The role of histone H2Av variant replacement and histone H4 acetylation in the establishment of *Drosophila* heterochromatin

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Activation and repression of transcription in eukaryotes involve changes in the chromatin fiber that can be accomplished by covalent modification of the histone tails or the replacement of the canonical histones with other variants. Here we show that the histone H2A variant of *Drosophila melanogaster*, H2Av, localizes to the centromeric heterochromatin, and it is recruited to an ectopic heterochromatin site formed by a transgene array. *His2Av* behaves genetically as a *PcG* gene and mutations in *His2Av* suppress position effect variegation (PEV), suggesting that this histone variant is required for euchromatic silencing and heterochromatin formation. *His2Av* mutants show reduced acetylation of histone H4 at Lys 12, decreased methylation of histone H3 at Lys 9, and a reduction in HP1 recruitment to the centromeric region. H2Av accumulation or histone H4 Lys 12 acetylation is not affected by mutations in *Su(var)3-9* or *Su(var)2-5*. The results suggest an ordered cascade of events leading to the establishment of heterochromatin and requiring the recruitment of the histone H2Av variant followed by H4 Lys 12 acetylation as necessary steps before H3 Lys 9 methylation and HP1 recruitment can take place.

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The basic unit of chromatin is the nucleosome, which is made up of 146 bp of DNA wrapped around a histone octamer composed of two molecules each of the histones H2A, H2B, H3, and H4. Activation of gene expression requires the transcriptional machinery to overcome the compaction of chromatin, and work in recent years has uncovered several strategies to accomplish this goal. ATP-dependent chromatin remodeling has been studied extensively as a mechanism to make the DNA accessible to the transcription apparatus (Narlikar et al. 2002). Covalent modification of the unstructured and solvent-exposed N-terminal tails of histones has also been shown to participate in processes such as activation or repression of transcription and chromosome condensation and segregation (Strahl and Allis 2000).

Replacement of canonical histones with other variants might provide an alternative mechanism for controlling transcription by inducing altered nucleosomal structures; in fact, recent work has implicated ATP-dependent chromatin remodeling complexes in the process of histone variant replacement (Krogan et al. 2003; Mizuguchi et al. 2004). The role of histone variants, and specially those of H3 and H2A, in various nuclear processes has been long appreciated (Wolffe and Pruss 1996; Ahmad and Henikoff 2002). There are at least three different families of H2A variants present in a variety of organisms from yeast to mammals, and the degree of conservation among members of each family is higher than to the canonical H2A (Jiang et al. 1998). H2AX is thought to play a role in DNA double-strand break repair; the serine in the SQEY motif of H2AX is phosphorylated at the site of the DNA damage and serves as a signal for the recruitment of repair proteins (Redon et al. 2002). Macro H2A1, another H2A variant, has been shown to have a role in X-chromosome inactivation and dosage compensation in mammals, where it is found to localize to the inactive X after silencing has been established (Ladurner 2003).

H2A.Z is a third histone H2A variant highly conserved across species and, therefore, likely to play an important role in chromatin function (van Daal et al. 1988; Stargell et al. 1993). H2A.Z is an essential protein in *Drosophila* and in mice (van Daal and Elgin 1992; Faast et al. 2001), and has been implicated in both activation and repression of transcription (Dhillon and Kamakaka 2000; Santisteban et al. 2000; Larochelle and Gaudreau 2003). Gene expression analyses using whole-genome microar-

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rays show that H2A.Z (named Htz1 in yeast) is involved in both activation and silencing of transcription in *Saccharomyces cerevisiae* (Meneghini et al. 2003). Recent studies have also shown that H2A.Z is enriched in the pericentric heterochromatin during early mammalian development (Rangasamy et al. 2003).

Heterochromatin consists of highly compacted DNA that is present around the centromeres and telomeres and is also dispersed at certain sites along the chromosomes (Grewal and Elgin 2002; Grewal and Moazed 2003). Heterochromatin has been found to be essential for proper chromosome segregation and genomic stability (Wallrath 1998) and for maintaining dosage compensation by inactivating one of the X chromosomes in female mammals (Avner and Heard 2001). Recent studies have helped elucidate several steps in the pathway leading to the formation of heterochromatin (Grewal and Moazed 2003). The earliest step established so far requires the RNAi machinery (Volpe et al. 2002; Pal-Bhadra et al. 2004) for targeting of small RNAs to heterochromatin by the RITS complex (Verdel et al. 2004). Subsequent steps require deacetylation of histone H3 Lys 9 followed by methylation of the same residue by Su(var)3-9 (Rea et al. 2000; Nakayama et al. 2001; Schotta et al. 2002). This modified histone then recruits HP1 (Bannister et al. 2001; Lachner et al. 2001; Nakayama et al. 2001), which in turns recruits the Suv4-20 methyltransferase to trimethylate histone H4 at Lys 20 (Kourmouli et al. 2004; Schotta et al. 2004).

Given the large number of suppressors and enhancers of PEV identified in *Drosophila* (Schotta et al. 2003), there must be additional steps involved in the establishment and maintenance of heterochromatin. Although acetylation of histone tails is generally correlated with transcription activation, and establishment of heterochromatin requires deacetylation of H3 Lys 9, acetylation of other residues in H3 or H4 might also play a role in silencing processes (Braunstein et al. 1993; Johnson et al. 1998). Histone acetyl transferases have been found to be physically associated with Su(var)3-9 (Vandel and Trouche 2001), and mutations in a MYST domain acetyltransferase behave as suppressors of PEV (Grienenberger et al. 2002). These results support the idea that acetylation of specific histone residues might be involved in the formation of heterochromatin. Here, we demonstrate that the H2A.Z variant of *Drosophila*, H2Av, plays a role in Pc-mediated silencing and in the establishment of centromeric heterochromatin. In addition, acetylation of H4 Lys 12 is required subsequent to H2Av replacement but before H3 Lys 9 methylation. The results highlight the complexity of the multistep process leading to heterochromatin formation in higher eukaryotes.

Results

The histone H2Av variant participates in Pc silencing

It has been previously shown that the *Drosophila* H2Av variant is distributed in a nonrandom manner in third instar polytene chromosomes (van Daal and Elgin 1992; Leach et al. 2000). H2Av is present in the heterochromatic chromocenter and is associated with both transcribed and nontranscribed genes in polytene chromosome bands and interbands (Leach et al. 2000). To gain further insights into the function of H2Av, we decided to test whether *His2Av* behaves genetically as a *trithorax-Group* (*trxG*) or *Polycomb-Group* (*PcG*) gene. In *Drosophila,* expression patterns of homeotic genes are maintained by the PcG and trxG proteins. Since H2Av is present in nontranscribed euchromatic regions (Leach et al. 2000), we first determined whether this histone variant is involved in Pc-mediated silencing by examining whether mutations in the *His2Av* gene enhance the phenotype of *Pc* mutants. Adult flies from a strain heterozygous for *Pc*, *Df(3R)Pc*/+, show a partial transformation of the second leg into the first leg, visualized by the appearance of sex combs in the second leg of male flies. When flies are also heterozygous for a mutation in the *His2Av* gene, the frequency and severity of these transformations increase dramatically (Table 1). Out of 100 flies of the genotype *Df(3R)Pc*+/+ $His2Av^{05146}$ examined, 33% had extra sex combs in all four second and third legs and 40% had extra sex combs in the second legs and one of the third legs. Out of 220 flies of the genotype *Df(3R)Pc*+/+ $His2Av^{810}$ tested, 18% showed transformations of second into first leg and 72% showed transformation of both second and third legs into first (Table 1). These results suggest that mutations in *His2Av* enhance the *Pc* phenotype and therefore *His2Av* might be classified as a *PcG* gene. To confirm this possibility, we examined genetic interactions between *His2Av* and *trxG* mutants. If *His2Av* is a *PcG* gene, mutations in *His2Av* should suppress the phenotype of *trxG* genes. We tested the effect of $His2Av^{05146}$ and $His2Av^{810}$ on two different combinations of *trG* genes, $ash1^{VF101}$ trx^{b11}/++ and brm^{2}

Table 1. *Interactions of* His2Av *with* PcG *and* trxG *genes*

Genotype of fly strains	Oregon R	$His2Av^{05146}$/*TM6B Tb*	$His2Av^{810}$/*TM6b Tb*
Df(3R)Pc/TM3 Sb Ser	400/14.5%	100/73%	200/90%
$ash1^{VF101}$ trx^{b11}/*TM3 Sb*	1000/66%	550/37%	219/29%
brm^{2} trx^{E2}/*TM3 Sb*	740/43%	690/22%	215/20%

Males or females of the genotypes indicated in the top row were crossed with females or males indicated in the first column. For *PcG* mutants, progeny were examined for second or third leg transformed toward first leg, that is, the presence of sex combs on the second and third legs. In the case of *trxG* mutants, the progeny were examined for homeotic transformations such as heltere to wing or third leg to second leg. Data are presented as number of flies examined/percentage of flies with transformations.

trx^{E2}/++. Flies of the genotype $ash1^{VF101}$ trx^{b11}/++ show transformations of third leg into second leg by the appearance of an apical bristle on the third leg in 66% of 1000 flies examined (Table 1). This frequency decreases to 37% in $ash1^{VF101}$ trx^{b11}+/++ $His2Av^{05146}$ flies and to 29% in $ash1^{VF101}$ trx^{b11}+/++ $His2Av^{810}$ flies (Table 1). Similarly, flies of the genotype brm^2, trx^{E2}/++ show a 43% frequency of haltere to wing or third leg to second leg transformations, and this frequency is reduced to 22% in brm^2 trx^{E2}+/++ $His2Av^{05146}$ flies and to 21% in brm^2 trx^{E2}+/++ $His2Av^{810}$ flies (Table 1). These data suggest that mutations in *His2Av* suppress the phenotype of *trxG* mutations and, together with the previously observed enhancement of the *Pc* phenotype, support the hypothesis that *His2Av* is a *PcG* gene.

PcG gene products repress transcription of homeotic genes outside of their normal expression boundaries (Paro 1990). If H2Av is a PcG protein, we can expect to observe ectopic expression of homeotic genes in *His2Av* mutants. To test this possibility, we examined the distribution of Antennapedia (Antp) protein in flies homozygous for the $His2Av^{810}$ mutation. Antp localizes in the ventral ganglion of wild-type larvae in three bands of cells corresponding to the three thoracic segments (Fig. 1A). In the case of the $His2Av^{810}$ mutant, this pattern is altered and the Antp protein is present further posteriorly through the ventral ganglion (Fig. 1B). A second homeotic protein, Ultrabithorax (Ubx), is involved in the development of the third thoracic and first abdominal segments, and it is expressed posterior to the Antp expression in the ventral ganglion of wild-type larvae (Fig. 1C). This pattern is not disrupted in the $His2Av^{810}$ mutant, as the band of Ubx expression appears to be similar in intensity and spatial distribution to that of wild-type larvae (Fig. 1D). These results suggest that H2Av might be required to maintain proper expression of homeotic genes in the anterior part of the animal, where Antp is expressed, but not in more posterior segments where Ubx expression occurs. The results also confirm the hypothesis suggesting that *His2Av* is a *PcG* gene.

Mutations in His2Av *result in reduced Pc accumulation in polytene chromosomes*

Recruitment of PcG complexes to silenced regions of the genome requires methylation of Lys 27 of histone H3 (Cao et al. 2002). To test whether H2Av replacement is required for Pc recruitment, we compared the distribution of this protein in wild type versus *His2Av* mutants. Pc localizes to ~100 sites on polytene chromosomes of wild-type-OR third instar larvae (Fig. 1F). In contrast, chromosomes from larvae homozygous for the $His2Av^{810}$ allele show a reduction in the number of Pc sites as well as in the amount of protein present at these sites (Fig. 1I). As a control, the Su(Hw) protein is present at similar levels in polytene chromosomes of wild-type and $His2Av^{810}$ flies (Fig. 1G,J). To test whether this decreased accumulation of Pc in polytene chromosomes was due to reduced synthesis of Pc protein or reduced recruitment of the protein to the chromosome, we carried out Western analyses of protein extracts obtained from wild-type and $His2Av^{810}$ mutant larvae. There is no significant difference in the levels of Pc protein between these two strains (Fig. 2A), suggesting that the observed effect is due to the inability of Pc to be recruited to the chromosomes in the absence of H2Av.

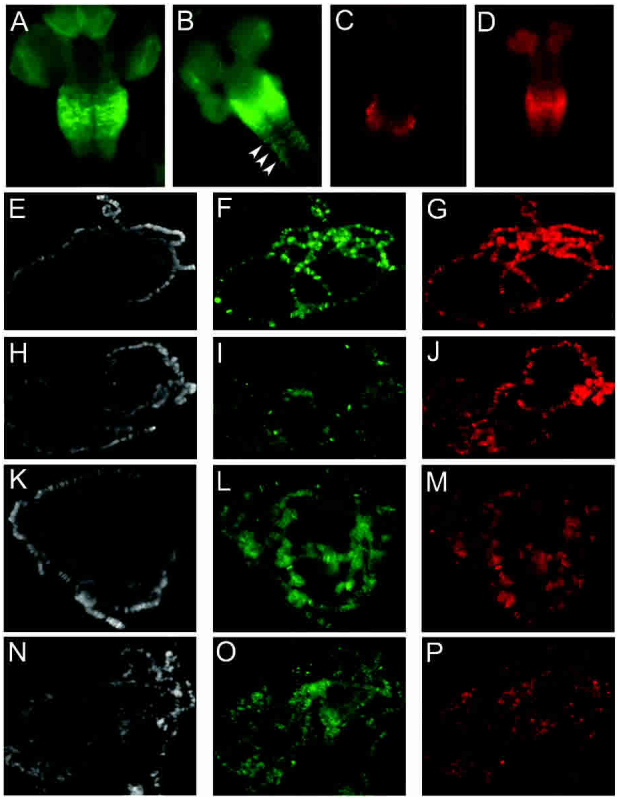

Figure 1. Effect of *His2Av* mutations on Pc-mediated silencing. (*A–D*) Expression of homeotic proteins in wild-type and mutant larvae. Brains and ventral ganglia were dissected from OR and $His2Av^{810}$ third instar larvae and stained with antibodies against Antp and Ubx. (*A*) Distribution of Antp in the central nervous system of OR larvae. (*B*) Distribution of Antp in the central nervous system of $His2Av^{810}$ larvae; arrows point to sites of ectopic expression. (*C*) Distribution of Ubx in the central nervous system of OR larvae. (*D*) Distribution of Ubx in the central nervous system of $His2Av^{810}$ larvae. (*E–P*) Effect of *His2Av* mutations on Pc and E(z) localization. Polytene chromosomes from third instar larvae were dissected from OR and $His2Av^{810}$ mutant larvae and stained with rabbit polyclonal anti-Pc and anti-E(z), and rat polyclonal anti-Su(Hw) antibodies. The bound antibodies were detected using FITC-labeled goat anti-rabbit (green) and Texas red-labeled goat anti-rat (red) secondary antibodies. (*E,H,K,N*) DNA stained with DAPI. (*G,J,M,P*) Chromosomes stained with Su(Hw) as a control. (*F*) Distribution of Pc in chromosomes from the wild-type strain OR. (*I*) Pc in chromosomes from $His2Av^{810}$ mutant larvae. (*L*) E(z) in wild-type OR. (*O*) E(z) in $His2Av^{810}$ mutant larvae.

Recent results suggest that H3 trimethylated at Lys 27 facilitates Pc binding to silenced regions (Cao et al. 2002) and this modification is carried out by the Enhacer of zeste [E(z)] protein present in the ESC–E(z) complex

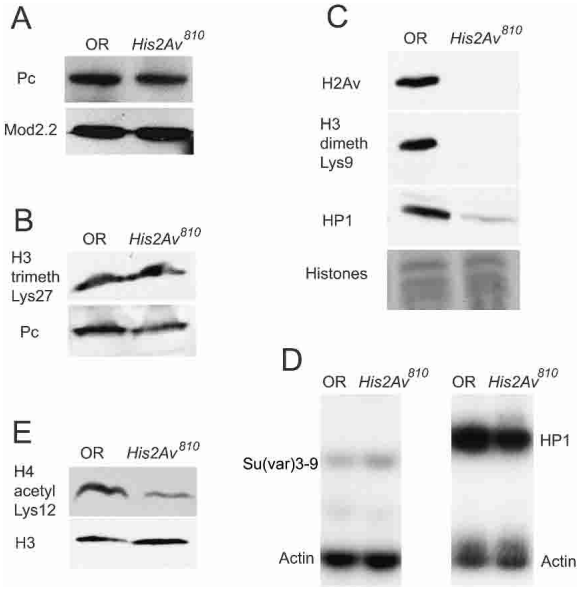

Figure 2. Western analysis of histone modifications in OR and *His2Av* mutants. Protein extracts were prepared from third instar larvae, run on polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies against various proteins or modified histones. (*A*) Levels of Pc in OR and $His2Av^{810}$ larvae; the same filter was probed with antibodies against the Mod(mdg4)2.2 (Mod2.2) protein as a loading control. (*B*) Levels of H3 trimethylated Lys 27 in OR and $His2Av^{810}$ larvae; the same filter was probed with Pc as a control. (*C*) Accumulation of H2Av, H3 dimethyl Lys 9, and HP1 in OR and $His2Av^{810}$ larvae; the *lower* panel (labeled as Histones) shows the gel stained with Coomassie to control for equal loading of the lanes. (*D*) RT–PCR analysis of RNA obtained from third instar wild-type OR or $His2Av^{810}$ mutant larvae using primers in the *Su(var)3-9* or *Su(var)2-5* (HP1) genes; actin is shown as a control for the amount of RNA used in each lane. (*E*) Accumulation of H4 acetylated Lys 12 in OR and $His2Av^{810}$ larvae; the *lower* panel shows levels of total histone H3 as a control.

(Czermin et al. 2002). Since we observed a reduction in Pc on polytene chromosomes in *His2Av* mutants, we examined whether recruitment of the ESC–E(z) complex is also impaired in these mutants. In wild type, E(z) can be observed at multiple sites throughout the genome (Fig. 1L). The levels and localization of E(z) do not appear to be altered in the $His2Av^{810}$ mutant compared to wild type (Fig. 1O). We then examined whether H3 Lys 27 methylation is affected by mutations in *His2Av*. The levels and distribution of this modification appear to be the same in polytene chromosomes from wild-type and $His2Av^{810}$ mutant larvae (data not shown). This result was confirmed by Western analysis, which shows equal levels of H3 trimethylated at Lys 27 in wild-type and $His2Av^{810}$ mutant larvae (Fig. 2B). These results suggest that H2Av is required upstream of Pc recruitment in the process of Pc-mediated silencing. Since neither recruitment of the E(z) complex nor H3 Lys 27 methylation seem to be affected in *His2Av* mutants, H2Av replacement might take place after H3 Lys 27 methylation and before Pc recruitment. Alternatively, Pc repression might require at least two parallel and independent pathways, one involving H2Av recruitment and a second one leading to H3 Lys 27 methylation, both of which might be required for proper Pc recruitment.

H2Av is essential for heterochromatin-induced silencing

Given the observed accumulation of H2Av in the centromeric heterochromatin (Leach et al. 2000), to test the possible involvement of H2Av in heterochromatic silencing we determined whether mutations in the *His2Av* gene can act as modifiers of variegated phenotypes caused by the presence of a gene next to heterochromatin. The $In(1)w^{m4}$ allele is caused by an inversion that positions the *white* gene next to the centromeric heterochromatin of the X chromosome (Muller 1930; Lewis 1950). This rearrangement results in the characteristic variegated phenotype shown in Figure 3A. Mutations in the *His2Av* gene act as dominant suppressors of this phenotype, with flies of the genotype $In(1)w^{m4}/In(1)w^{m4}$; $His2Av^{810}/+$ showing a dramatic increase in eye pigmentation when compared to $In(1)w^{m4}$ alone (Fig. 3B). The presence of the H2Av histone variant in the centromeric heterochromatin and its requirement for the variegated phenotype of the $In(1)w^{m4}$ mutation suggest that H2Av plays an important role in the establishment and/or maintenance of heterochromatin.

Mutations in His2Av *result in reduced H3 Lys 9 methylation and HP1 recruitment*

Formation of heterochromatin requires deacetylation of H3 Lys 9 followed by methylation of the same residue and recruitment of HP1 (Grewal and Moazed 2003). The heterochromatin of *Drosophila* chromosomes is enriched in dimethylated and trimethylated histone H3 in the Lys 9 residue (Fischle et al. 2003). To analyze the possible role of H2Av in heterochromatin assembly, we examined the localization of H3 dimethylated at Lys 9 in polytene chromosomes from larvae carrying a mutation in the *His2Av* gene. Antibodies against histone H3 dimethylated in Lys 9 stain the pericentric heterochromatin in wild-type larvae (Fig. 3F). Interestingly, polytene chromosomes from $His2Av^{810}$ mutants show a decrease in the amount of methylated H3 Lys 9 (Fig. 3I), whereas the presence of Su(Hw), used as a control, is the same in chromosomes from wild-type and $His2Av^{810}$ mutant larvae (Fig. 3G,J). Since modification of this residue is important for HP1 recruitment, we next analyzed whether localization of HP1 in heterochromatin is also affected by mutations in *His2Av*. In wild-type larvae, HP1 localizes preferentially to the pericentric heterochromatin of the chromocenter (Fig. 3L), but accumulation of HP1 is dramatically reduced in the $His2Av^{810}$ mutant (Fig. 3O).

To confirm these results, we carried out Western analyses of protein extracts obtained from wild-type and *His2Av* mutant larvae using antibodies against HP1 and histone H3 dimethylated in Lys 9. The results show

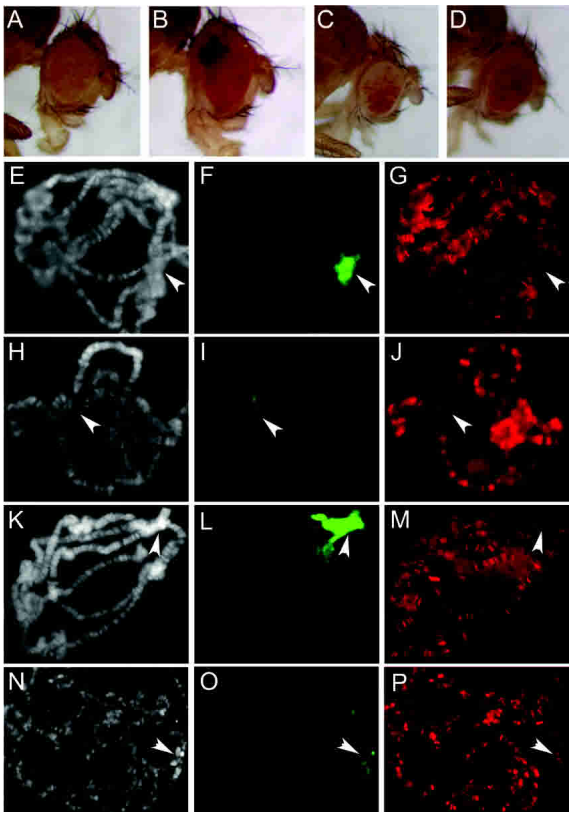

Figure 3. Effect of *His2Av* mutations on heterochromatin silencing. (*A–D*) Effect of *His2Av* mutations on PEV. (*A*) Eye from an adult fly of the genotype *In(1)w^{m4}*. (*B*) Eye from an adult fly of the genotype *In(1)w^{m4}; His2Av^{810}/+*. (*C*) Eye from an adult fly carrying a mutation in the *white* gene and six closely linked copies of a transgene containing the *white* gene. (*D*) The same fly as in panel *O* but also heterozygous for the *His2Av^{810}* mutation. (*E–P*) Histone H3 methylation and HP1 accumulation in wild-type and *His2Av* mutant chromosomes. Polytene chromosomes from third instar larvae were dissected from OR (*E–G,K–M*) and *His2Av^{810}* mutant (*H–J,N–P*) larvae and stained with rabbit polyclonal anti-H3 dimethylated Lys 9, mouse monoclonal anti-HP1, and rat polyclonal anti-Su(Hw) antibodies. The bound antibodies were detected using FITC-labeled goat anti-rabbit or anti-mouse (green) and Texas red-labeled goat anti-rat secondary antibodies (red). Arrows indicate the location of the chromocenter. (*E,H,K,N*) Chromosomes stained with DAPI to visualize DNA. (*G,J,M,P*) Chromosomes stained with anti-Su(Hw) as a control. (*F*) Accumulation of H3 dimethylated Lys 9 in OR chromosomes. (*I*) Accumulation of H3 dimethylated Lys 9 in chromosomes from *His2Av^{810}* larvae. (*L*) Accumulation of HP1 in chromosomes from OR larvae. (*O*) Accumulation of HP1 in chromosomes from *His2Av^{810}* larvae.

little or no accumulation of histone H3 methylated in Lys 9, and lower levels of HP1 in the *His2Av^{810}* mutant (Fig. 2C). Methylation of histone H3 at the Lys 9 residue is carried out by the Su(var)3-9 histone methyltransferase (Rea et al. 2000), and HP1 is encoded by the *Su(var)2-5* gene (Eissenberg et al. 1990, 1992). In order to ensure that the observed effects on the levels of HP1 or the methylation of H3 Lys 9 were not caused by alterations in transcription of *Su(var)3-9* or *Su(var)2-5* due to the *His2Av* mutation, we carried out quantitative RT–PCR analyses of RNA obtained from wild-type and *His2Av^{810}* mutant third instar larvae. The results show that there is no significant changes in the levels of Su(var)3-9 or HP1 RNAs in *His2Av^{810}* mutant larvae when compared to wild type (Fig. 2D). These results and those from immunocytochemistry analyses confirm a role for H2Av in the methylation of H3 Lys 9 and subsequent recruitment of HP1.

Based on the observed effects of *His2Av* mutations on H3 Lys 9 methylation and HP1 recruitment, it appears that the presence of H2Av in heterochromatin might be required prior to these two events. To confirm this hypothesis, we analyzed the pattern of H2Av distribution on polytene chromosomes from larvae carrying mutations in the *Su(var)2-5* and *Su(var)3-9* genes. In both cases, H2Av localization appears normal (Fig. 4), suggesting that the presence of H2Av is required prior to H3 Lys 9 methylation and HP1 recruitment during the establishment of heterochromatin.

H2Av localizes to an ectopic heterochromatin site formed by a transgene array

Dorer and Henikoff (1994) have shown that an ectopic heterochromatin domain could be created by insertion

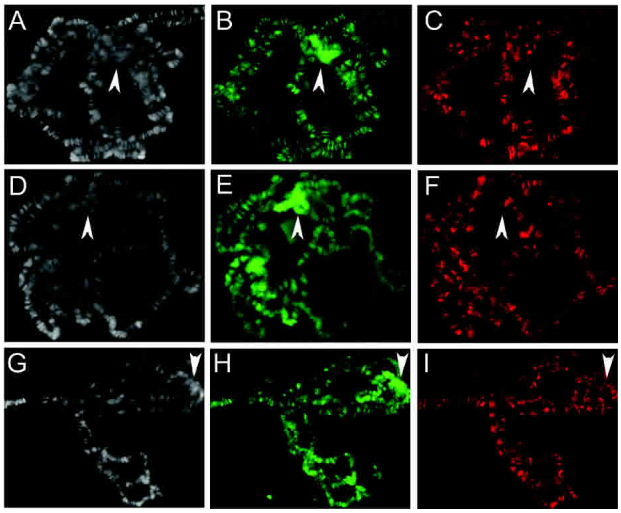

Figure 4. Accumulation of H2Av in chromosomes from wild type and mutant larvae. Polytene chromosomes from third instar larvae were dissected from OR (*A–C*), *Su(var)3-9^{evo}/Su(var)3-9^{06}* (*D–F*), and *Su(var)2-5^{05}/Su(var)2-5^{04}* (*G–I*) mutant larvae and stained with rabbit polyclonal anti-H2Av and rat polyclonal anti-Su(Hw) antibodies. The bound antibodies were detected using FITC-labeled goat anti-rabbit (green; H2Av) and Texas red-labeled goat anti-rat secondary antibodies [red; Su(Hw)]. Arrows indicate the location of the chromocenter. (*A,D,G*) Chromosomes stained with DAPI to visualize DNA. (*B,E,H*) Chromosomes stained with anti-H2Av antibodies in OR (*B*), *Su(var)3-9^{evo}/Su(var)3-9^{06}* (*E*), and *Su(var)2-5^{05}/Su(var)2-5^{04}* (*H*). (*C,F,I*) Chromosomes stained with anti-Su(Hw) antibodies in OR (*C*), *Su(var)3-9^{evo}/Su(var)3-9^{06}* (*F*), and *Su(var)2-5^{05}/Su(var)2-5^{04}* (*I*).

into euchromatin of closely linked multiple copies of a P-element transposon containing the *white* gene. HP1 is recruited to this site, suggesting that ectopic heterochromatin formation by the transgene array follows the same pathway as normal constitutive heterochromatin (Fanti et al. 1998). To test whether H2Av is also involved in ectopic heterochromatin formation or if its role is specific to centromeric heterochromatin, we analyzed the presence of H2Av at the site of integration of transgene repeats. In a strain carrying only one transgene insertion, the *white* gene present in the P transposon is expressed at normal levels, but in strains carrying an array of six closely linked transgenes, expression of the *white* gene shows a characteristic variegated phenotype (Fig. 3C). Mutations in *His2Av* suppress this variegated phenotype, showing a red pigmentation of the eye closer to that of wild-type flies (Fig. 3D). This result suggests a requirement for H2Av in the establishment of ectopic heterochromatin caused by transgene arrays.

To further test this conclusion, we determined whether H2Av is indeed present at the site of transgene insertion. For this, we performed simultaneous fluorescence in situ hybridization (FISH) using the *white* gene as a probe and immunolocalization using antibodies against H2Av. The FISH signal marks the site of insertion of the transgene, which can then be compared to that of H2Av immunostaining. Analysis of polytene chromosomes from a fly strain carrying a single-copy transgene (strain 6-2) shows that the site of insertion is located in an interband, where the chromatin is decondensed (Fig. 5A,C). In this strain, H2Av is not present at the site of insertion, in agreement with the normal expression of the *white* gene observed in these flies (Fig. 5B). When the same experiment was performed with polytene chromosomes from a strain carrying an array of six transposons at the same chromosomal location (strain DX1), the site of insertion was found to be associated with a DAPI-staining band as well as H2Av (Fig. 5E–H). This finding confirms a role for H2Av in ectopic heterochromatin formation, and suggests that compaction of chromatin at an ectopic site as a consequence of the presence of a transgene array follows the same pathway as that used for the formation of centromeric heterochromatin.

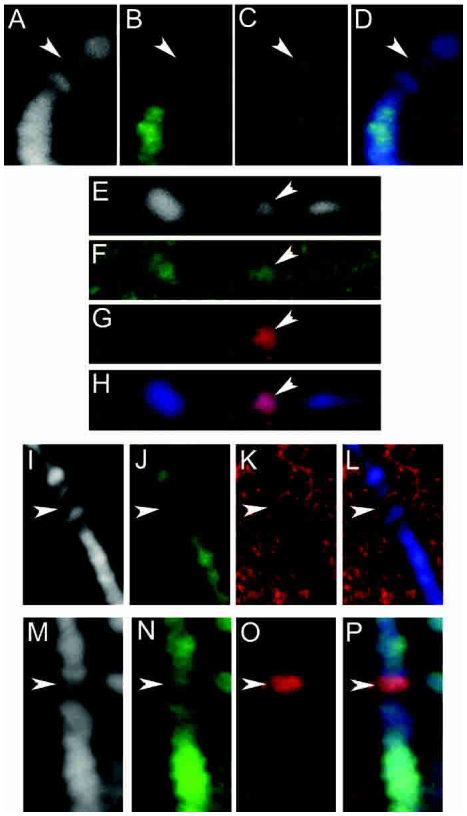

Figure 5. Localization of H2Av and histone modifications at sites of transgene arrays. Polytene chromosomes from third instar larvae were dissected from a strain carrying one copy (strain 6-2; *A–D*,*I–L*) or six copies (strain DX1; *E–H*,*M–P*) of a P-element transgene carrying the *white* gene. Chromosomes were subjected to FISH using sequences from the *white* gene as a probe and immunohistochemistry with rabbit polyclonal anti-H2Av or anti-H4 acetyl Lys 12 antibodies. The bound antibodies were detected using FITC-labeled goat anti-rabbit (green) and the DNA was detected using Texas red-labeled goat anti-mouse secondary antibodies (red). Arrows indicate the location of the insertion site of the transgene. (*A*,*E*,*I*,*M*) DNA stained with DAPI. (*B*) Localization of H2Av in the 6-2 strain at the site of transgene insertion. (*C*) FISH signal in the 6-2 strain at the site of transgene insertion. (*D*) Merge of panels *A*, *B*, and *C*. (*F*) Localization of H2Av in the DX1 strain at the site of transgene insertion. (*G*) FISH signal in the DX1 strain at the site of transgene insertion. (*H*) Merge of panels *E*, *F*, and *G*. (*J*) Localization of H4 acetylated in Lys 12 at the site of transgene insertion in strain 6-2. (*K*) FISH signal at the site of transgene insertion in strain 6-2. (*L*) Merge of panels *I*, *J*, and *K*. (*N*) Localization of H4 acetylated in Lys 12 at the site of transgene insertion in strain DX1. (*O*) FISH signal at the site of transgene insertion in strain DX1. (*P*) Merge of panels *M*, *N*, and *O*.

Histone H4 Lys 12 acetylation and heterochromatic silencing

The main covalent histone modification required for heterochromatin formation is the methylation of histone H3 at the Lys 9 residue. Based on the results described above, this process requires replacement of histone H2A for the H2Av variant. The presence of this variant might allow better access of Su(var)3-9 to the N-terminal tail of histone H3, but it is also possible that other steps not yet uncovered are required before modification of histone H3 can take place. Histone acetylation is usually thought to be involved in transcriptional activation, although there is also evidence for an involvement of this modification in silencing processes (Kelly et al. 2000). In *Drosophila*, mutations in the *chameau* gene, a member of the MYST HAT family of histone acetyltransferases, dominantly suppress position effect variegation, and the Chameau protein is required for Pc-induced silencing (Grienenberger et al. 2002). In addition, histone H4 acetylated in Lys 12 has been found in pericentric heterochromatin in both *Drosophila* and plants (Turner et al. 1992; Wako et al. 2003). We therefore decided to test whether H4 Lys 12 acetylation might

have a role in heterochromatin formation. As previously described (Turner et al. 1992), H4 Lys 12 acetylation is enriched in the centromeric heterochromatin and in euchromatic DAPI-intense bands (Fig. 6B). This pattern of localization is disrupted by mutations in *His2Av*, with a significant reduction in the overall acetylation pattern and specifically in the heterochromatin region (Fig. 6E). This result suggests that acetylation of H4 Lys 12 might play a role also in the formation of heterochromatin at a step subsequent to H2Av deposition. The pattern of H4 Lys 12 acetylation appears normal in polytene chromosomes of larvae carrying mutations in the *Su(var)3-9* and *Su(var)2-5* genes, suggesting that this acetylation event takes place before H3 Lys 9 methylation (Fig. 6H,K). Western analysis performed with third instar larval extracts confirm the immunofluorescence results, indicating that there is reduced H4 Lys 12 acetylation in the $His2Av^{810}$ mutant (Fig. 2E), but the level remains unchanged in $Su(var)3\text{-}9^{evo}/Su(var)3\text{-}9^{06}$ and $Su(var)2\text{-}5^{04}/Su(var)2\text{-}5^{05}$ mutants (data not shown).

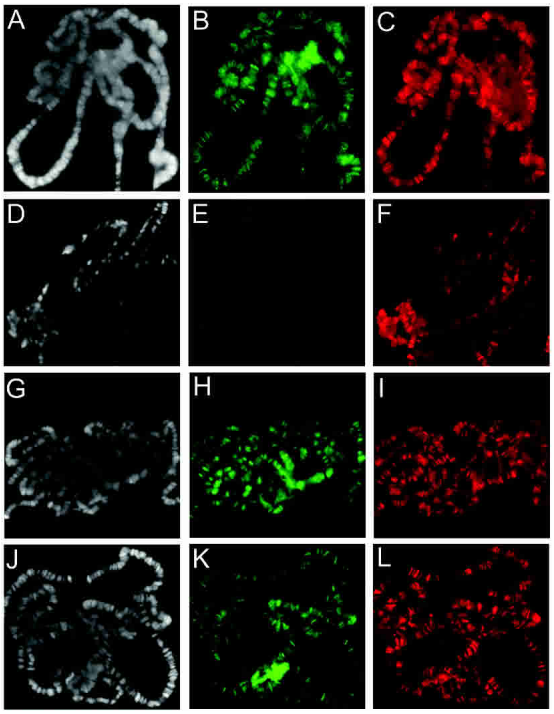

Figure 6. Acetylation of H4 Lys 12 in wild-type and mutant strains. Polytene chromosomes from third instar larvae were dissected from OR (*A–C*), $His2Av^{810}$ (*D–F*), $Su(var)3\text{-}9^{evo}/Su(var)3\text{-}9^{06}$ (*G–I*), and $Su(var)2\text{-}5^{05}/Su(var)2\text{-}5^{04}$ (*J–L*) mutant larvae and stained with rabbit polyclonal anti-H4 acetyl Lys 12 and rat polyclonal anti-Su(Hw) antibodies. The bound antibodies were detected using FITC-labeled goat anti-rabbit (green; H4 acetyl Lys 12) and Texas red-labeled goat anti-rat secondary antibodies [red; Su(Hw)]. (*A,D,G,J*) Chromosomes stained with DAPI to visualize DNA. (*B*) Localization of H4 acetyl Lys 12 in chromosomes from OR. (*C*) Localization of Su(Hw) in chromosomes from OR. (*E*) Localization of H4 acetyl Lys 12 in chromosomes from $His2Av^{810}$. (*F*) Localization of Su(Hw) in chromosomes from $His2Av^{810}$. (*H*) Localization of H4 acetyl Lys 12 in chromosomes from $Su(var)3\text{-}9^{evo}/Su(var)3\text{-}9^{06}$. (*I*) Localization of Su(Hw) in chromosomes from $Su(var)3\text{-}9^{evo}/Su(var)3\text{-}9^{06}$. (*K*) Localization of H4 acetyl Lys 12 in chromosomes from $Su(var)2\text{-}5^{05}/Su(var)2\text{-}5^{04}$. (*L*) Localization of Su(Hw) in chromosomes from $Su(var)2\text{-}5^{05}/Su(var)2\text{-}5^{04}$.

To confirm that acetylation of H4 Lys 12 is important for heterochromatin formation, we tested whether this modification is also involved in the formation of ectopic heterochromatin by transgene arrays. As previously seen for H2Av, chromosomes from a strain containing only one insertion of the transgene show no acetylation of H4 Lys 12 at the insertion site (Fig. 5J). Nevertheless, when six linked copies of the transgene are present at the same cytological location, a new band of H4 Lys 12 acetylation can be observed colocalizing with the new DAPI-positive band at the insertion site (Fig. 5N). Together, these results suggest that acetylation of H4 Lys 12 plays a key role in the formation of the heterochromatin domain at a step subsequent to H2Av replacement. This event might then help in the recruitment of an HDAC to deacetylate H3 Lys 9, which is then followed by methylation of this residue by Su(var)3-9.

Discussion

The establishment of heterochromatin has so far been defined as a four-step process initiated by the RNAi machinery through the production of small RNAs homologous to centromeric DNA repeats that are recruited to prospective heterochromatic regions as part of the RITS complex (Volpe et al. 2002; Verdel et al. 2004). The next step in this process described so far is the deacetylation and subsequent methylation of histone H3 Lys 9, which serves to recruit HP1 (Lachner et al. 2001; Nakayama et al. 2001). HP1 then recruits the Suv4-20 methyltransferase to trimethylate histone H4 at Lys 20 (Kourmouli et al. 2004; Schotta et al. 2004). The work described here suggests that heterochromatin formation is more complex than previously thought, and it involves at least two additional steps. One step requires recruitment of H2Av or replacement of the canonical histone H2A for the H2Av variant. This requirement is highlighted by the observation that mutations in the *His2Av* gene act as suppressors of position effect variegation by modulating the silencing effect of heterochromatin on the adjacent *white* gene.

The replacement of H2A for H2Av is not specific to heterochromatin, and it may also take place in silenced regions of the euchromatin, as it appears that *His2Av* behaves genetically as a *PcG* gene. PcG proteins are responsible for the maintenance of epigenetic silencing of the homeotic genes during *Drosophila* development (Paro 1990). The *His2Av* gene can be classified as a *PcG* gene, since mutations in *His2Av* enhance the phenotype of *Pc* mutants, suppress the phenotype of mutations in *trxG* genes, and cause ectopic expression of the *Ant* gene. The involvement of H2Av in Pc-mediated silencing is not completely unexpected, since H2Av is critical for the establishment of pericentric heterochromatin and both processes share similar strategies. Heterochromatin-induced silencing requires methylation of H3 at Lys 9 by

the Su(var)3-9 histone methyltransferase, whereas Pc-induced silencing involves the recruitment of the ESC–E(z) complex to methylate H3 at Lys 27. Although the modified residues are different, in both cases the modification serves as a tag to bind chromo domain-containing proteins, HP1 in the case of pericentric heterochromatin and Pc in euchromatic silencing. Given the parallels between the two processes, it was surprising to find that replacement of H2Av was required for subsequent H3 Lys 9 methylation in heterochromatin but not for H3 Lys 27 methylation in silenced regions of euchromatin. This later conclusion is supported by the observation that neither H3 Lys 27 methylation nor E(z) recruitment is affected by mutations in *His2Av*.

The requirement of H2Av for Pc recruitment but not for H3 Lys 27 methylation points to a slightly different strategy in the establishment of silencing in the euchromatin compared to heterochromatin. Heterochromatic silencing appears to involve a series of events that take place in a linear pathway; each event is dependent on the previous one for proper heterochromatin assembly. In this cascade of events, replacement of H2A by H2Av appears to be an early step in the process, although our results cannot distinguish among the possibilities that H2Av is recruited before, after, or in parallel to the recruitment of the RITS complex by the RNAi machinery. Surprisingly, the observed effects of H2Av on Pc-mediated silencing point to several possible mechanisms, all slightly different from that involved in heterochromatin formation. One formal possibility that would be consistent with the results but we consider less likely is that H2Av acts downstream of Pc; recruitment of this protein would be required for H2Av replacement, which would then in turn stabilize the binding of the Pc complex. A second possibility is that H2Av replacement is a relatively late event in the process, acting downstream of H3 Lys 27 methylation instead of being required for this modification. In this scenario, H2Av would not be required for the recruitment of the ESC–E(z) complex, but it would be required subsequently to alter chromatin structure and allow Pc recruitment. Alternatively, euchromatic Pc-mediated silencing might be accomplished by two relatively independent parallel pathways that converge at the end to ensure Pc binding to the chromatin. One pathway would alter chromatin structure by recruiting ESC–E(z) and methylating H3 at Lys 27; a second parallel but independent pathway would further alter chromatin structure by replacing H2A for H2Av. Both processes would then be required for the recruitment of the Pc-containing PRC-1 complex.

The apparent association of H2Av with silenced regions might appear puzzling in view of findings in other systems. In *Tetrahymena*, H2A.Z is present in the transcriptionally active macronucleus, but it is not detected in the silenced micronucleus (Allis et al. 1980). In *S. cerevisiae*, the H2A.Z histone variant Htz1 is required for the expression of SWI/SNF-dependent genes such as *PHO5* and *GAL1*; interestingly, although Htz1 is required for activation, it is present at higher levels in the promoters of these genes when they are repressed than when they are transcriptionally active (Santisteban et al. 2000). Nevertheless, Htz1 has also been shown to be present at silenced loci in yeast, and to be required for HMR and telomere-induced silencing (Dhillon and Kamakaka 2000). A detailed analysis of the role of Htz1 in gene expression in yeast using whole-genome microarrays resulted in the identification of 214 genes that are activated and 107 genes that are repressed by Htz1. Htz1-activated genes are located adjacent to heterochromatin; Htz1 is enriched in the promoters and coding regions of activated genes, where it appears to block the spreading of heterochromatic silencing (Meneghini et al. 2003).

One possible explanation for the functional differences between the yeast and *Drosophila* H2A.Z homologs is that *Drosophila* lacks H2A.X, and, therefore, H2Av plays the roles of both histone variants. Nevertheless, the localization of H2A.Z in heterochromatin is not limited to *Drosophila*. Studies in mice also show an association of this histone variant with pericentric heterochromatin (Rangasamy et al. 2003). The apparent contradiction between the two conflicting roles for H2A.Z in various organisms could be explained if the role of this histone variant is to assemble a chromatin that is more accessible to other chromatin-remodeling complexes, and that the final result depends on the type of factors recruited to H2A.Z-containing chromatin. This possible role is consistent with the finding that the functionally essential C-terminal domain of H2A.Z (Clarkson et al. 1999) is exposed on the surface of H2A.Z-containing nucleosomes, and therefore it could serve to recruit other factors (Suto et al. 2000). Analytical ultracentrifugation experiments suggest that nucleosomes reconstituted with H2A.Z have decreased stability compared to those reconstituted with the canonical H2A histone, and therefore might allow greater accessibility of various factors to H2A.Z-containing chromatin (Abbott et al. 2001). The decrease in stability is also supported by the crystal structure of H2A.Z-containing nucleosomes, which suggests an altered interface between the H2A.Z–H2B dimer and the H3–H4 tetramer (Suto et al. 2000). Alternatively, the exposed domain of H2A.Z could mediate internucleosome interactions to give rise to higher-order compacted chromatin structures (Fan et al. 2002). The H2A.Z-containing chromatin fiber contains regularly spaced nucleosomes, an arrangement that is characteristic of pericentric heterochromatin (Wallrath and Elgin 1995).

The results discussed here suggest the complex multistep model for heterochromatin assembly shown in Figure 7. The nature of the first step in the process might involve the RNAi machinery and the recruitment of the RITS complex, although it is also possible that H2Av recruitment takes place prior to this step. The mechanism by which H2Av recruitment is controlled is unclear. Recent studies in yeast have shown that a complex containing swr1, an Snf-2-related ATP-dependent chromatin-remodeling factor, is able to efficiently exchange H2A for H2A.Z (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). A *Drosophila* homolog of *SWR1* is *domino*, which has been characterized as a *PcG* gene

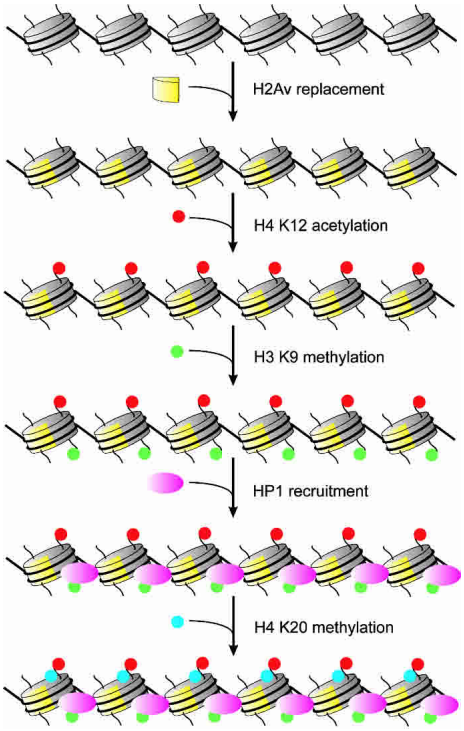

Figure 7. Possible pathway for heterochromatin formation in *Drosophila*. Initiation of silencing might occur by sequence-specific targeting of the RITS complex, although it is not clear whether this step is prior to or follows the exchange of H2A for H2Av in the nucleosome. This exchange triggers acetylation of H4 Lys 12, followed by deacetylation and methylation of H3 Lys 9. HP1 is then targeted to the methylated Lys 9 residue of histone H3, which recruits the Suv4-20 methyltransferase to trimethylate histone H4 at Lys 20.

and a dominant suppressor of PEV (Ruhf et al. 2001). It is plausible that RITS recruits a Domino-containing chromatin-remodeling complex, which in turn replaces histone H2A for H2Av. This alteration would then facilitate the recruitment of histone-modifying enzymes that would further change chromatin structure. Our results suggest that the next step in the process requires the acetylation of histone H4 Lys 12, but the nature of the enzyme responsible for acetylation of H4 Lys 12 is not known. A candidate for this role could be the product of the *chameau* gene, which encodes a MYST domain histone acetyltransferase. Mutations in *chameau* behave as *PcG* genes and suppress PEV (Grienenberger et al. 2002), but they fail to affect H4 Lys 12 acetylation (J. Swaminathan and V. Corces, unpubl.), suggesting that the Chameau protein affects a different step in the establishment of heterochromatin. Acetylation of H4 Lys 12 is then followed by methylation of H3 Lys 9 and recruitment of HP1, which in turn tethers Svar4-20 to methylate H4 Lys 20. Additional steps are likely to be required in this complex process. Analysis of the large collection of *Su(var)* and *E(var)* mutations identified in *Drosophila* should make possible the elucidation of additional steps in this pathway, resulting in a comprehensive understanding of the molecular events involved in heterochromatin formation.

Materials and methods

Fly stocks

Fly stocks were maintained in standard medium at 25°C, 75% humidity. Oregon R (OR) larvae were used as wild-type controls for all experiments. The *In(1)w^{m4}* strain was obtained from the Bloomington *Drosophila* Stock Center. The H2Av-null allele *His2Av^{810}/TM6B Tb* was obtained from Sarah Elgin (Department of Biology, Washington University, St. Louis, MO), and *His2Av^{05146}/TM6B Tb* was obtained from the *Drosophila* Bloomington Stock Center. The *His2Av^{05146}* allele is a hypomorph, and flies homozygous for this mutation die during the early stages of pupal development. The *His2Av^{810}* allele appears to be a null; the lethal period of flies homozygous for this mutation is late third instar/prepupae, and this lethal period is the same in flies of the genotype *His2Av^{810}/Df(3)Tl-I*, where *Df(3)Tl-I* is a deletion of the 97B–97E region containing the *His2Av* gene. *ash1^{VF101} trx^{b11}/TM3 Sb*, *brm^{2} trx^{E2}/TM3 Sb* and *Df (3R)Pc/TM3 Sb Ser* were obtained from Allen Shearn (Department of Biology, Johns Hopkins University, Baltimore, MD). The *Su(var)3-9^{evo}* and *Su(var)3-9^{06}* alleles were obtained from Allen Shearn, and *Su(var)2-5* alleles were from Joel Eissenberg (Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO) and Sergio Pimpinelli (Fondazione Cenci Bolognetti and Dipartimento, di Genetica e Biologia Molecolare, Universita "La Sapienza," Roma, Italy). Transgene array stocks were obtained from Steven Henikoff (Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, WA) and Doug Dorer (Department of Microbiology, Meharry Medical College, Nashville, TN). All other strains were obtained from the *Drosophila* Bloomington Stock Center.

Immunocytochemistry of polytene chromosomes

Wandering third instar larvae were collected, and salivary glands were removed and fixed in 3.7% formaldehyde, 45% acetic acid mix for 2 min and squashed in 45% acetic acid on subbed slides. The slides were frozen in liquid nitrogen and stored dry at –80°C until ready to use. Slides were blocked in antibody dilution buffer (1× PBS, 1% non-fat dry milk, 0.1% Triton X-100), and then were incubated overnight in dilution buffer containing primary antibodies at 1:1000 (anti-H2Av), 1:100 (anti-dimethyl histone H3, Lys 9), 1:100 (anti-HP1), 1:100 (anti-acetylated histone H4, Lys 12), 1:100 (anti-Pc), 1:100 [anti-Su(Hw)], 1:100 [anti-E(z)], 1:100 (anti-trimethylated histone H3, Lys 27). Following primary antibody incubation, slides were washed in antibody dilution buffer three times and incubated with FITC conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) at 1:200 dilution for 2 h at room temperature. Slides were again washed, rinsed briefly in 1× PBS, stained for 2 min with 4′,6-diamidino-2-phenylindole (DAPI), 0.5 µg/mL, and mounted in Vectashield anti-fade mounting medium (Vector Laboratories). Chromosomes were viewed using a Zeiss Axiophot microscope and a Photometrics cooled CCD camera. H2Av antibodies were obtained from Robert Glaser (Wadsworth Center, New York State Department of Health, State University of New York, Albany, NY), phosphorylated RNA polymerase II antibody from Joseph Gall (Department of Embryology, Carnegie Institution of Washington, Baltimore, MD, and Depart-

ment of Biology, Johns Hopkins University, Baltimore, MD), and HP1 antibody from Sarah Elgin. H3 dimethyl Lys 9 and H4 acetylated Lys 12 antibodies were obtained from Upstate USA, Inc. Antibodies against trimethylated H3 Lys 27 were obtained from Thomas Jenuwein (Research Institute of Molecular Pathology, The Vienna Biocenter, Vienna, Austria); the E(z) antibody was a gift of Richard Jones (Department of Biological Sciences, Southern Methodist University, Dallas, TX).

CNS preparation and immunocytochemistry

Wandering third instar larvae were dissected and the central nervous system was fixed with 2% paraformaldehyde for 20 min and then washed in antibody dilution buffer (1× PBS, 1% non-fat dry milk, 0.1% Triton X-100), three times for 30 min and then incubated with primary antibody overnight at 4°C. The rest of the procedure was the same as described above. The primary antibodies used were anti-Ubx (1:50) and anti-Antp (1:50), both of them mouse monoclonal antibodies; anti-mouse FITC or Texas red secondary antibodies (IgG) were obtained from Jackson Immunoresearch Laboratories.

Western and RT–PCR analyses

Wandering third instar larvae were collected, frozen in liquid nitrogen, and homogenized in either HEMGN buffer (25 mM HEPES-KOH at pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 mM DTT, 0.1 mM PMSF, 0.3 M KCl) or in cold sodium dodecyl sulfate (SDS) sample buffer (20 mM sodium phosphate, 2% SDS, 0.001% bromophenol blue, 0.2 M DTT, 2% glycerol). In the former case, an equal volume of 2× SDS reducing buffer was added and then boiled for 5 min. The extract was spun at room temperature in a microfuge for 10 min, and the supernatant was run on an SDS–15% polyacrylamide gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane. After transfer, the presence of H2Av, H3 dimethyl Lys 9, HP1, or H4 acetyl Lys 12 was detected by standard immunodetection methods using the Super signal West Pico chemiluminescent substrate from Pierce.

Total RNA from third instar larvae was prepared as previously described (Gerasimova et al. 1995). RT–PCR reactions were carried out using [^{32}P]dATP and the Titan One Tube kit (Roche) following the instructions provided by the manufacturer. Primers for the PCR reactions span exons of the *Su(var)2-5* and *Su(var)3-9* genes; the primers used were 5′-GCAAGGGAAAGGTGGAGTA-3′ and 5′-CCAGGATAGGC GCTCTTC-3′ for *Su(var)2-5*, and 5′-CATCACAATCAAGC TGGAAC-3′ and 5′-GATCTGCAGATTGAGTGGC-3′ for *Su(var)3-9*. RT–PCR experiments were carried out with increasing amounts of RNA, varying from 65 to 1000 ng, to ensure a linear range of response. The results shown in Figure 2D were obtained using 125 ng of RNA for Su(var)3-9 and 250 ng of RNA for Su(var)2-5.

In situ hybridization to polytene chromosomes

To detect the location of P-element transgenes on polytene chromosomes, we used a 3-kb DNA fragment from the *white* gene obtained by PCR. The primers used were 5′-CATGAGAG GTACGACAACCAT-3′ and 5′-GCCGAGCATCTGAACATG TG-3′, which amplify a 3-kb fragment. Digoxigenin-labeled DNA was prepared using the Prime-A-Gene random priming kit (Promega). The labeled probe was ethanol-precipitated and stored in hybridization buffer—4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 1× Denhardt's containing 0.4 mg/mL of salmon sperm DNA—until ready to use. Polytene chromosomes from third instar larvae were prepared as described above, except that the glands were fixed in acetic acid–lactic acid mixture (1 acetic acid:2 water:3 lactic acid). The slides were frozen in liquid nitrogen, and coverslips were removed and stored in ethanol at –70°C until ready to use. Slides were warmed to room temperature and heated in 2× SSC for 1 h, then dehydrated using an ethanol series and denatured in 0.07 M NaOH. The slides were again dehydrated and allowed to air dry. For hybridization of DNA, boiled probes were added to the slide and covered immediately with a cover slip. The slide and coverslip were sealed with rubber cement and incubated at 37°C overnight in a humidified chamber. Following hybridization, coverslips were removed, and the slides were washed in 2× SSC. Antibody detection and visualization were carried out as outlined above.

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