9 acting on adjacent histones would generate new binding sites, allowing HP1 to spread linearly along the chromatin fiber.

To determine whether HP1 is sufficient to nucleate silent chromatin and spread along the chromosome we used a tethering system to recruit HP1 to euchromatic sites within the genome of *Drosophila melanogaster* (Li et al., 2003; Robinett et al., 1996). In this system, sequences encoding the DNA-binding domain of the *E. coli* lacI repressor were fused to sequences encoding HP1 and placed under control of the heat shock inducible promoter, *hsp70*. HP1 tethering was achieved by expressing the lacI-HP1 fusion protein in *Drosophila* stocks carrying a single insertion of a transposon consisting of *lac* operator repeats upstream of two heat shock reporter genes. The association of lacI-HP1 with the *lac* repeats results in silent chromatin formation that spreads bi-directionally from the *lac* repeats and silences expression of reporter genes located 1.9 and 3.7 kb from the tethering site. Silencing correlated with alterations in chromatin structure that were similar to those observed in centric heterochromatin. In a *Su(var)3-9* mutant background, silencing was minimally affected at 1.9 kb, but eliminated at 3.7 kb, suggesting that HP1-mediated silencing operates in a SU(VAR)3-9-independent and -dependent manner.

Materials and methods

Plasmids

lacI-HP1 fusion

The DNA-binding domain of the *lacI* repressor from *E. coli* was fused to the N terminus of the full-length *Su(var)205* cDNA (Eissenberg et al., 1990) and cloned into pCaSpeR-hs-act under control of the heat shock inducible *hsp70* promoter (Li et al., 2003).

GFP-lacI fusion

The *GFP-lacI* fusion gene was isolated from pUdCE (gift from A. Belmont) and cloned into pCaSpeR-hs-act.

lac-hsp26-hsp70 reporter transposon

The 256 copy *lac* operator repeat array (10 kb) was excised from pSV2-dhfr.8.32 (gift from A. Belmont) and inserted into A412-plant (Wallrath and Elgin, 1995).

Germline transformation and genetic manipulation

Germline transformation

Stocks containing the *GFP-lacI* expressor transgene on the second chromosome and the *lac-hsp26-hsp70* reporter transposon on the X-chromosome were generated using standard P-element transformation (Rubin and Spradling, 1982). Stocks containing the *lacI-HP1* expressor transgene on the second chromosome have been previously described (Li et al., 2003).

P-element mobilization

To isolate additional insertions of the *lac-hsp26-hsp70* reporter transposon at different genomic positions, the transposon in stock hsp26-4D5 was mobilized using Δ2-3 transposase (Robertson et al., 1988).

Homozygous *hsp26-4D:lacI-HP1;Su(var)3-9^{06}* stock

To study the effects of *Su(var)3-9* on tethered HP1-induced gene silencing, fly stocks were generated through multiple crosses that resulted in a stock homozygous for the *lac-hsp26-hsp70* reporter transposon on the X-chromosome, *lacI-HP1* on the second chromosome and *Su(var)3-9^{06}* (a null allele) on the third chromosome.

Heat shock induction

Drosophila cultures were incubated at 37°C for 45 minutes once a day throughout development.

Inverse PCR

Inverse PCR (Li et al., 2003) was used to determine the insertion site for each *lac-hsp26-hsp70* reporter transposon. Sequences obtained were compared to the *Drosophila* Genome Database (Release 3.1) using Fly Blast at the Berkeley Drosophila Genome Project (http://www.fruitfly.org/blast/).

Northern analysis

Total RNA was isolated from 25 larvae or 15-20 adult flies using TRIzol Reagent (BRL Life Sciences) as described by the manufacturer. Total RNA (30 μg) was used in northern analyses and hybridized with radiolabeled fragments corresponding to the unique barley sequence tag fused to *hsp26* or sequences corresponding to the *white* transgene. Hybridization with sequences corresponding to the ribosomal gene, *rp49*, served as a loading control. Radioactive counts from each hybridization signal were quantitated using an Instant Imager (Packard).

Chromatin structure analysis

Nuclei were isolated from 1 g of third instar larvae or 5 ml of adult flies and digested with micrococcal nuclease or *Xba*I restriction endonuclease as previously described (Wallrath and Elgin, 1995). The digested DNA was assayed by Southern analysis and hybridized with fragments corresponding to the unique barley sequence tag fused to *hsp26*. Radioactive counts from each hybridization signal were quantitated using an Instant Imager (Pakard).

Chromatin immunoprecipitation (ChIP)

Salivary glands were dissected from third instar larvae in Ringers solution (8 g NaCl, 0.20 g KCl, 1 g $NaHCO_3$, 0.04 g NaH_2PO_4•$2H_2O$, 0.20 g $CaCl_2$•$2H_2O$, 0.05 g MgCl2•$6H_2O$ and 1.00 g glucose in 1 l H_2O) and crosslinked with 10% formaldehyde for 10 minutes. The tissue was rinsed three times in wash buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5 mM EGTA and 0.5 mM PMSF], frozen in liquid nitrogen and stored at –80°C. The frozen tissue was thawed on ice in 200 μl of SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8)]. One-third volume of glass beads (Sigma G-1277) was added to each sample and the tissue was sonicated using a Sonifier® Cell Disruptor (Heat Systems – Ultrasonics) four times for 25 seconds in an ethanol-ice bath using the micro tip. The tissue was diluted sixfold with IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA,

16.7 mM Tris-HCl pH8, 16.7 mM NaCl, 1 mM PMSF and 1 μg/μl aprotinin) and divided into 600 μl aliquots. One aliquot was set aside as the input sample and the rest of the aliquots were pre-cleared using 50 μl of 50% Protein A Sepharose™ CL-4B beads (Amersham Biosciences) resuspended in IP buffer. The samples were rotated at 4°C for 2 hours. The beads were removed by centrifugation and 4 μl of polyclonal HP1 antibodies (PRB-291, Covance) or 4 μl of polyclonal GFP antibodies (A-6455, Molecular Probes) or no antibody was added to the samples. The samples were rotated overnight at 4°C.

Multiple attempts to perform ChIP with HP1 monoclonal antibody C1A9 were unsuccessful. Three polyclonal HP1 antibodies are currently commercially available (Covance); however, these antibodies recognized multiple bands on westerns of nuclear extracts from whole larvae and are designated for use with only salivary glands by the manufacturer. Staining with HP1 antibody PRB291C (Covance) showed colocalization with HP1 on polytene chromosomes and gives a predominant band corresponding to the correct molecular weight of HP1 on westerns of nuclear extracts from third instar salivary glands. Therefore, salivary gland tissue was used as a source of starting material for the ChIP analyses. The heat-shock reporter genes are robustly expressed in salivary glands upon heat shock (Cryderman et al., 1998). Furthermore, salivary glands allow for cytological analyses that provide supporting data for ChIP results.

Thirty microlitres of 50% Protein A sepharose beads were added to each chromatin/antibody sample and rotated at 4°C for 2 hours. Supernatants were collected into fresh tubes; the beads were washed with low salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl (pH 8), 150 mM NaCl]; high salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl (pH 8), 500 mM NaCl]; LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid (disodium salt), 1 mM EDTA (pH 8), and twice with TE buffer [10 mM Tris-Cl (pH 8) and 1 mM EDTA (pH 8)]. The beads were resuspend in 100 μl of TE buffer (pH 8) and RNase A was added to all samples (beads, supernatants and input) to a final concentration of 50 μg/ml and incubated at 37°C for 30 minutes. Then, SDS was added to a final concentration of 0.5% and Proteinase K to a final concentration of 100 μg/ml and the samples were incubated at 37°C overnight. The samples were then incubated at 65°C for 6 hours. The DNA was extracted once with phenol:chloroform:isoamyl alcohol (24:24:1) and twice with chloroform and then ethanol precipitated. The DNA pellet was suspended in 25 μl of TE buffer and assayed using PCR. The unique primers sets used were hsp26-1.9 kb, forward (5′ CGAGGAAGAGCGTGTTGTAGG 3′) and reverse (5′ ACAAC-ACCGACATGCTCTACAG 3′); hsp70-3.7 kb, forward (5′ GCAACC-AAGTAAATCAACTGC 3′) and reverse (5′ GTTTTGGCACAGCA-CTTTGTG 3′); 4D5-10.6 kb, forward (5′ GAGCCAAGAAGATAAA-CACAC 3′) and reverse (5′ GAATAACAAAACGTTGTACCG 3′); 4D5-0.5 kb, forward (5′GCAACGTGTGCAACAAGAAG 3′) and reverse (5′ GTCTTCATGTGCGTATGCAG 3′); 4D5-3.1 kb, forward (5′ GGAAGCACTCTCTAATTCAC 3′) and reverse (5′ CGCCGA-CTGATGGAAGTTGG 3′). Triplicate PCR reactions were performed with 26, 27 and 28 extension cycles to ensure that the PCR amplification was in the linear range. A Student's *t*-test was performed to determine the statistical significance between samples.

Results

Generating reporters to study HP1 silencing in euchromatin

To investigate the effects of HP1 on both gene expression and chromatin structure, we modified our previously described HP1 tethering system (Li et al., 2003). The HP1 tethering system has two components. The first component is an expressor stock that expresses either a lacI-HP1 fusion protein or a GFP-*lacI* fusion protein (control) and the second component is a reporter stock that contains a transposon consisting of *lac* operator repeats cloned upstream of reporter genes. Modifications of the system included introduction of reporter genes that are easily monitored for inducible gene expression and changes in chromatin structure. Specifically, two heat shock reporter genes (Wallrath and Elgin, 1995) were cloned in tandem downstream of 256 copies of the *lac* operator repeats (Robinett et al., 1996) (Fig. 1A). Heat-shock genes were selected because they are well characterized and can be induced in all tissues throughout development. The first reporter gene consists of nucleotide positions –1917 to +490 (relative to transcription start at +1) of the *hsp26* gene fused to a partial cDNA of the barley *sip1* gene. The *sip1* fragment serves as a unique sequence tag (designated *hsp26-tag*). The second reporter gene consists of nucleotide position –259 to +195 of the *hsp70* heat-shock promoter driving expression of the *white* gene (designated *hsp70-white*). The *lac* repeats are 1.9 kb upstream of the *hsp26* transcription start site, and 3.7 kb upstream of the *hsp70* start site. This construct was designated the *lac-hsp26-hsp70* transposon. The *lac* repeat array appeared to have no effect on robust heat-shock induction of either reporter (Fig. 1B,C). Flies carrying an expressor transgene and a reporter transgene were raised with a daily heat shock treatment to drive expression of the fusion protein and study the effects of HP1 tethering on chromatin structure and gene expression.

In order to determine whether tethering HP1 alters expression and chromatin structure of the heat shock reporter genes, we needed to recover insertions of the *lac-hsp26-hsp70* transposon within euchromatic regions of the genome that were permissible for transcription. Five independent insertion stocks were recovered. The genomic insertion site of the transposon was determined using inverse PCR. In cases where inverse PCR indicated an insertion within repetitive DNA sequences making the chromosomal assignment difficult, the site of insertion was determined by in situ hybridization to polytene chromosomes. In total, insertions were identified at cytological position 4D5, 52, 54F1, 60F5 and 87C1 (Table 1). Two of these positions are within intronic regions. The hsp26-4D5 insertion is within the first intron of *CG32772*, placing the *lac* repeat array 11.1 kb from the *CG32772* predicted transcription start site and 9.1 kb from *CG4041*, an adjacent 3′ gene. The hsp26-54F1 insertion is within the first intron of *CG30111*, a gene that is nested within the second intron of the *grainy head* (*grh*) locus, placing the *lac* repeat array 0.7 kb from the *CG30111* predicted transcription start site. Three stocks contained insertions adjacent to repetitive sequences. The hsp26-52 insertion is adjacent to sequences similar to those of a *BS element,* a LINE-like transposable element. The hsp26-60F5 insertion is adjacent to a partial *Invader4* LTR-type transposon and is ~4 kb 3′ from the predicted transcription start site of *CG30428,* the most distal gene on chromosome 2R. No TAS elements characteristic of sub-telomeric regions are present within the 700 bp of sequences that are currently available in FlyBase distal to the insertion site, suggesting the transposon is not within telomeric chromatin. Finally, the hsp26-87C1 insertion is within an ~40 kb region of repeated $\alpha\beta$ elements located within the *hsp70* heat shock locus. These $\alpha\beta$ elements are transcribed along with the four endogenous heat shock genes at 87C1 during heat shock induction (Lis et al., 1981; Lis et al., 1978). In summary, the intronic insertions recovered

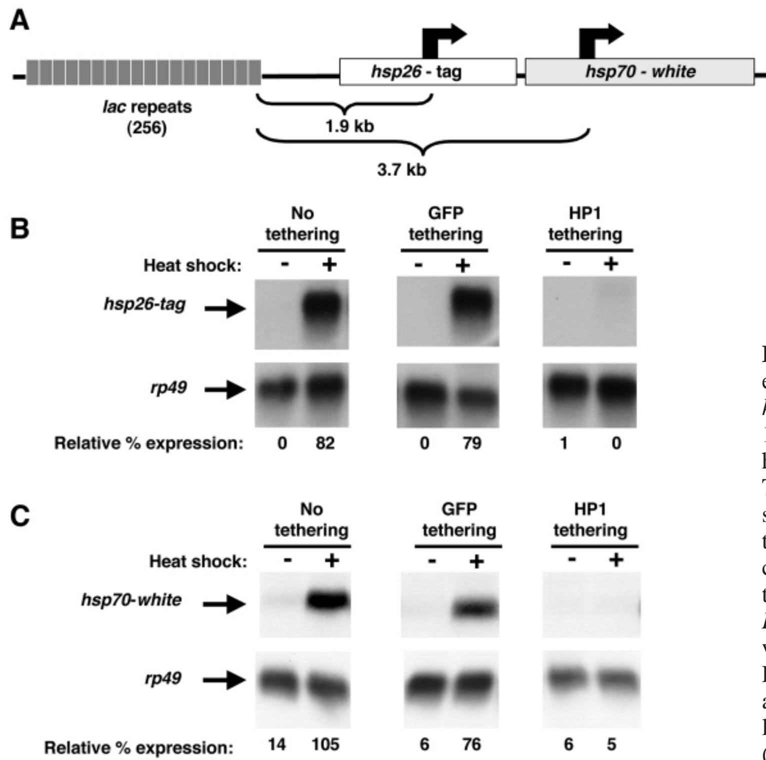


Fig. 1. lacI-HP1 association silences heat shock inducible expression of *hsp26* and *hsp70* reporter genes. (A) The *lac-hsp26-hsp70* reporter contains 256 *lac* operator sites positioned 1.9 and 3.7 kb upstream from the transcriptional start site of the heat shock inducible promoters *hsp26* and *hsp70*, respectively. The *hsp26* gene is fused to a barley cDNA fragment as a unique sequence tag and *hsp70* is fused to the *white* reporter gene as a transformation marker. (B) RNA was isolated from adult flies carrying either the *lac-hsp26-hsp70* reporter transposon alone, the *GFP-lacI* expressor and the reporter transposon, or the *lacI-HP1* expressor and the reporter transposon. The flies were raised with daily heat shock treatments or non-heat shock conditions. RNA was analyzed for *hsp26-tag* expression (upper autoradiograph) and *rp49* expression (lower autoradiograph) as a loading control. (C) Northern analysis of *hsp70-white* expression (upper autoradiograph) and *rp49* expression (lower radiograph).

at 4D5 and 54F1 are ideal to test the effects of HP1 tethering on gene expression because they are composed of unique copy sequences in gene rich regions. We were also intrigued by the insertion at 87C1 because this region exhibits robust transcriptional activity during heat shock induction, begging the question of whether HP1 could silence in a highly active region. Although insertions at positions 52 and 60F5 are adjacent to repetitive elements, they exhibit robust transcriptional activity under heat shock, therefore they were included in the study.

HP1 tethering silences adjacent reporter genes

To determine whether HP1 tethering is sufficient to silence heat-shock-induced gene expression, northern analysis was performed on the *hsp26* and *hsp70* reporter genes located 1.9 and 3.7 kb from the tethering site, respectively. RNA from adults carrying an expressor and reporter transgene were analyzed after daily heat shock treatments. The lacI-HP1 fusion protein was expressed 2.5- to 3.0-fold higher than endogenous levels of HP1, as determined by western analysis (data not shown). The control GFP-lacI fusion protein was expressed at similar levels to the *lacI*-HP1 protein (data not shown). Both fusion proteins localize to the *lac* repeats and *lacI*-HP1 also localizes to sites of endogenous HP1 (Li et al., 2003) (data not shown). Association of GFP-*lacI* to the *lac* repeats resulted in levels of *hsp26*-induced expression similar to that observed under non-tethering conditions (Fig. 1B). By contrast, when lacI-HP1 was associated with the *lac* repeats in stock hsp26-4D5, there was a decrease in *hsp26* expression (Fig. 1B, Table 1). Similar results were observed for all five *lac-hsp26-hsp70* insertion stocks (Table 1). Among these five stocks, the *hsp70-white* reporter positioned 3.7 kb from the *lac* repeat array also exhibited a decrease in expression during HP1 tethering compared with GFP tethering and non-tethering conditions (Fig. 1C; Table 1). Thus, HP1 tethering is sufficient to induce silencing at least 3.7 kb from the *lac* repeat array.

Table 1. The effects of HP1 tethering

Insertion stock	Cytological position	Nearest 5′ promoter*	Nearest 3′ promoter*	Fold repression		Fold decrease in accessibility	Regular nucleosome array
				hsp26	*hsp70*		
hsp26-4D5	4D5	11.1 kb	9.1 kb	25	7	11.5	Yes
hsp26-52	52	ND	ND†	18	11	ND	ND
hsp26-54F1	54F1	12.3 kb	<34 kb	35	10	4.0	ND
hsp26-60F5	60F5	4 kb	ND‡	16	5	ND	ND
hsp26-87C1	87C1	ND†	ND†	32	11	3.0	Yes

*Distances are calculated from the 5′ or 3′ end of the *lac* repeat array.
ND, not determined.
†The precise transgene insertion site was not determined because the integration site is adjacent to repetitive DNA sequences.
‡The transgene is 3′ of the most distal predicted gene on chromosome 2R.

However, silencing strength appears to decrease as the distance from the tethering site increases. For example, in stock hsp26-4D5 the *hsp26* reporter is repressed 25-fold, but the *hsp70* reporter is only repressed sevenfold; a trend observed in all five insertion stocks (Table 1).

To determine whether continual production of the lacI-HP1 fusion protein (produced from daily heat shock) was required for silencing, experiments were performed in which a single heat shock was given during embryogenesis and reporter genes expression was assayed during the third instar larval stage. Northern analysis revealed that 20% and 57% expression was observed for the *hsp26* and *hsp70* reporters, respectively, relative to expression in the absence of lacI-HP1, set at 100% (data not shown). These values are between the 5% expression observed with daily heat shock treatments and 100% expression in the absence of lacI-HP1. Multiple explanations could account for these results. First, partial silencing might be due to semi-stability of the silent chromatin state through mitosis. Second, partial silencing could be achieved from leaky expression of the *hsp70* promoter that produces lacI-HP1 during times of non-heat shock. Low levels of lacI-HP1 could maintain the silent chromatin established during the embryonic heat shock. Third, partial silencing might be due to expression of lacI-HP1 during the larval heat shock treatment required to assay for heat-shock-inducible expression of the reporter genes. Given these experimental caveats, it is unclear whether silencing induced by the lacI-HP1 tethering system is stable through mitosis.

HP1 tethering alters chromatin structure

Heterochromatin-mediated gene silencing correlates with changes in chromatin structure (Wallrath and Elgin, 1995). The chromatin structure of the *hsp26* promoter has been well characterized in both euchromatic and heterochromatic locations; the accessibility of the chromatin correlates with heat-shock inducibility (Lu et al., 1993; Wallrath and Elgin, 1995). In euchromatin, the *hsp26* promoter is potentiated for transcription under non-heat shock conditions. Two DNase I hypersensitive sites are located over the heat shock elements, RNA Pol II is paused downstream of transcription start, TFIID is bound to the TATA box, GAGA factor is bound to $GA_{(n)}$ elements and the coding region of the gene is packaged with irregularly spaced nucleosomes (Cryderman et al., 1998; Cryderman et al., 1999; Wallrath and Elgin, 1995). In a heterochromatic context, however, the DNase I hypersensitive sites, transcription factors and polymerase are not detectable at the promoter (Cryderman et al., 1999; Wallrath and Elgin, 1995). Furthermore, the coding region is packaged into a regular nucleosome array (Sun et al., 2001; Wallrath and Elgin, 1995).

To determine whether silencing by lacI-HP1 alters the chromatin structure of the *hsp26* reporter transgene, a restriction enzyme accessibility assay was performed. Nuclei were isolated from the hsp26-4D5 reporter stock under non-tethering, GFP tethering or HP1 tethering conditions and treated with an excess amount of *Xba*I. The *hsp26* promoter contains three *Xba*I restriction sites that reside within two DNase I hypersensitive regions (Fig. 2A). After *Xba*I digestion, genomic DNA was purified and digested with *Sal*I, which cleaves on either side of *hsp26* (Fig. 2A). The purified DNA was used for Southern analysis and hybridized with radiolabeled sequences corresponding to the unique *hsp26-tag*. The digestion products consisted of a 3.0 kb *Sal*I-*Sal*I fragment (generated by cleavage only at the *Sal*I sites), a 1.2 kb *Xba*I^{distal}-*Sal*I fragment (generated from cleavage at either or both *Xba*I sites and the *Sal*I site 3′ of the *hsp26* transgene), and a 0.8 kb *Xba*I^{proximal}-*Sal*I fragment (generated by cleavage at the proximal *Xba*I site and the *Sal*I site 3′ of the *hsp26* transgene) (Fig. 2A,B). The percent accessibility of the *hsp26* promoter was determined by calculating the percent of signal detected in the *Xba*I^{proximal}-*Sal*I fragment compared with the total signal produced by all three fragments. The proximal *Xba*I site in stock hsp26-4D5 was 45.9±6.7% (*n*=3) accessible during non-tethering conditions and 44.4±3.6% (*n*=3) accessible during GFP tethering. By contrast, the *Xba*I site was only 4.2±2.0% (*n*=3) accessible under HP1 tethering conditions (Fig. 2B). Similar accessibility results were demonstrated for stocks hsp26-87C1 and hsp26-54F5 (Table 1). Together, these data support the hypothesis that association of lacI-HP1 alters chromatin structure by forming a less accessible configuration.

One possible explanation for this closed chromatin structure is a change in the nucleosome arrangement. Micrococcal nuclease (MNase) digestions were performed to examine the nucleosome positioning over the *hsp26* transgene in stock hsp26-4D5. After heat shock induction, nuclei were isolated from homozygous third instar larvae raised under HP1 tethering and non-tethering conditions and treated with increasing amounts of MNase, which cleaves chromatin in the linker region between nucleosomes. DNA was purified and hybridized with radiolabeled sequences corresponding to the unique *hsp26-tag* by Southern analysis. The MNase digestion under the non-tethering condition or GFP tethering produced a smeared hybridization pattern, demonstrating irregular nucleosome positioning (Fig. 2C and data not shown). By contrast, the MNase digestion under HP1 tethering conditions produced a defined ladder of digestion products consistent with the presence of a regular nucleosomal array (Fig. 2C). To verify that comparable digestion was achieved between samples, membranes were washed and re-hybridized with single copy sequences from a centric location that produce a MNase digestion pattern characteristic of a regular nucleosome array (Sun et al., 2001). The result gave comparable patterns, indicating that the samples were digested to similar extents (Fig. 2C). Similar results showing regular nucleosome positioning upon HP1 tethering were observed over *hsp26* in stock hsp26-87C (data not shown). Thus, the differential digestion patterns observed between the HP1 tethering and non-tethering conditions is due to HP1 association at the *hsp26* reporter. Taken together, the nuclease sensitivity assays indicate that HP1 nucleates silent chromatin by facilitating the reorganization of nucleosomes into more regular arrays.

HP1 spreads bi-directionally and associates with silenced transgenes

Genes brought into juxtaposition with heterochromatin are thought to be silenced by the spread of heterochromatin-associated factors (Weiler and Wakimoto, 1995). To determine whether silencing of the *hsp26* and *hsp70* reporter genes by tethered HP1 correlated with the spread of HP1 from the tethering site, chromatin immunoprecipitation (ChIP) experiments were performed. A PCR primer set corresponding

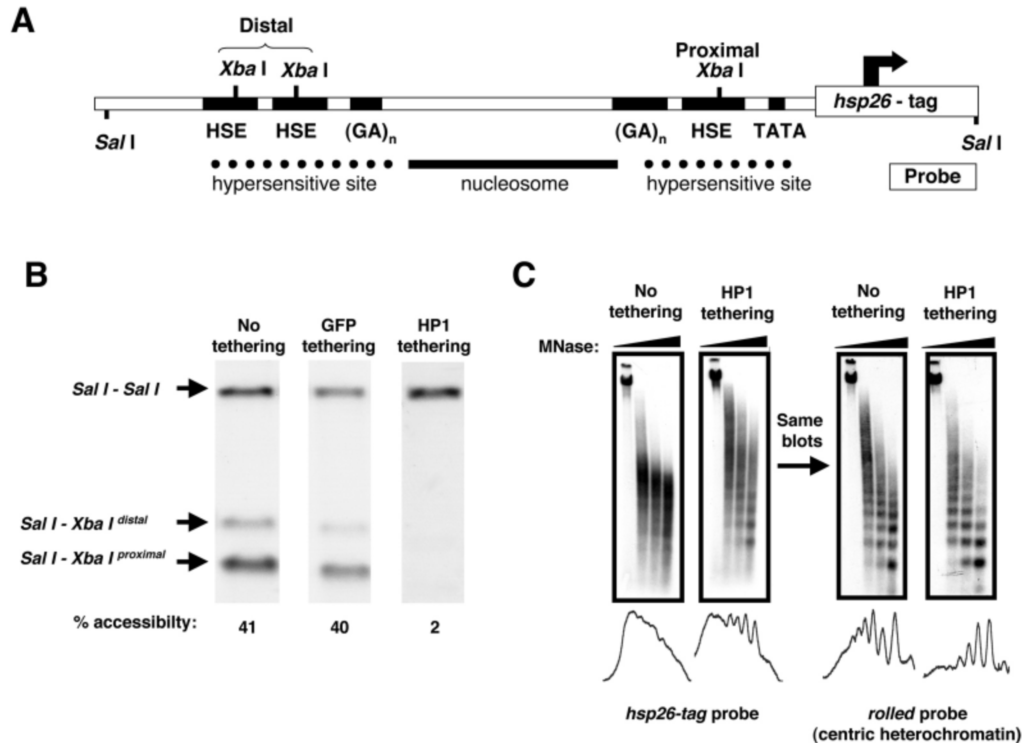


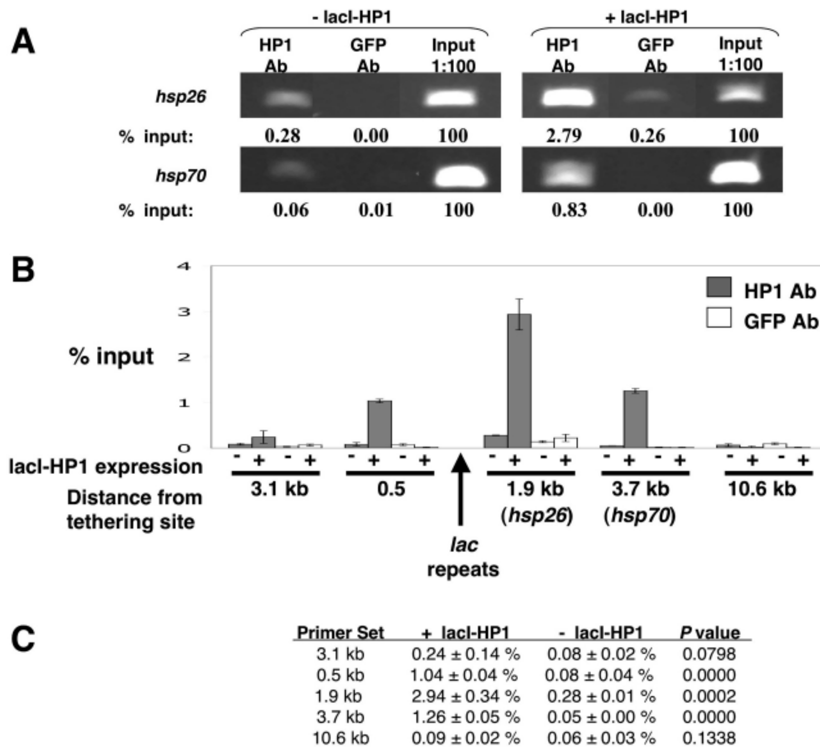
Fig. 2. lacI-HP1 association renders the *hsp26* promoter less accessible. (A) The *hsp26* reporter gene (not to scale) contains a TATA box, HSEs and $(GA)_n$ elements within two DNaseI hypersensitive regions. *Xba*I sites within the HSEs were used for restriction enzyme accessibility. (B) Accessibility of the *hsp26* promoter region was determined under non-tethering, GFP-tethering and HP1-tethering conditions. Nuclei were isolated and treated with an excess of *Xba*I. The DNA was purified, digested to completion with *Sal*I, and analyzed by Southern analysis using the unique *hsp26*-tag sequences for hybridization. The percent accessibility is shown below each lane. (C) MNase accessibility of the *hsp26* promoter was determined in homozygous larvae containing either the *lac-hsp26-hsp70* reporter and the lacI-HP1 expressor transgene, or the reporter gene alone. Nuclei isolated from homozygous third instar larvae were treated with increasing amounts of MNase. The DNA was purified and assayed by Southern analysis using the unique *hsp26* tag sequences for hybridization. The left pair of membranes was re-hybridized with heterochromatic sequences upstream of the *rolled* locus (shown on right). A densitometry trace through the fourth lane (top to bottom) of each membrane is plotted below.

to unique barley sequences within the *hsp26*-tag, positioned 1.9 kb in the 3′ direction from the *lac* repeats, was used to amplify immunoprecipitated material from stock hsp26-4D5. Two negative controls were used for these experiments. First, anti-GFP antibodies were used during the immunoprecipitation of material isolated from stocks expressing the lacI-HP1 fusion protein. This antibody does not recognize endogenous proteins and serves as a non-specific antibody control (NS). As a second control, HP1 antibodies were used during immunoprecipitation of material isolated from stocks that did not express the *lacI*-HP1 fusion protein, i.e. non-tethering (NT). At the *hsp26*-tag sequences, 0.22±0.08% and 0.28±0.01% of input material was immunoprecipitated for the NS and NT controls, respectively (Fig. 3). By contrast, 2.94±0.34% of the input material was immunoprecipitated with HP1 antibodies when lacI-HP1 was associated with the *lac* repeats (Fig. 3). These results indicate that HP1 associates with the silenced *hsp26* promoter region.

To determine whether HP1 was associated with the *hsp70* reporter, a primer set corresponding to sequences at the junction between the *hsp70* promoter and the *white* transgene, 3.7 kb in the 3′ direction from the *lac* repeats, was used to amplify immunoprecipitated material from stock hsp26-4D5. The results showed that 0.01±0.01% and 0.05±0.00% of the input material was immunoprecipitated for the NS and NT negative controls, respectively. By contrast, 1.26±0.05% of input material was immunoprecipitated with HP1 antibodies when lacI-HP1 was expressed (Fig. 3). Thus, HP1 was found in association with both the silenced *hsp26* and *hsp70* promoter regions and appeared to spread at least 3.7 kb from the tethering site. Consistent with this data, HP1 was found in association with both reporter genes inserted at 87C1 (data not shown).

To demarcate the limits of HP1 spreading, additional primer sets were designed to amplify endogenous sequences 5′ and 3′ of the *lac* repeats in stock hsp26-4D5. At sequences 10.6 kb in the 3′ direction, 0.01±0.01% and 0.06±0.03% of the input material was immunoprecipitated for the NS and NT controls, respectively. Using HP1 antibodies, 0.09±0.02% of input material was immunoprecipitated when lacI-HP1 was associated with the repeats (Fig. 3B,C). These values are statistically similar to the negative controls. Thus, HP1 associates with sequences at least 3.7 kb, but not 10.6 kb, from the *lac* repeats in the 3′ direction.

To determine the extent of HP1 association in the 5′ direction, two primer sets were designed to sequences 0.5 kb and 3.1 kb from the *lac* repeats. At 0.5 kb, 0.01±0.01% and 0.08±0.04% of the input material was immunoprecipitated for the NS and NT controls, respectively. By contrast, 1.04±0.04%



Primer Set	+ lacI-HP1	- lacI-HP1	*P* value
3.1 kb	0.24 ± 0.14 %	0.08 ± 0.02 %	0.0798
0.5 kb	1.04 ± 0.04 %	0.08 ± 0.04 %	0.0000
1.9 kb	2.94 ± 0.34 %	0.28 ± 0.01 %	0.0002
3.7 kb	1.26 ± 0.05 %	0.05 ± 0.00 %	0.0000
10.6 kb	0.09 ± 0.02 %	0.06 ± 0.03 %	0.1338

Fig. 3. HP1 associates with the silenced *hsp26* and *hsp70* reporter genes. Chromatin immunoprecipitation experiments were performed to determine whether the *lac-hsp26-hsp70* transposon in stocks carrying the lacI-HP1 expressor and the transposon (+ lacI-HP1) or the reporter transposon alone (– lacI-HP1) was associated with HP1. Polyclonal HP1 antibodies were used and polyclonal GFP antibodies served as a negative control. (A) Primer sets corresponding to unique sequences within the reporter genes were used to PCR amplify the immunoprecipitated material. The amount of immunoprecipitated material (designated % input) was quantitated by dividing its signal intensity by the signal intensity generated from a 1:100 dilution of the input material. (B) The limits of HP1 spreading were determined using primer sets corresponding to sequences over the reporter transposon in stock hsp26-4D5. Primers corresponding to sequences located 1.9 (*hsp26*), 3.7 (*hsp70*) and 10.1 kb from the 3′ end of the *lac* repeat array and 0.5 and 3.1 kb from the 5′ end of the *lac* repeat array were assayed for HP1 association. (C) The amount of HP1 associated material (% input) determined from immunoprecipitation with anti-HP1 antibodies, with or without lacI-HP1 expression, was compared at each primer site (n=3). A probability (P value) of less than 0.05 was considered to be a significant change.

of the input material was immunoprecipitated with HP1 antibodies when lacI-HP1 was associated with the *lac* repeats indicating HP1 association at 0.5 kb in the 5′ direction. When examining sequences at 3.1 kb, 0.07±0.02% and 0.08±0.02% of the input material were immunoprecipitated for the NS and NT controls, respectively. When HP1 was associated with the *lac* repeats 0.24±0.14% of input material immunoprecipitated with HP1 antibodies under tethering conditions. This value is statistically similar to that obtained for the NS and NT controls. Thus, HP1 associates at 0.5 kb, but not at 3.1 kb in the 5′ direction. Collectively, the ChIP results demonstrate that HP1 associates with sequences in both the 3′ and 5′ direction from the *lac* repeats, suggesting bi-directional spreading (Fig. 3B,C). Furthermore, the amount of HP1 association decreases as distance from the *lac* repeats increases (Fig. 3B,C).

Effects of SU(VAR)3-9 on silencing

One model to explain the heterochromatin spreading relies on an interaction between HP1 and SU(VAR)3-9 (Bannister et al., 2001; Lachner et al., 2001). As HP1 interacts directly with SU(VAR)3-9, HP1 has been proposed to recruit SU(VAR)3-9, thereby causing methylation of adjacent nucleosomes and subsequent binding of HP1 (Bannister et al., 2001; Lachner et al., 2001; Schotta et al., 2002). This process allows HP1 to bind the newly modified nucleosomes and spread linearly along the chromosome. According to this model, lacI-HP1 induced silencing is predicted to be dependent upon SU(VAR)3-9 activity. To test for this, the expression of the *hsp26* reporter transgene was analyzed in flies homozygous for *Su(var)3-9*^{06} (a null allele) (Schotta et al., 2002). The heat-shock-induced expression of the *hsp26* reporter (positioned 1.9 kb from the *lac* repeats) increased from 2% in the wild-type background to 17% in the *Su(var)3-9*^{06} mutant background when lacI-HP1 was associated with the *lac* repeats (Fig. 4A,C). This indicates that substantial silencing persists even in the absence of SU(VAR)3-9. By contrast, heat-shock-induced expression of the *hsp70* transgene (positioned 3.7 kb from the *lac* repeats) under HP1 tethering conditions increased from 11.5% to 100% in the *Su(var)3-9*^{06} mutant background, equalling expression levels of the non-tethering conditions (Fig. 4). These data suggest that the mechanism of HP1-mediated silencing at the *hsp26* promoter positioned 1.9 kb from the *lac* repeats is largely independent of SU(VAR)3-9, whereas the mechanism of HP1-mediated silencing at the *hsp70* promoter positioned 3.7 kb from the *lac* repeats is completely dependent on SU(VAR)3-9.

Discussion

HP1 is sufficient to generate silent chromatin in transcriptionally permissive regions of the genome

The molecular basis of silent chromatin spreading is not well understood, but is hypothesized to involve the propagation of heterochromatic factors from initiation sites (Tartof et al., 1984). This process has been difficult to study because initiation sites are not well defined and spreading occurs over long stretches of repetitive DNA sequences. Previous studies using mammalian cell culture have used HP1 tethering systems on plasmids and episomes; however, these systems are unlikely to recapitulate a native chromosomal environment (Lehming et al., 1998; Seeler et al., 1998; van der Vlag et al., 2000). To overcome this issue, we developed an HP1 tethering system that targets HP1 to large (10 kb) regions within euchromatin (Li et al., 2003). The lacI-HP1 tethering system uses single copy reporter insertions at multiple euchromatic sites, allowing for in vivo HP1 association in a native chromosomal context.

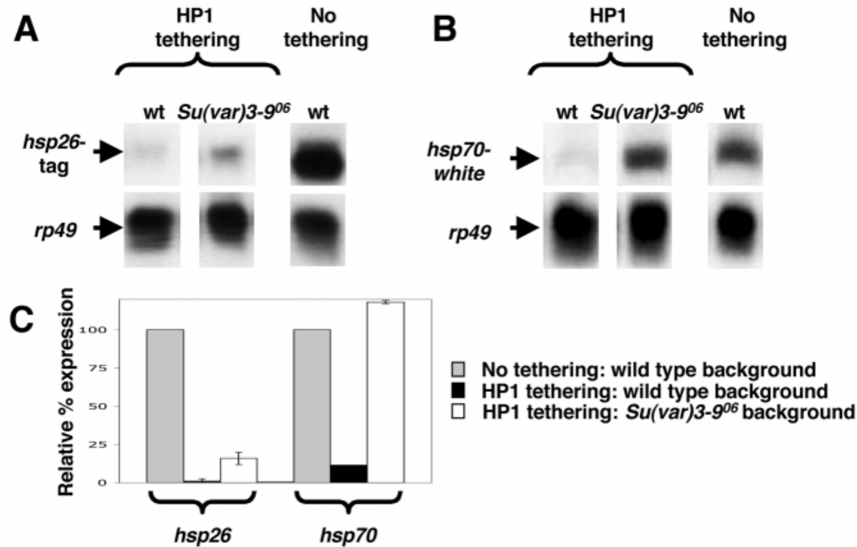


Fig. 4. Effects of *Su(var)3-9^{06}* on silencing. The expression levels of the *hsp26* and *hsp70* reporters in adult flies carrying the *lac-hsp26-hsp70* transposon and the lacI-HP1 expressor were compared to adults carrying the *lac-hsp26-hsp70* transposon, the lacI-HP1 expressor, and *Su(var)3-9^{06}*. (A) Northern analysis of RNA isolated from adults showing *hsp26* expression (upper autoradiograph) and *rp49* expression (lower autoradiograph). (B) Northern analysis of RNA isolated from adults showing *hsp70* expression (upper autoradiograph) and *rp49* expression (lower autoradiograph). (C) Summary of the expression data (*n*=3).

Upon daily production of the lacI-HP1 fusion protein, silencing of the reporter genes is observed at ectopic locations, even within regions of robust transcriptional activity. By contrast, a single pulse of lacI-HP1 in the embryo results in at best, partial silencing at the larval stage. This lack of mitotic stability is reminiscent of results obtained using tethered Polycomb (Pc), a protein required for the stable silencing of homeotic loci (Müller, 1995). Faithfully inherited silencing was observed with a single pulse of Gal4-Pc only when the transgene included a PRE (Polycomb Response Element), thought to stabilize the silencing complex. To date, HP1-mediated gene silencing has been shown to be relatively independent of DNA sequences; therefore, the continued presence of HP1 appears to be required for heritability of the silenced state.

Upon association of HP1 at these ectopic locations, we observe changes in gene expression and chromatin structure at least 3.7 kb from the *lac* repeat array. Sequences adjacent to the tethering site are relatively inaccessible to nuclease digestion and packaged into regular nucleosome arrays, mimicking a heterochromatic state. Such chromatin features are similar to those that form over euchromatic genes when placed into juxtaposition with heterochromatin. HP1 might cause chromatin reorganization through the recruitment of chromatin remodeling factors. An interaction between HP1 and chromatin remodeling machines has been documented in mammalian systems (Nielsen et al., 2002). Chromatin reorganization might also occur through the spread of HP1 along the chromosome. Our data clearly demonstrate HP1 association within the promoter regions of silenced reporter genes up to 3.7 kb from the tethering site.

In contrast to the silencing over several kb shown here, an HP1 tethering system using a stably integrated reporter gene in mammalian cell culture demonstrated only short range effects over a few hundred base pairs (Ayyanathan et al., 2003). In this case, HP1$^{Hs\alpha}$ was recruited to a reporter transgene through an interaction with tethered KRAB/KAP1 interaction partners. Silencing and a less accessible chromatin structure were apparent at 0.28 kb from the tethering site, but not at 2.78 kb. One possible explanation for the difference between these two tethering studies might be that human HP1$^{Hs\alpha}$ and *Drosophila* HP1 have distinctly different silencing mechanisms. We think this is unlikely as human HP1$^{Hs\alpha}$ localizes appropriately and rescues the lethality of *Su(var)2-5* mutants when expressed in *Drosophila* (Ma et al., 2001) (Norwood et al., 2004). A second possibility is that lacI-HP1 overexpression enhances spreading, whereas the KRAB/KAP1 system operates under endogenous levels of HP1. A third explanation to account for the different results might be the manner in which the HP1 proteins are recruited to the reporter gene. Using the lacI-HP1 tethering system, recruitment occurs through a heterologous DNA-binding domain fused to the N terminus of HP1, thus leaving the CSD available for homodimerization and/or interaction with other partners. In the KRAB/KAP1 tethering system, recruitment occurs through an interaction between the HP1$^{Hs\alpha}$ CSD and the transcriptional co-repressor KAP1, which may limit its availability for interactions with partners that are required for long distance spreading.

A model for HP1 in silent chromatin spreading

Transcriptional repressors can regulate gene expression over both short and long distances. Short-range repressors such as Giant and Krüppel operate at distances of less than 100 bp (Arnosti, 2003; Nibu and Levine, 2001). These repressors frequently bind to sites within the promoter region and recruit histone deacetylases that locally deacetylate histone tails (Shi et al., 2003; Subramanian and Chinnadurai, 2003). By contrast, long-range silencing is hypothesized to involve the spread of silencing factors along the chromatin fiber, deacetylation of histone tails and generation of the MeH9K3 modification throughout the region (Litt et al., 2001; Noma et al., 2001). In experiments described here, silencing was observed 3.7 kb from the HP1 tethering site, implying that HP1 acts as a long-range silencer. Evidence of HP1 spreading is demonstrated by chromatin immunoprecipitation experiments that place HP1 near the promoter region of the silenced reporter genes. As the distance from the tethering site increases, the amount of HP1 association decreases, supporting a linear spreading model (Fig. 3B; Table 1). However, these data do not exclude the

possibility that HP1 association and silencing occur through a looping mechanism that is mediated by the 'stickiness' of silencing proteins (Li et al., 2003; Seum et al., 2001; Talbert and Henikoff, 2000).

One proposed linear spreading model involves the association of HP1, subsequent recruitment of SU(VAR)3-9, and methylation of adjacent histones, forming new HP1-binding sites (Bannister et al., 2001; Lachner et al., 2001). We tested this model by examining the effects of HP1 tethering in a *Su(var)3-9* mutant background. In the absence of SU(VAR)3-9, HP1 induced silencing of the *hsp26* reporter persisted at 1.9 kb from the tethering site. Consistent with this finding, *Su(var)3-9*^{06} also had virtually no effect on silencing of a mini-*white* transgene positioned 0.5 kb from the HP1 tethering site (Li et al., 2003). Taken together, these data suggest that silencing up to 1.9 kb is not heavily dependent upon SU(VAR)3-9 activity. We speculate that HP1 might self-propagate for a limited distance along the chromosome, perhaps by multimerization through the CSD (Cowieson et al., 2000; Yamada et al., 1999) or by MeK9H3-independent interactions with histones (Meehan et al., 2003; Smothers and Henikoff, 2001; Zhao et al., 2000). The introduction of HP1 mutants that abolish homodimerization into the tethering system will shed light on this issue.

In contrast to the persistence of silencing at 1.9 kb in the *Su(var)3-9* mutant, a substantial loss of silencing was observed at 3.7 kb. Heat shock-induced expression of *hsp70* during HP1 tethering in a *Su(var)3-9*^{06} mutant background was equal to expression levels observed in the non-tethering and GFP-tethering conditions. Several explanations could account for the different SU(VAR)3-9 requirements observed for silencing the *hsp26* and *hsp70* reporters. First, the *hsp70* transgene promoter might be stronger than the *hsp26* transgene promoter. We think this is unlikely as the *hsp26* transgene appears to show greater fold induction than *hsp70* at all five of the genomic insertion sites tested here under non-tethering conditions (Fig. 1B,C; data not shown). Second, the two heat shock genes could have different mechanisms of transcriptional activation. This idea is inconsistent with years of research demonstrating that the regulatory elements and trans-activators for these two genes are nearly identical (Amin et al., 1994; Glaser et al., 1990; Leibovitch et al., 2002; Lu et al., 1993; Mason and Lis, 1997; O'Brien and Lis, 1991; Thomas and Elgin, 1988). Alternatively, the differences observed might be due to multiple mechanisms of HP1-mediated silencing. Silencing at long distances (between 1.9 and 3.7 kb) may require SU(VAR)3-9, as current models for HP1 spreading would predict (Bannister et al., 2001; Lachner et al., 2001). By contrast, silencing at short distances (less than 1.9 kb) is relatively independent of SU(VAR)3-9, and would suggest alternate mechanisms of HP1 spreading that might involve self-propagation (Yamada et al., 1999). We favor this model as several recent reports demonstrate that HP1 can be found independently of SU(VAR)3-9 and MeK9H3 on chromosomes (Cowell et al., 2002; Greil et al., 2003; Li et al., 2003). In particular, others have demonstrated that several genes silenced in *Drosophila* Kc cells were associated with HP1, but not SU(VAR)3-9 (Greil et al., 2003). Thus, our understanding of the role of HP1 in gene regulation will depend upon knowledge about the method of localization and the interaction partners at a given genomic site.

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