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HP1 Is Distributed Within Distinct Chromatin Domains at Drosophila Telomeres

Radmila Capkova Frydrychova,* James M. Mason* and Trevor K. Archer[†,1]

*Laboratory of Molecular Genetics and †Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences/National Institutes of Health/Department of Health and Human Services, Research Triangle Park, North Carolina 27709

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ABSTRACT

Telomeric regions in Drosophila are composed of three subdomains. A chromosome cap distinguishes the chromosome end from a DNA double-strand break; an array of retrotransposons, *HeT-A*, *TART*, and *TAHRE* (HTT), maintains telomere length by targeted transposition to chromosome ends; and telomere-associated sequence (TAS), which consists of a mosaic of complex repeated sequences, has been identified as a source of gene silencing. Heterochromatin protein 1 (HP1) and HP1-ORC-associated protein (HOAP) are major protein components of the telomere cap in Drosophila and are required for telomere stability. Besides the chromosome cap, HP1 is also localized along the HTT array and in TAS. Mutants for *Su(var)205*, the gene encoding HP1, have decreased the HP1 level in the HTT array and increased transcription of individual *HeT-A* elements. This suggests that HP1 levels directly affect *HeT-A* activity along the HTT array, although they have little or no effect on transcription of a *white* reporter gene in the HTT. Chromatin immunoprecipitation to identify other heterochromatic proteins indicates that TAS and the HTT array may be distinct from either heterochromatin or euchromatin.

TELOMERES are nucleoprotein structures at the ends of eukaryotic chromosomes with important roles in chromosome replication, stability, segregation, and position within the nucleus (Hochstrasser *et al.* 1986; Blackburn 1991; Hari *et al.* 2001; Chan and Blackburn 2002; Biessmann and Mason 2003; Abad *et al.* 2004). In most eukaryotes, chromosomes terminate in an array of simple repeats that is synthesized by telomerase (Blackburn 1991). The terminal arrays at Drosophila telomeres, however, are composites of three telomere-specific nonlong terminal repeat (non-LTR) retrotransposons, *HeT-A*, *TAHRE*, and *TART* (Mason and Biessmann 1995; Mason *et al.* 2008), whose stochastic transposition creates an array (HTT) that differs in length at different chromosomal ends in a range of 147–26 kb in one stock (Abad *et al.* 2004). Telomeric retrotransposons maintain chromosome length by targeted transposition to chromosome tips and by terminal recombination/gene conversion (Kahn *et al.* 2000; Biessmann and Mason 2003). The attachment of the elements by their 3′ oligo (A) tails to the chromosome end probably occurs via target-primed reverse transcription (Luan *et al.* 1993) and does not depend on the DNA sequence at the terminus (Biessmann *et al.* 1992; Biessmann and Mason 2003). The *HeT-A* element is the most abundant telomeric retroelement; it has a promoter located at its 3′ end that directs transcription of a downstream sequence (Danilevskaya *et al.* 1997; Capkova Frydrychova *et al.* 2007).

The terminal part of the HTT array is covered by protein complex, termed the chromosome cap, that protects chromosome ends from telomeric fusions. The telomere capping complex is formed by a special interaction of heterochromatin protein 1(HP1) with the HP1/ORC-associated protein (HOAP) (Cenci *et al.* 2005). The formation of the cap is mediated by a sequence-independent mechanism regardless of the presence of telomeric retroelements (Biessmann and Mason 1988; Biessmann *et al.* 1990). Analysis of chromosome ends broken within the *yellow* upstream region suggested that there is a special chromatin structure that interferes with enhancer function when the chromosome end is within ∼4 kb of the enhancer (Mikhailovsky *et al.* 1999; Melnikova *et al.* 2004), suggesting that the chromosome cap may extend up to this distance from the chromosome end.

To date, mutations in several genes have been implicated in the control of telomere elongation: the HP1-encoding gene *Su(var)205*, *Tel*, *E(tc)*, *spn-E*, *aub*, and the Drosophila orthologs of *Ku70* and *Ku80* (Melnikova and Georgiev 2002; Savitsky *et al.* 2002, 2006; Cenci *et al.* 2005; Melnikova *et al.* 2005). Although all these genes act as negative regulators of telomere length, so far only mutations in *Su(var)205*, *spn-E*, and *aub* have been shown to increase retroelement transcripts and transposition of the retroelements to chromosome ends (Savitsky *et al.* 2002, 2006). *Su(var)205*, *Tel*, and *E(tc)* regulate telomere length by controlling terminal gene

[1]*Corresponding author:* Laboratory of Molecular Carcinogenesis, NIEHS/NIH/DHHS, 111 T. W. Alexander Dr., Research Triangle Park, NC 27709. E-mail: archer1@niehs.nih.gov

conversion (Melnikova and Georgiev 2002; Savitsky *et al.* 2002; L. Melnikova and P. Georgiev, personal communication).

The terminal retrotransposon arrays are adjacent to the subterminal *t*elomere-*a*ssociated *s*equence (TAS), which in turn borders euchromatic transcribed genes (Karpen and Spradling 1992; Abad *et al.* 2004). The TAS region covers ~20 kb and consists of several kilobases of complex satellite sequences, which, in spite of some sequence similarities, vary among telomeres (Mason *et al.* 2008). Drosophila telomeres have been considered heterochromatic, as they contain repetitive DNA sequences and have the ability to repress gene activity (Gehring *et al.* 1984; Karpen and Spradling 1992; Wallrath and Elgin 1995; Cryderman *et al.* 1999; Zhimulev and Belyaeva 2003). However, recent detailed genetic analysis of *white* (*w*) transgenes inserted into distal and proximal sites within a telomere region identified TAS as the primary source of telomeric silencing (Mason *et al.* 2003; Biessmann *et al.* 2005a,b). TAS-induced silencing is unidirectional (Kurenova *et al.* 1998) toward the chromosome end and shows decreasing effect with increasing distance. Transgenes in TAS or the HTT array close to TAS displayed repressed and variegated expression, whereas expression of transgenes inserted into HTT >10 kb from TAS was comparable to that of control euchromatic insertions (Biessmann *et al.* 2005a,b). As gene silencing is considered to be a feature of closed chromatin and telomeric retroelements seem to lack silencing potential, TAS and the HTT array may be two distinct chromatin domains resembling closed chromatin and open chromatin, respectively (Biessmann *et al.* 2005a,b; Mason *et al.* 2008). These genetic results agree with immunostaining data that indicate distinct protein components in the chromosome cap, and the HTT and TAS arrays of Drosophila polytene chromosomes (Andreyeva *et al.* 2005), with proteins associated with interband regions found at HTT and Polycomb group proteins found at TAS.

HP1 is a chromosomal protein that is predominantly associated with heterochromatin. It has been shown that HP1 is a component of the telomere capping complex and is required for telomere elongation and transcriptional repression of telomeric retrotransposons (Fanti *et al.* 1998; Savitsky *et al.* 2002; Cenci *et al.* 2003, 2005; Perrini *et al.* 2004). On the basis of several studies it has been proposed that heterochromatin formation and epigenetic gene silencing is a result of a multistep process including replacement of histone H2A with the histone variant H2A.v, deacetylation, and subsequent methylation of Lys9 on histone H3, and binding of HP1 (Nakayama *et al.* 2001; Volpe *et al.* 2002; Schotta *et al.* 2003, 2004; Verdel *et al.* 2004; Swaminathan *et al.* 2005).

Using ChIP analysis, we show the presence of HP1 at the promoter of *w* transgenes inserted into the HTT array and TAS. We mapped the effect of HP1 mutations on the transcriptional activity of individual *HeT-A* elements located along the HTT array. Transcription at three specific sites in the HTT array was measured by quantification of readthrough transcripts that were transcribed from a *HeT-A* element into the adjacent *P*-element insertion. In HP1 mutants we observed elevated levels of the readthrough transcripts. These data suggest that the presence of HP1at telomeres and HP1 regulation of *HeT-A* transcription are not restricted to a specific region, such as the chromosome cap, but rather extend along the whole length of the HTT array. A mutation in *caravaggio* (*cav*), the gene encoding HOAP, however, does not affect transcriptional activity of *HeT-A* elements located along the HTT array, suggesting that the cap itself has no role in the regulation of telomere elongation via retroelement transcription.

MATERIALS AND METHODS

Drosophila stocks: Drosophila stocks were raised and crosses performed at 25° on cornmeal-molasses medium with dry yeast added to the surface. Stocks were obtained from a *P*-element mobilization screen by the Berkeley Drosophila Genome Project described previously (Bellen *et al.* 2004; Biessmann *et al.* 2005a; Capkova Frydrychova *et al.* 2007) and from the Bloomington Stock Center. All original stocks were converted into similar *y* w^{67c23} genetic backgrounds by crossing with *y* w^{67c23}; *Sco/SM1*; *Sb/TM6* and then with control *y* w^{67c23}; *Sco/SM1* or *y* w^{67c23}; *Sb/TM6* before establishing new stocks. *P{*w^+*}EY00453* (hereafter *EY00453*) carries the *Epgy2* element at the 3′ end of a *TART* element in the telomere at the left end of chromosome *3* (*3*L), 656 bp from its (A) tail and >20 kb from TAS. *P{*w^+*}EY03383* (hereafter *EY03383*) has an *Epgy2* inserted into *2*R TAS (Biessmann *et al.* 2005a). As controls, *P{*w^+*}EY00630* and *P{*w^+*}EY06734* (hereafter *EY00630* and *EY06734*) carry an *Epgy2* element in euchromatin at 59D8 or in *2*R pericentric heterochromatin, respectively. *P{*w^{var}*}11-5* (hereafter *11-5*) has a copy of the genomic *w* gene inserted between the HTT and TAS arrays on *2*L (Golubovsky *et al.* 2001; Capkova Frydrychova *et al.* 2007). *P{*w^+*}KG01591* (hereafter *KG01591*) carries a *SuPor-P* element inserted into a *HeT-A* element 5 kb from *3*R TAS.

Micrococcal nuclease digestion: Nuclei were isolated from third instar larvae and treated with 0.1, 0.2, and 0.3 units of MNase as previously described (Cryderman *et al.* 1998). The DNA was purified, separated on a 1.5% agarose/TAE gel, transferred to a nylon membrane, and hybridized to a DNA probe labeled by PCR with [^{32}P]dCTP.

Chromatin immunoprecipitation (ChIP): Drosophila HP1 polyclonal antibody was purchased from Covance (cat. no. PRB-291C), the other antibodies from Abcam: rabbit polyclonal antibody to histone H2A.Z (cat. no. ab4174), rabbit polyclonal antibody to histone H2A (cat. no. ab13923), rabbit polyclonal antibody to tri-methyl K9 of histone H3 (cat. no. ab8898), rabbit polyclonal antibody to di-methyl K9 of histone H3 (cat. no. ab7312). Specificity of the antibodies was checked by Western blot. For the ChIP assay we used nuclei isolated from 100 mg of third instar larvae and followed a protocol described by Upstate Biotechnology. Crosslinking reactions were performed by 1% formaldehyde, nuclei were lysed, the DNA was fragmented by sonication, and 50 μl of the chromatin solution was saved as input. Five microliters of each antibody were added to tubes containing 1000 μl of

chromatin solution. Following incubation, the antibody complexes were captured using protein A-agarose beads. The beads were pelleted and washed. The chromatin was extracted and reverse crosslinked, and the DNA was purified using phenol-chloroform. Samples were analyzed using real-time PCR. Threshold cycle (Ct) was used for assessing the relative level of each amplification product *vs.* the amplification product of 5% of input DNA.

RNA isolation and cDNA synthesis: RNA samples were made using RNasy mini kit (QIAGEN) according to the manufacturer's instructions and reverse transcribed using oligo dT and the SuperScript first-strand synthesis system for RT–PCR (Invitrogen).

Real-time PCR: Quantitation was performed in two independent experiments of three samples for each strain. Relative levels of transcripts were compared by real-time PCR using an Mx3000P real-time PCR system. The reactions were prepared using SYBR Green PCR master mix (Stratagene) and Ct was used to assess relative levels of target transcripts *vs.* reference *RpL32* transcripts. Normalization of *HeT-A* transcript levels was done by calculating mean transcript levels and dividing by mean *HeT-A* copy number. A reverse transcriptase-minus control was included for each sample; in all cases the control gave undetectable Ct value. Primer sequences are given in supplemental Table S1.

RESULTS

HP1 has been shown to play a role in the control of telomere length via regulation of gene conversion and transcription of telomeric retroelements. HP1 has repressive effect on the telomeric retroelements and its mutations lead to dramatic increase in transcriptional activity of the elements (Savitsky *et al.* 2002; Perrini *et al.* 2004).

Despite the role of HP1 in transcriptional regulation of telomeric retroelements, immunostaining of polytene chromosomes of the *Tel* mutant in previous studies surprisingly failed to reveal localization of HP1 along the HTT array and showed HP1 localized only at chromosome cap, *i.e.*, in a region at the extreme chromosome ends (Siriaco *et al.* 2002; Andreyeva *et al.* 2005). This led us to three alternative hypotheses. First, localization of HP1 specifically to the chromosome cap may indicate that only the retroelements under telomere cap are affected by HP1 and that these retroelements make the major contribution to the increase in overall retroelement transcription and telomere elongation seen in HP1 mutants. Second, the cap is a structure with extensive repressive effect on transcriptional activity of retroelements located along the HTT array both inside and by some unknown mechanism outside of the telomere cap. Finally, we could not exclude the possibility that immunostaining of polytene chromosomes might not reflect the general telomeric localization of HP1, perhaps because of the character of polytene chromosomes or the unusual features of exceedingly long HTT arrays in the *Tel* mutant or because of low sensitivity of immunostaining. Thus HP1 might be present along the HTT array outside chromosome cap. This led us to retest for the presence of HP1 at telomeric

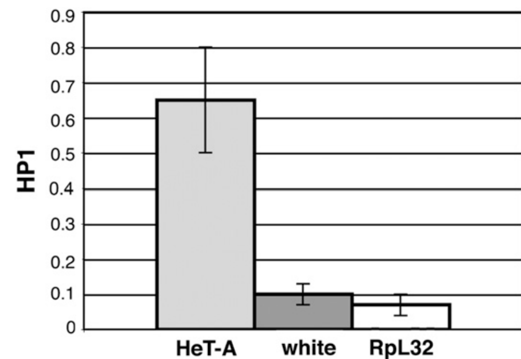

Figure 1.—Chromatin immunoprecipitation (ChIP) analysis of HP1 at the 5′ end of *HeT-A*, the *white* promoter, and coding sequence of *RpL32* in Oregon R. Quantitation of HP1 was performed using real-time PCR. ChIP samples were normalized to 5% of input DNA. Error bars represent standard deviations.

retroelements located in the HTT array outside of the chromosome cap using a ChIP assay performed on nuclei isolated from whole third instar larvae.

Presence of HP1 at Drosophila telomeres: We first measured the average level of HP1 located at the 5′ end of the *HeT-A* elements in larvae of wild-type Oregon R with quantification of coprecipitating DNA by real-time PCR. As controls we used primers to the promoter of the *w* gene residing in its nontelomeric, euchromatic position on the *X* chromosome and primers to the coding sequence of the ribosomal protein gene *RpL32*. We found a 7-fold enrichment of HP1 at *HeT-A* compared to *w* and a 12-fold enrichment compared to *RpL32* (Figure 1).

HP1 binding in the HTT array: To distinguish between HP1 associated with retroelements in the chromosome cap and retroelements located outside of the cap, we could not probe any DNA sequence that is common in telomeric retroelements and we needed to test some unique sequence in telomeres. Assuming that HP1 can spread into adjacent transgenes (Danzer and Wallrath 2004) we looked for HP1 at *P* elements inserted into specific telomeric regions outside of the telomere cap (Bellen *et al.* 2004; Biessmann *et al.* 2005a,b). First, we compared HP1 at the *w* promoter of an insertion line *11-5* (Figures 2 and 3A), which has a copy of the genomic *w* gene inserted precisely between the HTT array and TAS at the 2L telomere (Golubovsky *et al.* 2001; Capkova Frydrychova *et al.* 2007), and at the wild-type *w* promoter of Oregon R. The distance between the *w* promoter of *11-5* and the chromosome end is estimated to be at least 30 kb on the basis of a correlation of $P\{w^{var}\}$ variant eye color with HTT length (Golubovsky *et al.* 2001; Mason *et al.* 2003). HP1 showed a ninefold higher level at the telomeric *w* of *11-5* compared to the nontelomeric *w* gene of Oregon R (Figure 3B).

We also compared the HP1 level at the *w* promoter in *11-5* with HP1 at the promoter of a mini-*w* reporter

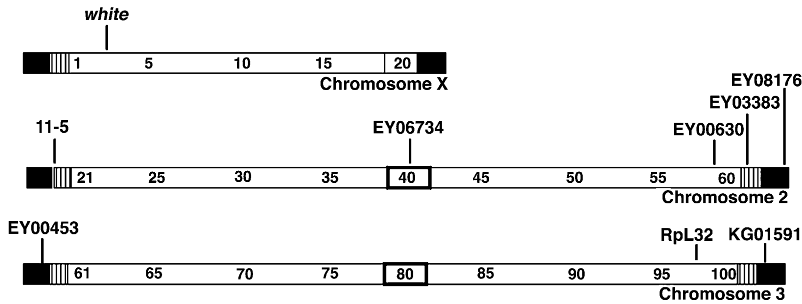

FIGURE 2.—Schematic map showing locations of the wild-type *white* gene, the *RpL32* gene, and the insertion sites of *P* elements used here. Boxes around 20, 40, and 80, indicate pericentric heterochromatin; striped boxes, TAS; filled boxes, HTT. Numbers indicate cytological map positions.

transgene of the *EY06734* insertion in pericentric heterochromatin of 2L (Figures 2 and 3B). The HP1 level at *11-5* was approximately twofold lower than at mini-*w* of the pericentric insertion. Further analyses were performed at *EPgy2* elements inserted into the HTT array, TAS, euchromatin, and pericentric heterochromatin (Figure 2) to examine HP1 levels at the promoter of a mini-*w* reporter transgene and at the 3′ end of *EPgy2* insertions immediately adjacent to the insertion site (Figure 3, C–E). In *EY00453*, the distance between the chromosome end and the *w* promoter is estimated to be at least 6.6 kb (including 3.8 kb between the *w* promoter and the 5′ end of the *P* element), and the distance between the chromosome end and the 3′ end of the *P* element is estimated to be at least 12.3 kb. The length estimation was based on a 2.8-kb PCR product generated with primers to *HeT-A* coding sequence and the *P*-element 5′ end (Het_seq1F, Car1P5_seq1B primers; specificity of the PCR product was checked by sequencing). Consistently, at both the *w* promoter and the 3′ end of the *P* element we found distinct HP1 levels showing an increase in the direction of *EY00630* (euchromatin) < *EY00453* (HTT) < *EY03383* (TAS) < *EY06734* (pericentric heterochromatin) (Figure 3, D and E), although HP1 levels at *EY00453* and *EY03383* are not significantly different from each other at either site. That is, HP1 is present in the HTT array and TAS, and the levels of HP1 in these regions are intermediate between euchromatin and pericentric heterochromatin.

Mutations in *Su(var)205* decrease HP1 levels in *EY00453* and *11-5*: By genetic crosses we introduced *Su(var)205*^{04}, which encodes a truncated HP1 protein that lacks part of the domain required for its nuclear localization (Powers and Eissenberg 1993), into the *EY00453* and *11-5* insertion lines and quantified HP1

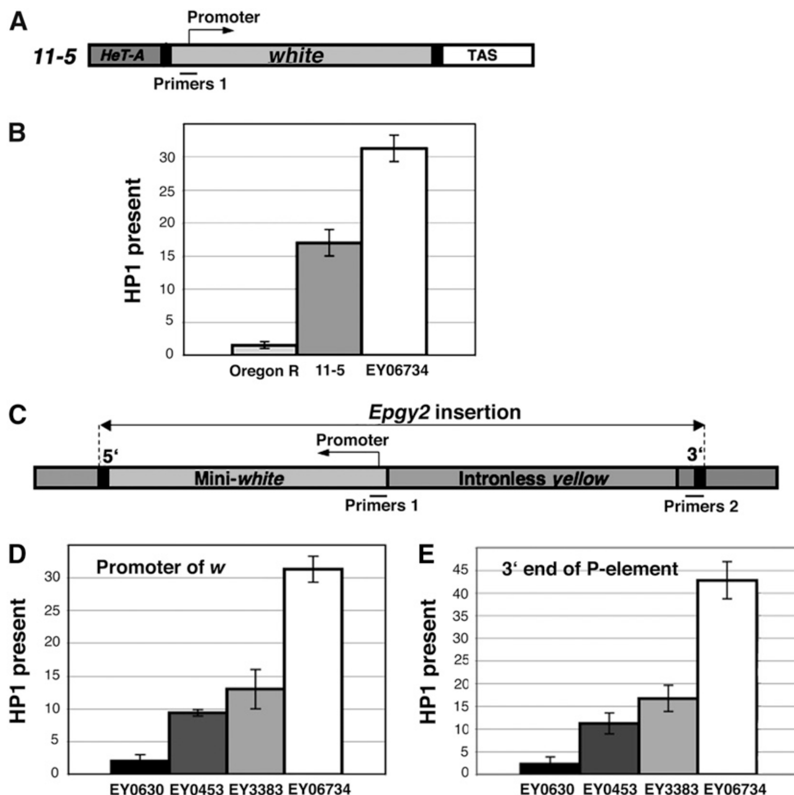

FIGURE 3.—HP1 is found at telomeric insertions. (A) *11-5* bears a complete *white* gene inserted between the terminal retrotransposon array and TAS at the 2L telomere. Primers 1 used in the ChIP experiment surround the promoter. (B) ChIP analysis of HP1 at the *w* promoter of Oregon R, *11-5*, and *EY06734*. Graphs represent real-time PCR results obtained after ChIP. HP1 measurements were normalized to 5% of input DNA and further normalized to the *RpL32* locus. Error bars represent standard deviations. (C) The *EPgy2* construct of EY insertions has a mini-*white* gene (mini-*w*) and an intronless *yellow* gene. Primers 1 and Primers 2, which correspond to the mini-*w* promoter and the 3′ end of the *P*-element insertion, respectively, were used for PCR analysis after ChIP. (D and E) The level of HP1 binding at the *w* promoter (D) and the 3′ end of the *P* element of EY insertions (E) analyzed by ChIP. Error bars represent standard deviations.

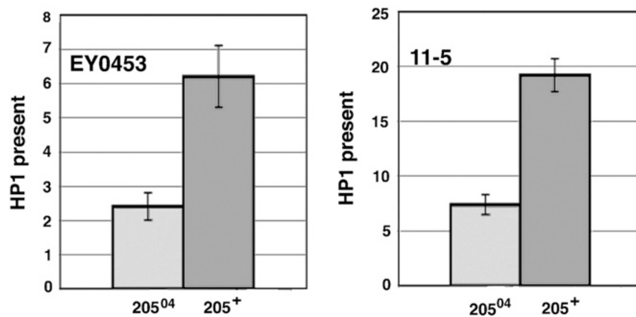

Figure 4.—HP1 levels at the *w* the promoter in *EY00453* and *11-5* insertion lines bearing a wild type or mutant *Su(var)205* gene. Graphs represent real-time PCR results after ChIP. Measurements for each antibody were normalized to 5% of input DNA and further normalized to the results from the *RpL32* locus. Error bars represent standard deviations.

at the *w* promoter. Larvae heterozygous for the $Su(var)205^{04}$ mutation showed a 2.5-fold decrease in HP1 at both insertions compared to wild-type larvae (Figure 4). These results indicate that the HP1 level in the HTT array is affected by the $Su(var)205^{04}$ mutation, and it confirmed that HP1 is present in the internal region of the telomere and is not limited only to the telomere cap.

Mutations in HP1 stimulate *HeT-A* transcription along the HTT array: Localization of HP1 in the internal region of the HTT array suggests that expression of telomeric retroelements is regulated by local binding of HP1 to these elements. This led us to investigate the impact of *Su(var)205* mutations on the transcriptional activity of individual *HeT-A* retroelements located in different positions of the HTT array. Promoter activity at the 3′ end of each *HeT-A* element may result in transcription into a downstream *P*-element insertion, which can be identified as a *HeT-A/P*-element readthrough transcript (Capkova Frydrychova *et al.* 2007). This allows us to measure transcriptional activity of individual *HeT-A* elements by quantitative real-time PCR with primers specific to a *HeT-A/P*-element transcript. We used the *11-5*, *KG01591*, and *EY08176* lines with *P*-element insertions in or adjacent to the HTT array (Figures 2 and 5) and compared levels of the *HeT-A/P*-element readthrough transcript between the *Su(var)205* mutant and $Su(var)205^{+}$ control flies (Figure 6). For the test we used two *Su(var)205* mutants: $Su(var)205^{02}$, with a point mutation in the conserved chromodomain and $Su(var)205^{04}$ (Eissenberg *et al.* 1992; Platero *et al.* 1995). The stocks were kept for two generations before they were analyzed. Compared to the $Su(var)205^{+}$ controls, larvae that were heterozygous for *Su(var)205* displayed significantly increased levels of *HeT-A/P*-element readthrough transcripts compared to $Su(var)205^{+}$ (Figure 6). $Su(var)205^{02}$ exhibited a fourfold increase of the readthrough transcript in *EY08176* and a fivefold increase in *KG01591*. The increase in the transcript level in $Su(var)205^{04}$ was

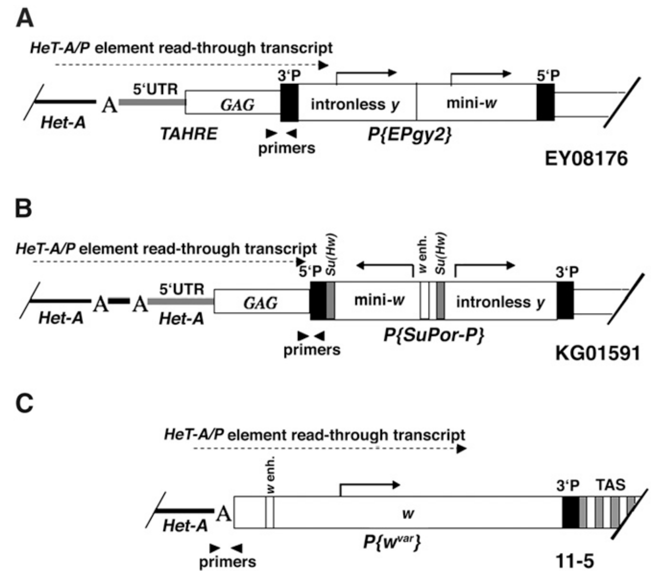

Figure 5.—Diagram showing the structure of *P*-element insertions used in the experiment to measure *HeT-A/P*-element readthrough transcript levels. (A) EY08176 has a single *EPgy2* element, containing an intronless *yellow* (*y*) gene and a mini-*white* (mini-*w*) gene. The *EPgy2* construct is inserted in inverted orientation into the GAG open reading frame (ORF) of a *TAHRE* element >8 kb from the 2R chromosome end and >15 kb from TAS. The *TAHRE* bearing the insertion is bordered by an upstream *HeT-A* element. (B) KG01591 carries a *SuPor-P* element with a mini-*w* gene containing the *w* enhancer and an intronless *y* gene inserted 5 kb from 3R TAS. The mini-*w* is bordered by *Su(Hw)* insulators. The *SuPor-P* is inserted into the ORF of a *HeT-A* element. Directly upstream of this *HeT-A* lies a 168-bp *HeT-A* fragment with an oligo (A) tail followed by a 3′ *HeT-A* UTR with another oligo (A) tail. (C) *11-5* contains $P\{w^{var}\}$ carrying a *w* transgene inserted between the HTT array and a truncated 2L TAS region. The *P*-element construct lacks its 5′ end (Biessmann *et al.* 2005a; Capkova Frydrychova *et al.* 2007). Arrowheads indicate the positions of primers used to quantify *HeT-A/P*-element readthrough transcript. "A" indicates the *HeT-A* oligo(A) tail. The presence of the *HeT-A/P*-element readthrough transcripts in all three insertions was reported previously (Capkova Frydrychova *et al.* 2007).

sixfold in *EY08176*, eightfold in *KG01591*, and fourfold in *11-5*. As the same degree of increase was seen for all three of the insertions independent of position, it appears that upregulation of *HeT-A* transcription by *Su(var)205* mutations is spread along the HTT array and is not limited to one specific region of the array.

Using primers specific to the *HeT-A* coding sequence, we also measured overall *HeT-A* transcript level and *HeT-A* genomic copy number, allowing us to calculate *HeT-A* transcript per genomic element. Comparison of genomic *HeT-A* copy numbers showed almost no differences between *Su(var)205* mutants and the $Su(var)205^{+}$ control (supplemental Table S2). This was probably due to the low number of generations since the *Su(var)205* mutations were introduced into the insertion lines. When we analyzed the same stocks of *EY08176; $Su(var)205^{02}$* and *EY08176; $Su(var)205^{04}$* after 24 gener-

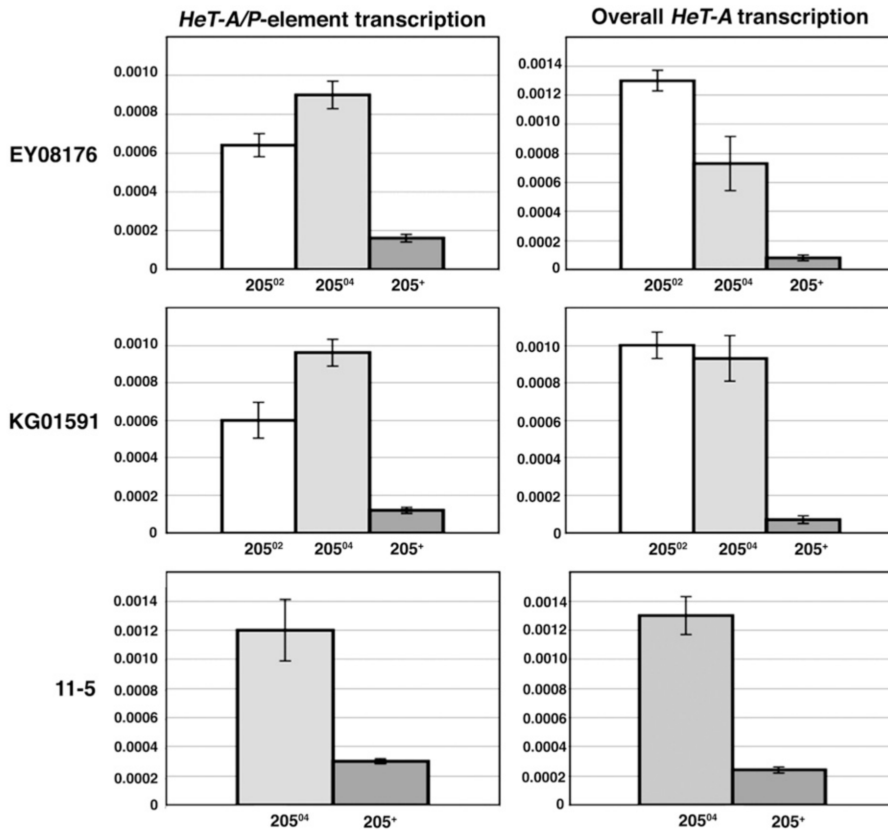

Figure 6.—Levels of *HeT-A* and *HeT-A/P*-element readthrough transcripts are increased in *Su(var)205* mutants. Levels of *HeT-A/P*-element and *HeT-A* transcripts in *EY08176*, *KG01591*, and *11-5* insertions heterozygous for *Su(var)* 205^{02} or *Su(var)*205^{04} were compared with a *Su(var)205* wild type. The transcript levels were normalized to *RpL32* transcripts and insertion copy number. Overall *HeT-A* transcription was further normalized to genomic *HeT-A* copy number of each tested genotype. Error bars represent standard deviations.

ations, we found twice the genomic *HeT-A* copy number compared to the *EY08176 Su(var)205*$^+$ control. Despite little difference in genomic *HeT-A* copy number we found the levels of overall *HeT-A* transcript elevated 7.5- to 16-fold in mutant flies compared to the *Su(var)205*$^+$ controls (Figure 6). The increase in overall *HeT-A* transcript in *Su(var)205* mutants is more than the increase we observed in individual *HeT-A/P*-element transcript levels (Figure 6), which may indicate that the effect of *Su(var)205* mutations on *HeT-A* transcription varies in different positions of the HTT array. These data suggest that regulation of *HeT-A* transcriptional activity by HP1 is not restricted to the telomere cap or any specific region of the HTT array, but affects the transcription of *HeT-A* elements along the HTT array.

Although we saw stimulation of *HeT-A* transcriptional activity in the presence of a mutation in *Su(var)205*, the mutation had no effect on transcription of the *w* transgene in any tested genotype. The *w* transcript was measured using real-time PCR with primers to coding sequence of the *w* gene (supplemental Table S3).

The capping complex has no significant effect on overall *HeT-A* transcription: The telomere-capping complex is comprised of HP1 and HOAP. Mutants for the HOAP-encoding gene, *cav*, display a telomere fusion phenotype and a defect in HP1 localization at telomeres (Cenci *et al.* 2003). Formation of the telomere-capping complex may be disrupted by mutations in several telomere protective genes, such as *tefu*, which encodes the ATM kinase. ATM plays a role in DNA repair and telomere function and is required to recruit or maintain HP1 and HOAP at chromosome ends. Loss of ATM leads to telomeric fusions and significant reduction of HP1 and HOAP association with telomeres (Oikemus *et al.* 2004; Bi *et al.* 2005; Cenci *et al.* 2005). As HP1 acts as a repressor of transcription of telomeric retroelements, we asked whether *cav* and *tefu* mutations, through their effect on formation of the capping complex and association of HP1 with telomeres, lead to an increase in *HeT-A* transcriptional activity. As *tefu* homozygotes are viable during the third larval instar, and as the loss of ATM has been reported to reduce HP1 and HOAP localization at telomeres, we measured *HeT-A* transcript levels in *tefu* homozygotes. To distinguish homozygous larvae, we balanced *tefu* with the *TM3* balancer chromosome marked with GFP. We simultaneously measured the *HeT-A* transcript level and *HeT-A* genomic copy number using the same set of primers specific to coding sequence of *HeT-A* and calculated *HeT-A* transcription per genomic element. *tefu* homozygotes showed no difference in normalized *HeT-A* transcription from *tefu/TM3, GFP* heterozygotes (Figure 7). As we saw an 8- to 10-fold increase in *HeT-A* transcriptional activity per *HeT-A* element caused by *Su(var)205* mutations (Figure 6) the lack of an effect exhibited by the *tefu* mutant may indicate first, that ATM within the telomere interacts solely with the telomere cap and binding of HP1 in the HTT array outside of the telomere cap is ATM in-

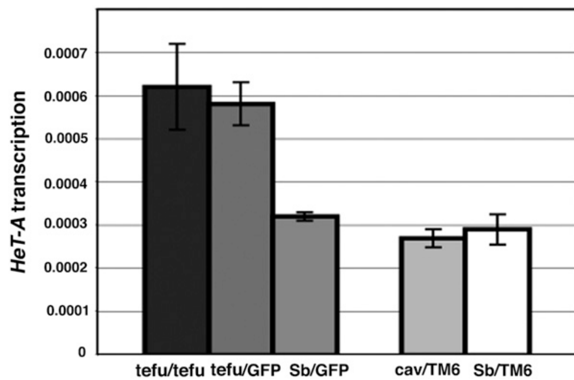

FIGURE 7.—Relative levels of *HeT-A* transcripts in homozygous and heterozygous *tefu* and heterozygous *cav* larvae. The levels of *HeT-A* transcript were normalized to transcript levels of *RpL32* and to genomic *HeT-A* copy number of each tested strain. To minimize effects due to different genetic backgrounds, original mutant strains were crossed into the same *y* w^{67c23} background. Error bars represent standard deviations.

dependent, and second, that a change in HP1 level in the cap due to loss of ATM is limited in distance and thus has no or a limited effect on retroelement activity in the HTT array outside of the telomere cap. We did not observe a significant change in *HeT-A* transcript level due to mutation in the *cav* heterozygote compared to a control (Figure 7), which is consistent with the idea that formation of the capping complex and association of HP1 at the end of telomeres do not have extensive effects on the overall level of transcriptional activity of telomeric retroelements.

Chromatin domains in Drosophila telomeres: Expression analysis of telomeric *w* transgenes suggested two distinct chromatin domains in Drosophila telomeres: the heterochromatic TAS and the euchromatic HTT array (BIESSMANN *et al.* 2005a,b). In our study, however, HP1 levels at the *w* promoter in the *EY00453* and *EY03383* insertions, in the HTT array, and TAS, respectively, revealed no significant differences and are intermediate compared to HP1 levels at *EPgy2* elements located in euchromatin (*EY00630*) and pericentric heterochromatin (*EY06734*). This led us to look at levels of other chromatin proteins, histone modifications, and nucleosome organization at the *w* promoter and the 3′ end of these insertions (Figure 8) to better understand any difference between expression data and the presence of HP1 at tested transgenes.

Mutations in *His2Av* are dominant suppressors of PEV in Drosophila, and exchange of histone H2A for H2A.v is implicated in heterochromatin formation (SWAMINATHAN *et al.* 2005). Histone H2A and H2A.v levels did not show significant differences between TAS-located *EY03383* and pericentric *EY06734*, with the exception of a slightly lower level of H2A.v in the *w* promoter region of *EY06734* compared to *EY03383* (Figure 8). H2A at these two insertions showed significantly lower levels in comparison to both euchromatic *EY00630* and HTT-located EY00453. In contrast, H2A.v levels show significant elevation in *EY03383* and *EY06734*. *EY00453* shows a lack of proportionality in the transition between levels of H2A and H2A.v. Although H2A.v levels in *EY00630* and *EY00453* are comparable, H2A in *EY00453* is intermediate between *EY00630* and *EY03383* (Figure 8). These data indicate that the TAS and pericentric domains contain H2A/H2A.v levels that are similar to each other, but distinct from those in HTT and the euchromatic control.

We tested levels of di- and trimethylated histone H3 at Lys9 (Me2K9H3 and Me3K9H3), as histone H3-Lys9 methylation plays a role in gene silencing (SCHOTTA *et al.* 2003; EBERT *et al.* 2006). In both the *w* promoters and the 3′ ends of the insertions, the levels of Me2K9H3 resemble HP1 levels in that they show an increase in the direction of *EY00630* < *EY00453* < *EY03383* < *EY06734*, and at the 3′ ends of these insertions the level of Me2K9H3 in *EY00453* is comparable to that of *EY03883*. Me3K9H3 levels, on the other hand, did not show a significant difference between *EY00630* and *EY00453*, or between *EY03383* and *EY06734* at the *w* promoter and only a relatively minor twofold difference between the latter pair and the former. More strikingly, the levels of Me3K9H3 at the 3′ end of these insertions was found to be similar in *EY00630*, *EY03383*, and *EY00453*, while *EY06734* showed an approximately sevenfold increase relative to the others (Figure 8). Thus, although levels of HP1 do not distinguish the HTT array from TAS, other chromatin markers show that HTT more closely resembles open chromatin, while TAS resembles more closed chromatin. Of the chromatin marks examined here, the level of histone H2A.v, most closely (inversely) corresponds to the expression of the tested *w* transgenes as assayed by transcript levels (Figure 9) or by eye color (BIESSMANN *et al.* 2005a).

Nucleosome organization at telomeres: To examine a possible difference between the *EY00453*, *EY03383*, and *EY00630* transgenes at the level of nucleosome organization we treated nuclei from third instar larvae with micrococcal nuclease, an enzyme that preferentially cuts between nucleosomes. DNA fragments were analyzed using Southern hybridization with probes to the *w* promoter and the 3′ end of the insertion. The probe to the 3′ end was used to study nucleosome organization in regions adjacent to the insertions. Hybridization showed regular nucleosome spacing without significant differences among the different insertions (supplemental Figure S1). Thus, the functional differences in HP1 binding and *white* gene transcription between the TAS and HTT do not lie at the level of nucleosome organization.

DISCUSSION

On the basis of expression of telomeric *white* and *yellow* transgenes Drosophila telomeres have been proposed to

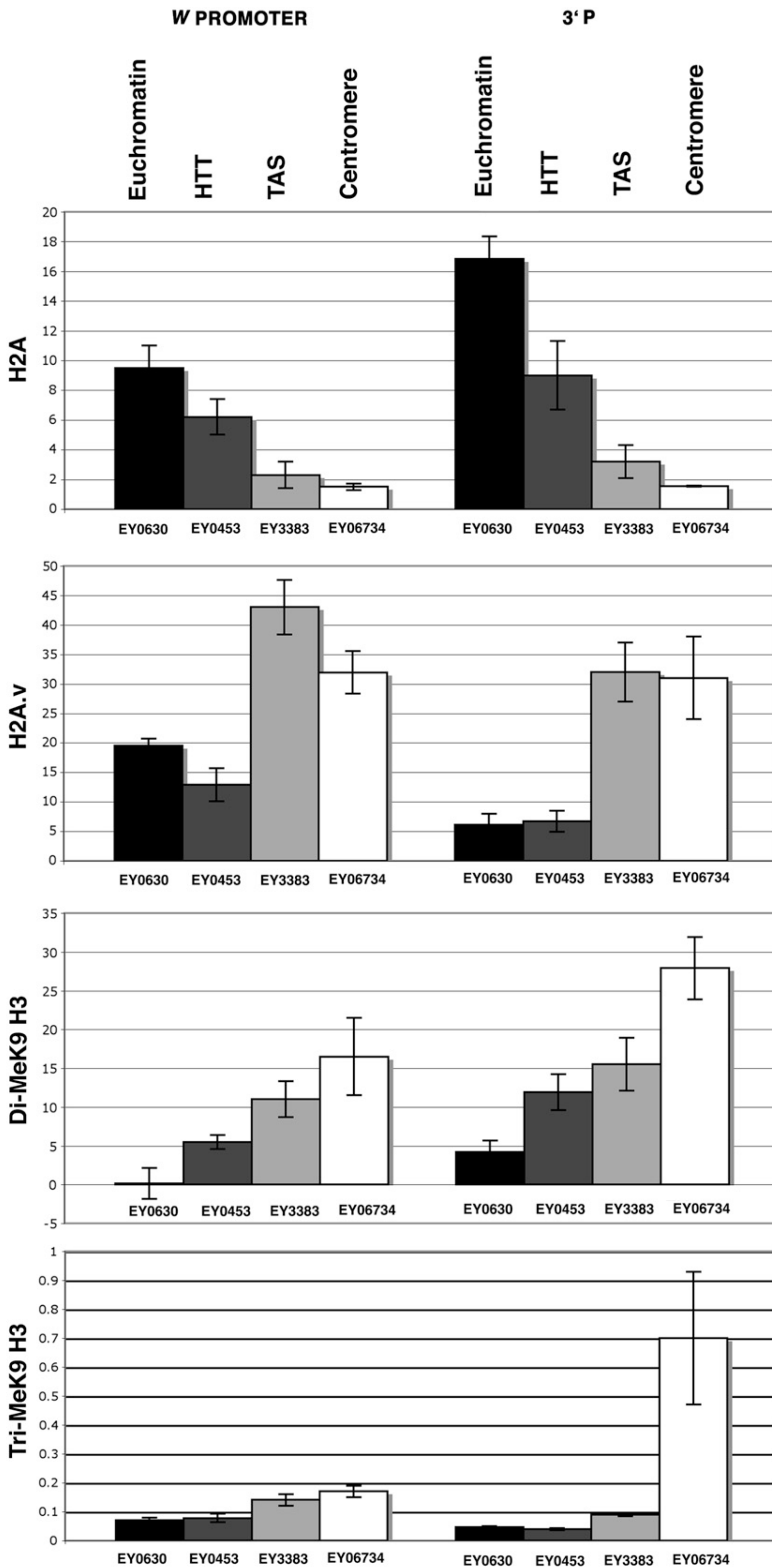

Figure 8.—ChIP analysis of histones and histone modifications at *EPgy2* insertion sites. Antibody quantification was performed using real-time PCR with primers specific to the promoter of the mini-*w* transgene and the 3′ end of the *EPgy2* insertion. Graphs show real-time PCR results. The level of each antibody was normalized to 5% of input DNA and further normalized to the *RpL32* locus. The data were obtained from four to six independent experiments, each of which included all of the strains compared. *EY00630* carries an insertion in euchromatin of chromosome *2*R. The *EY00453* insertion is located in *3*L HTT, *EY03383* carries an insertion in *2*R TAS, and the *EY06734* insertion is located in *2*R pericentric heterochromatin. Error bars represent standard deviations.

have two distinct domains: TAS, which resembles heterochromatin and the HTT array, which behaves like euchromatin (Biessmann *et al.* 2005a,b). According to the pattern of chromatin proteins revealed by immunostaining of extended polytene chromosomes in a *Tel* mutant, telomeres consist of three distinct and nonoverlapping domains: the chromosome cap, the HTT array, and TAS (Andreyeva *et al.* 2005). The immunostaining results indicate that HP1 in telomeres is restricted to the cap region (Siriaco *et al.* 2002; Andreyeva *et al.* 2005).

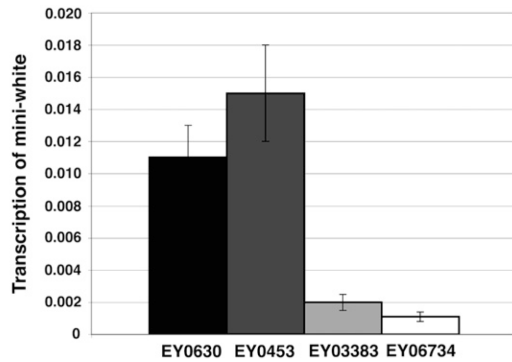

FIGURE 9.—Levels of mini-*w* transcripts in *EY00630* (euchromatin), *EY00453* (*3*L HTT), *EY03383* (*2*R TAS), and *EY06734* (*2*R pericentric heterochromatin). Mini-*w* transcript was measured by real-time PCR with primers specific to coding sequence of the *w* gene. The levels of the transcript were normalized relative to transcript levels of *RpL32*. Error bars represent standard deviations.

Using ChIP, we show here that HP1 is also present along the HTT array outside of the cap as well as in TAS. The difference between our observations and previous reports might be due to a higher abundance of HP1 in the telomere cap than in the internal HTT region or better accessibility of antibodies to the telomere cap, and thus the difference in the reports may be explained by higher sensitivity of ChIP compared to immmnostaining of polytene chromosomes. The difference may be caused also by different properties of long telomeres of a *Tel* mutant or different biological properties of polytene salivary chromosomes compared to diploid or other polyploid cells. In any case, ChIP data on whole animals are more likely to be generalizable than immunostaining data on a specific cell type.

Su(var)205 belongs to a group of suppressor of variegation [Su(var)] genes, many of which encode chromosomal proteins or modifiers of chromosomal proteins. Mutations in Su(var) genes lead to suppression of position-effect variegation (PEV), which is repressed and variegated expression of genes placed in or near pericentric heterochromatin (EBERT *et al.* 2006). Despite phenotypic similarities between PEV and telomere position effect (TPE), TPE does not respond to Su(var) mutations (CRYDERMAN *et al.* 1999; MASON *et al.* 2004). Although TAS was identified as a source of telomeric silencing, and the retrotransposon array genetically resembles euchromatin (BIESSMANN *et al.* 2005a,b), we found comparable levels of HP1 at transgenes inserted in these two telomeric domains. The levels of other marks for silent chromatin, such as histone H2A.v and MeK9H3, however, did vary between these two regions in a manner consistent with proposals in previous reports that HTT is associated with open chromatin and TAS is associated with closed chromatin. TPE may thus be caused by a silencing system different from HP1-mediated heterochromatin. One candidate is Polycomb silencing, as Polycomb group proteins were found associated with TAS (BOIVIN *et al.* 2003; ANDREYEVA *et al.* 2005). As levels of the chromatin markers in all tested regions, including euchromatin and pericentric heterochromatin, showed significant differences, interpretation of HTT and TAS as either heterochromatin or euchromatin is rather difficult. It may suggest that HTT and TAS are in a category of some transitional type of chromatin between euchromatin and heterochromatin, such as closed/inactive euchromatin, or it suggests the existence of additional chromatin types.

The relatively high level of HP1 on a transgene inserted into pericentric heterochromatin compared with transgenes in either HTT or TAS may suggest that failure of telomeric HP1 to silence telomeric transgenes is caused by its relative paucity. HP1, however, is a negative regulator of telomere length; its mutations lead to an increase in the transcriptional activity of *HeT-A* and *TART,* as well as an accumulation of these elements at the chromosome end (SAVITSKY *et al.* 2002; PERRINI *et al.* 2004). We showed previously that the promoter activity of a telomeric *w* transgene inserted between the HTT array and TAS significantly exceeds the activity of a single *HeT-A* promoter (CAPKOVA FRYDRYCHOVA *et al.* 2007). Here we show that *Su(var)205* mutations lead to a severalfold increase in the transcriptional activity of *HeT-A*, however we did not see any increase in transcription of a *w* gene inserted into the HTT array. In particular, using *HeT-A/P*-element readthrough transcripts in three *P*-element insertion lines, we found that *Su(var)205* mutations lead to stimulation of *HeT-A* elements along the HTT array in all regions assayed. With regard to the low level of HP1 in telomeric regions compared to pericentric heterochromatin, as observed by ChIP experiments, it is conceivable that the relatively weak *HeT-A* promoter is more sensitive to HP1concentration than the more robust *w* promoter. However, HP1 *per se* cannot be considered as a signal for silencing. An analysis of genome-wide correlations between the HP1 binding pattern and the pattern of gene expression revealed that recruitment of the protein is not sufficient to repress transcription completely (GREIL *et al.* 2003). Moreover, some euchromatic genes in Drosophila are activated by the presence of HP1 (CRYDERMAN *et al.* 2005). With respect to these observations, it is difficult to predict the effect of HP1 recruitment on the transcription pattern in any specific region.

HP1, by interaction with HOAP, forms capping complexes at the ends of Drosophila chromosomes (CENCI *et al.* 2005). Formation or maintenance of the HP1-HOAP capping complex requires ATM. Loss of ATM reduces localization of HP1 and HOAP at telomeres and leads to frequent telomeric fusions (OIKEMUS *et al.* 2004). *tefu* and *cav* mutations, however, did not lead to a profound increase in *HeT-A* transcription, as was

observed in *Su(var)205* mutants. This suggests that HP1 presence in the cap does not significantly participate in overall *HeT-A* transcriptional activity, and that *HeT-A* transcription is regulated mainly by HP1 in the HTT array outside the cap. Our data are consistent with Perrini *et al.* 2004, who suggested two distinct mechanisms for HP1 control of telomere capping and telomere elongation by retroelement transcription. They proposed that the capping function of HP1 is due to its direct binding to telomeric DNA, while the silencing of telomeric sequences and control of transcription of telomeric retroelements is due to interaction of HP1 with MeK9H3 and spreading of HP1 and repressive chromatin along the telomere.

Collectively, our data show that HP1 is present along the HTT array as well as in TAS and plays a role as a negative regulator of transcription of telomeric retroelements. The present data also support the observation that the *HeT-A* promoter is relatively weak compared with a mini-*w* promoter and more sensitive to local HP1 concentration and suggest that telomeric chromatin in Drosophila may be distinct from either euchromatin or heterochromatin.

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