The *lawc* Gene Is a New Member of the *trithorax*-Group That Affects the Function of the *gypsy* Insulator of Drosophila

Izanne D. Zorin, Tatiana I. Gerasimova and Victor G. Corces

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Manuscript received November 16, 1998
Accepted for publication April 7, 1999

ABSTRACT

Mutations in the *lawc* gene result in a pleiotropic phenotype that includes homeotic transformation of the arista into leg. *lawc* mutations enhance the phenotype of *trx*-G mutations and suppress the phenotype of *Pc* mutations. Mutations in *lawc* affect homeotic gene transcription, causing ectopic expression of Antennapedia in the eye-antenna imaginal disc. These results suggest that *lawc* is a new member of the *trithorax* family. The *lawc* gene behaves as an enhancer of position-effect variegation and interacts genetically with *mod(mdg4)*, which is a component of the *gypsy* insulator. In addition, mutations in the *lawc* gene cause alterations in the punctated distribution of mod(mdg4) protein within the nucleus. These results suggest that the lawc protein is involved in regulating the higher-order organization of chromatin.

INSULATOR elements are DNA sequences that interfere with the ability of an enhancer to act on a promoter when placed between the two (Jack *et al.* 1991; Geyer and Corces 1992; Kellum and Schedl 1992). It has been hypothesized that insulators define separate areas of gene activity by establishing higher-order chromatin domains. In such a way, promoters from one gene could be sheltered from action of enhancers from a neighboring gene (Gdula *et al.* 1996; Gerasimova and Corces 1996). In addition, transgenes flanked by insulator elements are expressed regardless of the integration site in the chromosomes, *i.e.*, insulators can block repression of transgene expression due to adjacent sequences (Kellum and Schedl 1991; Roseman *et al.* 1993). Several insulator elements have been identified, including the scs and scs' sequences of Drosophila (Kellum and Schedl 1991), a subset of the *Fab-7* DNA sequences of the *Drosophila bithorax* complex (Hagstrom *et al.* 1996; Zhou *et al.* 1996; Mihaly *et al.* 1997), and sequences present in the *gypsy* retrotransposon (Jack *et al.* 1991; Geyer and Corces 1992). In vertebrates, a DNA sequence at the 5′ end of the chicken β-globin gene (Chung *et al.* 1997) and sequences present in the human apolipoprotein B gene (Kalos and Fournier 1995) have also been shown to affect enhancer-promoter communication.

The *gypsy* insulator is present in the 5′ transcribed untranslated region of the *gypsy* retrotransposon of Drosophila (Gdula *et al.* 1996). Three components of this insulator have been identified: a 350-bp sequence of the *gypsy* retrotransposon, the su(Hw) protein that binds to *gypsy* DNA via its zinc fingers, and the mod(mdg4) protein that interacts with su(Hw). In the case of the *yellow*^{2} (*y*^{2}) mutation, the *gypsy* insulator causes a unidirectional repression of *yellow* gene enhancers such that those distal to the *yellow* promoter with respect to the *gypsy* insulator are repressed, whereas the enhancers proximal to the promoter relative to the insulator are active. In the absence of su(Hw) protein, the enhancers are all active and the *y* phenotype reverts to wild type, indicating that su(Hw) is an essential component of the *gypsy* insulator. In the presence of hypomorphic mutations in the *mod(mdg4)* gene, the *gypsy* insulator is only partially functional, and all enhancers (both upstream and downstream of the insulator) become partially active; for example, in the posterior abdominal segments, the *y* body cuticle enhancer is active in some cells and pigment is produced, whereas the enhancer is inactive in other cells, resulting in a loss of pigmentation. This effect manifests itself in a variegated abdominal pigment phenotype in *y*^{2} *mod(mdg4)* flies (Gerasimova *et al.* 1995). This phenotype is reminiscent of the position-effect variegation (PEV) seen when a gene is juxtaposed to heterochromatin through chromosomal rearrangements (Casperson and Schultz 1938). In agreement with this effect, mutations in *mod(mdg4)* act as typical *Enhancer of variegation* [*E(var)*] mutants by enhancing the phenotype of the *white-mottled4* (*w*^{m4}) allele, suggesting that the mod(mdg4) protein acts at the level of chromatin organization (Dorn *et al.* 1993; Gerasimova *et al.* 1995).

In support of this function at the level of chromatin structure, *mod(mdg4)* has been shown to display the properties characteristic of members of the *trithorax*-Group (*trx*-G; Gerasimova and Corces 1998) whose products [as well as those of the *Polycomb*-Group (*Pc*-G)] are thought to maintain homeotic gene expression through stable chromatin conformation changes (re-

Corresponding author: Victor G. Corces, Department of Biology, The Johns Hopkins University, 3400 North Charles St., Baltimore, MD 21218. E-mail: corces@jhu.edu

viewed by Kennison 1995). Homeotic genes determine segment identity along the anterior-posterior axis (Lewis 1978; Kaufman *et al.* 1980). The gap and pair-rule genes initially determine the expression domains of the homeotic genes (Ingham 1983); for example, *fushi tarazu* is necessary for the activation of both Antennapedia complex and bithorax complex genes (Ingham and Martinez-Arias 1986). However, gap and pair-rule genes are not expressed beyond early stages of embryogenesis. The *trx*-G and *Pc*-G gene products help maintain homeotic gene expression domains beyond the time when the gap and pair-rule genes themselves are no longer expressed, and indeed, throughout development (Kennison and Tamkun 1988; Kuziora and McGinnis 1988). Null mutations in *Pc*-G and *trx*-G genes are lethal, but phenotypes can be observed in heterozygous flies. The *trx*-G and *Pc*-G gene products act antagonistically and mutations in one group suppress mutations in the other (Ingham 1983; Capdevila *et al.* 1986). The *trx*-G gene products maintain homeotic gene expression within their normal domains (Breen and Harte 1993) and, when mutant, cause a variety of homeotic phenotypes, for example, haltere-to-wing transformation (Shearn 1989). The *Pc*-G gene products repress homeotic gene expression outside the normal domains (Kuziora and McGinnis 1988) and, when mutant, cause an extra sex combs phenotype indicative of transformation toward the first leg (Duncan 1982). *trx*-G and *Pc*-G gene products are thought to form large protein complexes (see Paro and Harte 1996 for a review) and these complexes compete for the same binding sites in and around homeotic genes called Pc response elements (PREs; Orlando and Paro 1993; Gindhart and Kaufman 1995).

Members of the *trx*-G include *trithorax* (*trx*; Capdevila and Garcia-Bellido 1981), *brahma* (*brm*; Kennison and Tamkun 1988), *absent, small, or homeotic discs1* and *2* (*ash-1* and *ash-2*; Shearn 1989), and *mod(mdg4)* (Gerasimova and Corces 1998). Another member, *trithorax-like* (*Trl*), encodes the GAGA factor (Farkas *et al.* 1994), which has been shown to participate in chromatin remodeling during transcription (Tsukiyama *et al.* 1994). The *Pc*-G includes *Polycomb* (*Pc*; Lewis 1978), *polyhomeotic* (*ph*; Dura and Brock 1985), *Polycomblike* (*Pcl*; Duncan 1982), and *extra sex combs* (*esc*; Struhl 1981). The Pc protein has a domain homologous to the nonhistone heterochromatin-associated protein HP1, which is encoded by the suppressor of PEV gene *Su(var)205* (James and Elgin 1986; Eissenberg *et al.* 1990).

For a gene to be classified as a member of the *trx*-G, certain genetic criteria (described by Shearn 1989) need to be met: (1) the gene should have a homeotic mutant phenotype; (2) mutations in the gene should enhance the severity and frequency of homeotic transformations due to other *trx*-G members (*e.g.*, flies doubly heterozygous for *trx* and *ash-1* have a higher number of, and more extreme, transformations than either *trx* or *ash-1* heterozygotes alone); (3) *trx*-G mutants should suppress the dominant extra sex comb phenotype of *Pc* mutants. The *lawc* mutation has been mapped to position 23.0 on the X chromosome (Simonova *et al.* 1992). We noted that the homeotic phenotype of *lawc* mutations is enhanced by *mod(mdg4)* and decided to investigate the relationship between these two genes further. Using the criteria listed above, we have determined that *lawc* is a *trx*-G member. *lawc* enhances the *mod(mdg4)* phenotype and vice versa, and like *mod(mdg4)* and *Trl*, *lawc* acts as an enhancer of position-effect variegation. In addition, mutations in the *lawc* gene affect the subnuclear distribution of *gypsy* insulator components. These results suggest that the lawc protein might play a general and fundamental role in chromatin organization.

MATERIALS AND METHODS

Drosophila stocks and crosses: Flies were kept in standard medium and grown at 23°, 75% humidity. The $mod(mdg4)^{u1}$ allele is a spontaneous mutation caused by a Stalker transposable element insertion (Gerasimova *et al.* 1995), which is viable and behaves genetically as a hypomorph. $lawc^{+10}$ is a revertant of $lawc^{P1}$ that was generated by crossing $lawc^{P1}$ to a transposase-supplying line and the subsequent excising of the *P* element. *l(1)EF520/FM7* was obtained from Dr. Norbert Perrimon; $ash\text{-}1^{VF101}$/*TM3*, trx^{B11}/*TM3*, trx^{B11} $ash\text{-}1^{VF101}$/*TM1*, and *Df(3L)Pc-Mk/TM6* were received from Dr. Allen Shearn; brm^{2} trx^{E2}/*TM3* was obtained from Dr. Jim Kennison. Pc^{4}/*TM3*, brm^{2}/*TM6*, $In(1)w^{M4}$, and *Df(1)RA2/TM3* were obtained from the Bloomington Fly Stock Center. The cytological limits of *Df(3L)Pc-Mk* are 78A2-78C9.

Immunolocalization of proteins: Antibodies were obtained from the following investigators. Antp antibodies were received from Dr. Matt Scott, Ubx antibodies from Dr. Juan Botas and Dr. Javier Lopez, and labial antibodies from Dr. Bill McGinnis. Immunolocalization of proteins on fly tissues was performed as described by LaJeunesse and Shearn (1995). Third instar larvae were dissected in PBS and fixed in 4% formaldehyde for 25 min. The tissue was incubated in 0.1% Triton X-100, 0.3% BSA, and 0.5% sheep serum in PBS for 30 min. The primary antibody was then added and the samples were incubated on a shaker overnight at room temperature. The samples were washed six times for 30 min in PBT (PBS, 0.1% Triton X-100, and 0.3% BSA). FITC-conjugated secondary antibodies were then added and the samples were incubated for 2 hr at room temperature. Samples were washed again and the tissue was examined in a Zeiss (Thornwood, NY) microscope.

For the analysis of the nuclear distribution of mod(mdg4) protein, larvae were dissected in Cohen's buffer (25 mm glycerophosphate, 10 mm potassium phosphate, 30 mm potassium chloride, 10 mm magnesium chloride, 3 mm calcium chloride, 160 mm sucrose, and 0.5% NP40) and tissues were fixed for 25 min at room temperature in 0.1 m sodium chloride, 2 mm potassium chloride, 10 mm phosphate, 2% NP40, and 2% paraformaldehyde and transferred to 45% acetic acid. After 10 min, the imaginal discs were dissected out and placed in a drop of 45% acetic acid on a polylysine-treated slide. A siliconized coverslip was placed over the sample and then firmly pressed down to squash the disc tissue. The slides were then frozen at −80° for an hour. The coverslips were then removed and the slides placed into a Coplin jar containing

antibody dilution buffer (130 mm sodium chloride, 10 mm sodium phosphate, 0.1% Triton X-100, and 1% BSA) for 5 min. The buffer was changed twice and the slides were incubated with 20 μl of a 1:250 dilution of mod(mdg4) antirat antibody in a humidity chamber overnight at 4°. The slides were then washed three times in antibody dilution buffer and incubated in 20 μl of Texas Red-conjugated secondary antibodies diluted 1:200 in antibody dilution buffer. After incubation at room temperature in the dark for 1 hr, the slides were washed three times in antibody dilution buffer and then stained with 4′,6-diamidino-2-phenylindole (DAPI). Antifade mounting medium (Vectashield) was placed on the slides and covered with a coverslip. Slides were viewed under a Zeiss microscope at ×100 magnification.

RESULTS

The *lawc^{P1}* mutation results in homeotic transformations: Mutations in the *lawc* gene result in an arista-to-leg homeotic transformation. The *lawc^{P1}* mutation was generated by Simonova and coworkers (1992) and is ~1% penetrant for complete transformation of the arista into leg, such that leg segments and tarsal claws can be observed instead of arista tissue (Figure 1B). *lawc^{P1}* is fully penetrant for partial transformation into leg, which is manifested by a thickening of the arista (Figure 2B). In addition, *lawc^{P1}* mutants often have ectopic bristles on the scutellum and thorax (Figure 1D) and the wings are held apart with multiple incisions in the margins, especially along the posterior margin (Figure 1F). Due to this pleiotropic phenotype, the mutation was called *leg, arista, wing complex* (*lawc*). The mutant phenotype is stronger in males than in females. The *lawc^{P1}* mutation is caused by the insertion of a *P* element at approximately position 7E on the X chromosome (Simonova *et al.* 1992). We used overlapping deficiencies to map *lawc* and found that *Df(1)KA14*, which lacks 7F1 to 8C6, complements *lawc^{P1}*, whereas *Df(1)RA2*, which deletes 7D10 to 8A4,5, fails to complement the *lawc* gene. Most *lawc^{P1}/Df(1)RA2* females die as early larvae. The 10% that do eclose have an enhanced *lawc* phenotype (Figure 2C) that includes strong arista-to-leg transformation, many ectopic bristles, and larger wing margin incisions. *l(1)EF520* (Lefevre 1976) is an EMS-induced allele that causes early larval lethality. The 3% of *lawc^{P1}/l(1)EF520* females that eclose show an enhanced *lawc* phenotype, including arista-to-leg transformation (Figure 2D), indicating that *l(1)EF520* is an allele of *lawc*. Because the phenotype of *lawc^{P1}/l(1)EF520* females is the same as that of *lawc^{P1}/Df(1)RA2*, the *l(1)EF520* mutation is an allele of *lawc* that behaves genetically as a null. We will refer to this mutation as *lawc^{EF520}*.

***lawc* has the properties of a *trx*-G gene:** On the basis of homeotic transformation and the pleiotropic phenotype of the *lawc^{P1}* mutation, we hypothesized that *lawc* may be a new member of the *trx*-G family. For a gene to be classified as a *trx*-G member, mutations in that gene should enhance the phenotype of other *trx*-G members

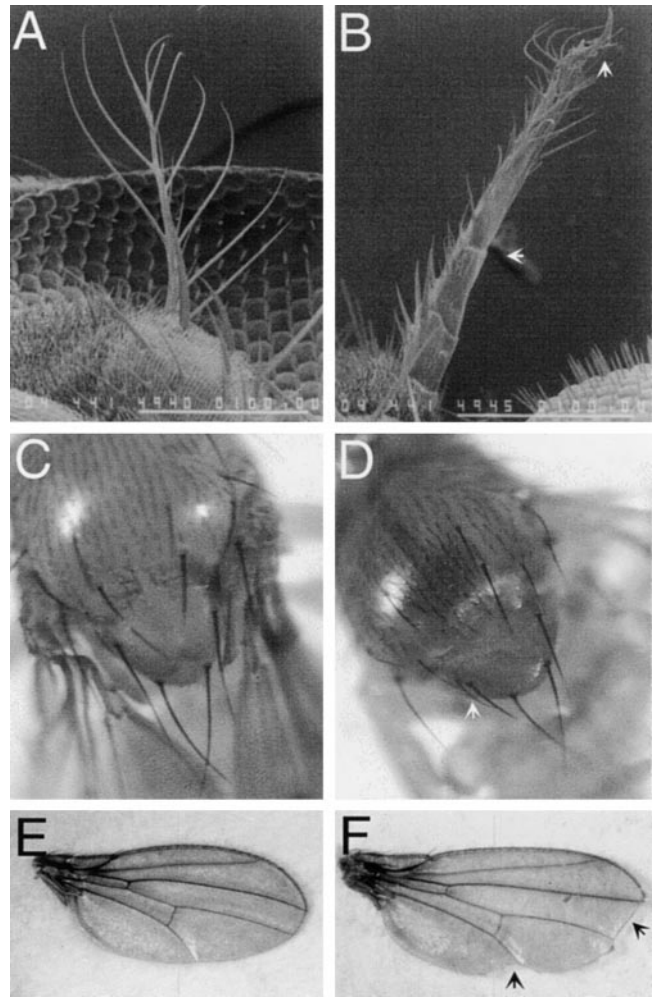

Figure 1.—Phenotypes of *lawc* mutants. (A, C, and E) Wild-type Oregon R flies. (A) Arista with characteristic branched appearance. (C) Scutellum with four macrochaete. (E) Wing with smooth, entire wing margins. (B, D, and F) *lawc^{P1}* flies. (B) Complete transformation of arista to leg; note the leg segments and tarsal claw (arrows). (D) Scutellum with five instead of four macrochaete (arrow). (F) Wing with incisions in the posterior wing margin (arrows).

(Shearn 1989). To test this possibility, *lawc^{P1}/lawc^{P1}* females were crossed to *trx^{B11}/TM3* males and the resulting progeny were analyzed for transformations of haltere-to-wing, posterior abdominal segments into more anterior, or third leg into second leg. No additional transformations were seen in the hemizygous *lawc^{P1}; trx^{B11}/+* males of the F_1 progeny compared to the control *lawc^{P1}; +/+* (Table 1). An increase in transformation frequency or severity was also not observed in *lawc^{P1}; brm^{2}/+* or *lawc^{P1}; ash-1^{VF101}/+* males (Table 1). We speculated from these results that *lawc^{P1}* might be too weak an allele to show an effect in combination with single heterozygous mutant *trx*-G members such as *trx^{B11}*, *ash-1^{VF101}*, or *brm^{2}*. To test this possibility, we analyzed whether combinations of two *trx*-G mutations could enhance the *lawc* phenotype. *trx^{B11} ash-1^{VF101}/++* and *brm^{2}*

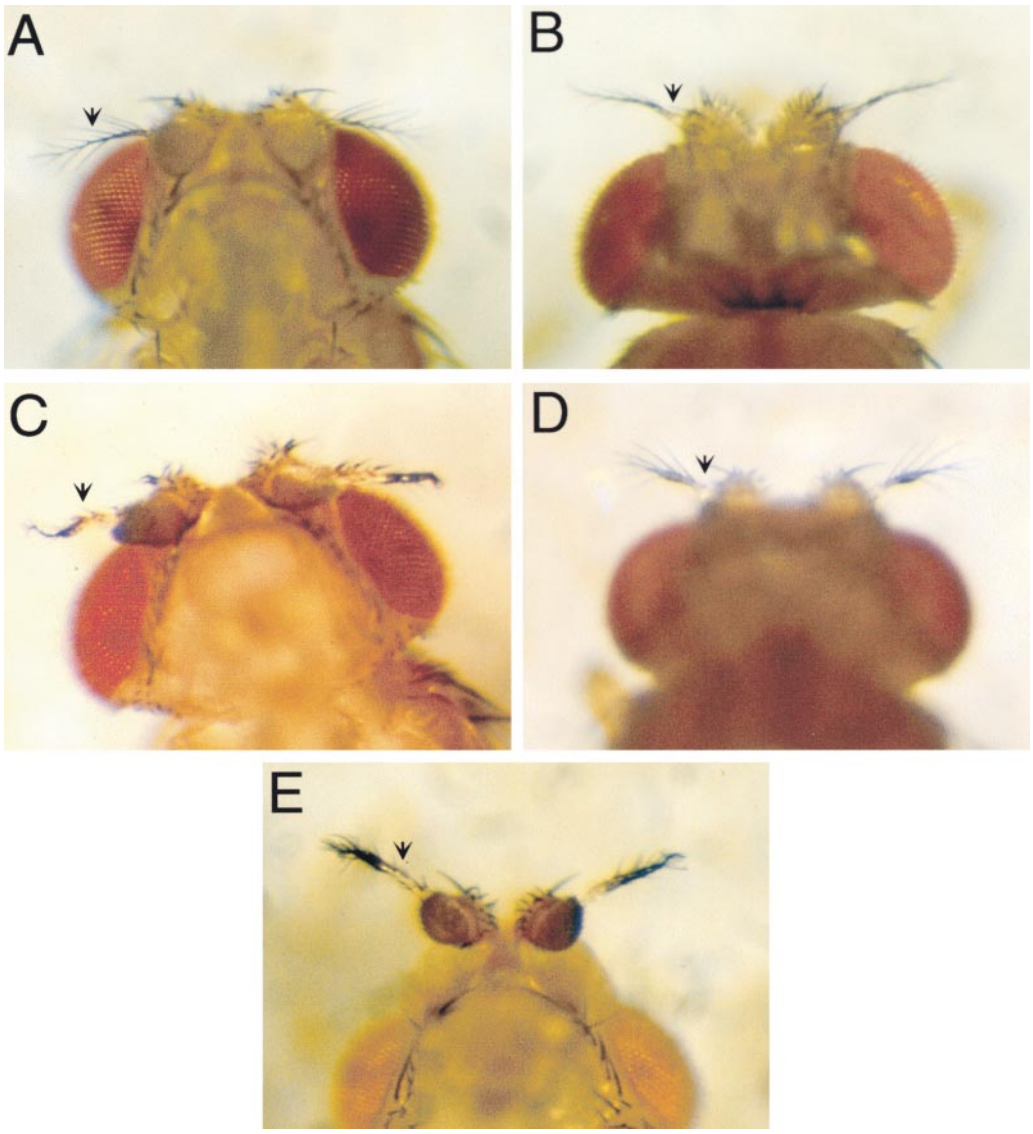

Figure 2.—Homeotic transformations in *lawc* mutants. (A) Oregon R. (B) $lawc^{P1}$ (partial transformation). (C) $Df(1)RA2/lawc^{P1}$. (D) $lawc^{EF520}/lawc^{P1}$. (E) $lawc^{P1}$; $mod(mdg4)^{u1}$.

TABLE 1

Interactions of *lawc* alleles with *trx*-G and *Pc*-G genes

	Females						
		$lawc^{P1}$	$lawc^{EF520}$	*Df(1)RA2*	$Lawc^{+10}$	$trx^{B11}ash\text{-}1^{VF101}$	$brm^{2}trx^{E2}$
Males	Oregon R	$lawc1^{P1}$	*FM7*	*FM7*	$lawc^{+10}$	*TM1*	*TM3*
$trx^{B11}/TM3$	211/0	249/0	213/0	250/0	234/0		
$brm^{2}/TM6$	217/0	238/0	210/0	249/0	198/0		
$ash\text{-}1^{VF101}/TM3$	292/0	372/0	297/0	212/0	254/0		
$trx^{B11}ash\text{-}1^{VF101}/TM1$	272/14	338/64	218/69	274/27	261/12		
$brm^{2}trx^{E2}/TM3$	192/10	268/29	215/28	249/18	229/9		
$lawc^{P1}$						270/44	245/16
$Df(3L)Pc\text{-}Mk/TM3$	276/83	258/3			232/80		
$Pc^{4}/TM3$	192/57	124/2			244/59		

Homozygous females of the genotypes indicated in the top row were crossed to males of the genotypes listed in the first column. Resulting progeny were examined for homeotic transformations such as haltere to wing, or third leg to second leg. In the case of *Pc*-G mutants, the progeny were examined for second or third legs transformed toward first legs, *i.e.*, sex combs on the second or third legs. Data are presented as number of flies examined/percentage of flies with transformations.

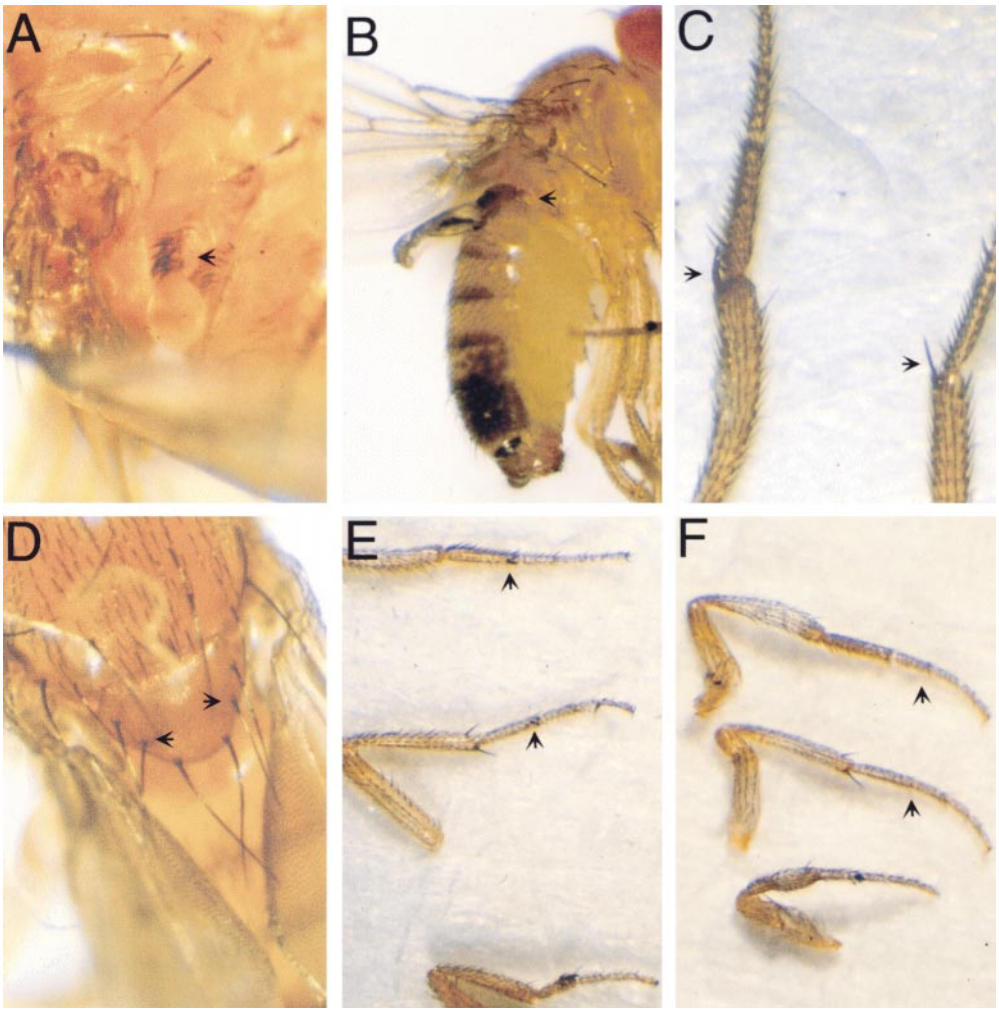

Figure 3.—Genetic interactions between $lawc^{P1}$ and *trx*-G and *Pc*-G genes. (A) Partial haltere-to-wing transformation in an $ash\text{-}1^{VF101}$ trx^{B11}/++ male. (B) More complete haltere-to-wing transformation in a $lawc^{P1}$; $ash\text{-}1^{VF101}$ trx^{B11}/++ male. (C) Third leg to second leg transformation in a $lawc^{P1}$/+; $ash\text{-}1^{VF101}$ trx^{B11}/++ female; note the apical bristle on upper third leg (arrow). (D) Ectopic macrochaete on the scutellum of a male of the genotype $lawc^{P1}$; $ash\text{-}1^{VF101}$ trx^{B11}/++. (E) *Df(3L)Pc-Mk*/+ male with ectopic sex combs on second and third leg (arrows). (F) $lawc^{P1}$; *Df(3L)Pc-Mk*/+ male with no ectopic sex combs on the second and third legs.

trx^{E2}/++ double transheterozygous mutants result in homeotic transformations such as haltere to wing or third leg to second leg manifested by bristles on the haltere and apical bristles on the third leg, respectively (Figure 3A). In the case of $lawc^{P1}$; trx^{B11} $ash\text{-}1^{VF101}$/++ or $lawc^{P1}$; brm^2 trx^{E2}/++ males, both the frequency and severity of the homeotic transformations are increased (Figure 3 and Table 1). The occurrence of homeotic transformations increases at least threefold, from 14 to 64% in the case of trx^{B11} $ash\text{-}1^{VF101}$ and from 10 to 29% in the case of brm^2 trx^{E2}. In addition, there is a dramatic increase in the severity of the homeotic transformations, with many halteres completely transformed into wings (Figure 3B). This increase in frequency and severity of homeotic transformations is also seen in $lawc^{P1}$/+; trx^{B11} $ash\text{-}1^{VF101}$/++ or $lawc^{P1}$/+; brm^2 trx^{E2}/++ females (Figure 3C). This enhancement of the *trx*-G mutant phenotypes was stronger in a $lawc^{P1}$ maternal background (Table 1). In addition to the homeotic transformations described, the arista-to-leg transformation characteristic of *lawc* mutants is enhanced in flies heterozygous for two *trx*-G mutations, which show transformed aristae and many ectopic bristles (Figure 3D and Table 2), suggesting that $lawc^{P1}$ enhances the phenotype of *trx*-G genes and vice versa.

When females carrying the null $lawc^{EF520}$ allele are crossed to single *trx*-G mutant males, the resulting $lawc^{EF520}$/+; *trx*-G/+ females show no homeotic transformations (Table 1). The number of transformations increases relative to the control in $lawc^{EF520}$/+; trx^{B11} $ash\text{-}1^{VF101}$/++ or $lawc^{EF520}$/+; brm^2 trx^{E2}/++ females (Table 1). The $lawc^{EF520}$ allele is lethal by itself, but $lawc^{EF520}$/$lawc^{P1}$ females are viable. In combination with single *trx*-G mutants, no $lawc^{EF520}$/$lawc^{P1}$; *trx-G*/+ females eclose, suggesting that heterozygosity in a *trx-G* gene such as *trx*, *ash1*, or *brm* is sufficient to cause lethality in combination with strong *lawc* alleles (Table 3). A similar effect can be observed in females carrying *Df(1)RA2*, which uncovers the *lawc* gene. Females heterozygous for this deficiency in combination with two *trx*-G mutations show an increase in the rate of transformations with respect to the control (Table 1). In addition, *Df(1)RA2*/$lawc^{P1}$ females are viable, but they are also lethal in combination with single or double mutants in *trx*-G genes (Table 3). The ability of *lawc* mutations to enhance the phenotype of *trx*-G mutants suggests that *lawc* might be a new *trx*-G gene.

To further test this hypothesis, we analyzed the possibility of genetic interactions between *lawc* and *Polycomb* (*Pc*). The ability to suppress the dominant phenotype

TABLE 2

Interactions of *trx*-G genes with *lawc*^{P1}

Males	Females: *lawc*^{P1}		
	No. of flies examined	Flies with strongly transformed aristae (%)	Flies with ectopic bristles (%)
Oregon R	52	25.0	26.9
trx^{B11} *ash-1*^{VF101}	169	61.5	79.2
brm^{2} *trx*^{E2}	67	35.8	50.7

Homozygous *lawc*^{P1} females were crossed to heterozygous males of the genotypes listed in the first column. The male progeny were examined for strong transformation of the aristae to legs, *i.e.*, a very thickened arista base, and for ectopic macrochaete on the scutellum.

of *Pc* mutants is another criterion for *trx*-G membership (Shearn 1989). About 83% of *Df(3L)Pc-Mk*/+ heterozygous males have ectopic sex combs on the second and/or third legs, denoting a transformation toward the first leg (Figure 3E). The vast majority of *lawc*^{P1}; *Df(3L)Pc-Mk*/+ males (97.4%) have no ectopic sex combs on the second or third legs (Figure 3F, Table 1), indicating that *lawc*^{P1} is capable of suppressing the phenotype of *Df(3L)Pc-Mk*. The same result was obtained using the *Pc*^{4} mutation (Table 1), *i.e.*, *lawc*^{P1} suppresses the *Pc*^{4} phenotype. Flies carrying the *Pc*^{4} chromosome had a 57% frequency of ectopic sex combs and this was reduced to 2% in *lawc*^{P1}; *Pc*^{4}/+ males. These results further support the hypothesis that *lawc* might be a new *trx*-G gene.

To confirm that the *lawc*^{P1} mutation is responsible for the observed interactions with *trx*-G and *Pc*-G members, *lawc*$^{+10}$/*lawc*$^{+10}$ revertant females (see materials and methods) were crossed to *trx*^{B11} *ash-1*^{VF101}/*TM1* males and the subsequent *lawc*$^{+10}$; *trx*^{B11}*ash-1*^{VF101}/++ male offspring were examined for homeotic transformations. The rate and severity of transformation were on a par with that seen when Oregon R females were used in the same cross (12% *vs.* 14% in the control; Table 1). The same result was obtained when *lawc*$^{+10}$/*lawc*$^{+10}$ females were crossed to *brm*^{2} *trx*^{E2}/*TM3* males, *i.e.*, there was no significant increase in the frequency of homeotic transformations in the F_1 males (9% *vs.* 10% in the control; Table 1). The number of ectopic sex combs was not reduced in the male offspring of *lawc*$^{+10}$/*lawc*$^{+10}$ mothers crossed to *Df(3L)Pc-Mk*/*TM3* males (80% compared to 83% of male offspring from Oregon R mothers; Table 1). The frequency of 59% ectopic sex combs in *lawc*$^{+10}$; *Pc*^{4}/+ males was similar to the 57% frequency seen in controls (Table 1). *lawc*$^{+10}$ was therefore unable to suppress the phenotype of *Pc* mutants or enhance the phenotype of *trx*-G mutants, confirming that the results shown in Table 1 are due only to the *lawc* mutation.

TABLE 3

Interactions of *lawc* with combinations of *trx*-G genes

Males	Females	
	lawc^{EF520}	*Df(1)RA2*
	FM7	*FM7*
lawc^{P1}	421/3	461/10
law^{P1}; *trx*^{B11}/*TM3*	433/1	430/5
lawc^{P1}; *brm*^{2}/*TM6*	354/2	385/6
lawc^{P1}; *ash-1*^{VF101}/*TM3*	405/2	494/5

Females of the genotypes indicated in the top row were crossed to males of the genotypes listed in the first column. Resulting progeny were examined for viability. Data are presented as number of flies examined/percentage of flies hatching to adults.

Effect of *lawc* mutations on the expression of homeotic genes: *trx*-G members are positive regulators of homeotic genes, and, therefore, if mutant, result in a decrease of homeotic gene expression. If *lawc* is a member of the *trx*-G, the level of homeotic gene expression may be influenced in the background of *lawc* mutations. To test this possibility, we determined the level of tissue-specific expression of various homeotic gene products using immunofluorescence microscopy. Immunostaining of *Df(1)RA2*/*lawc*^{P1} female larval imaginal discs and brains was performed with various antibodies to homeotic proteins. No effect of the *lawc* mutation was found on the level of Scr protein in the central nervous system (CNS) or first leg discs (data not shown). The level of Ubx protein was slightly reduced in the CNS and haltere discs (Figure 4, A and B, and data not shown) of *Df(1)RA2*/*lawc*^{P1} larvae. Labial protein was present but reduced in the eye-antenna disc (Figure 4, C and D) and deformed protein was likewise reduced (data not shown). Antp protein levels were not reduced in the brain or leg discs, but ectopic expression of Antp protein was observed in the eye-antenna disc (Figure 4, E and F). The ectopic accumulation of Antp protein in the eye-antenna disc correlates with the arista-to-leg transformation seen in *lawc*^{P1} adults. The level of some homeotic gene products is therefore influenced by *lawc* mutations, whereas other homeotic gene product levels are not altered. This variable ability to affect levels of

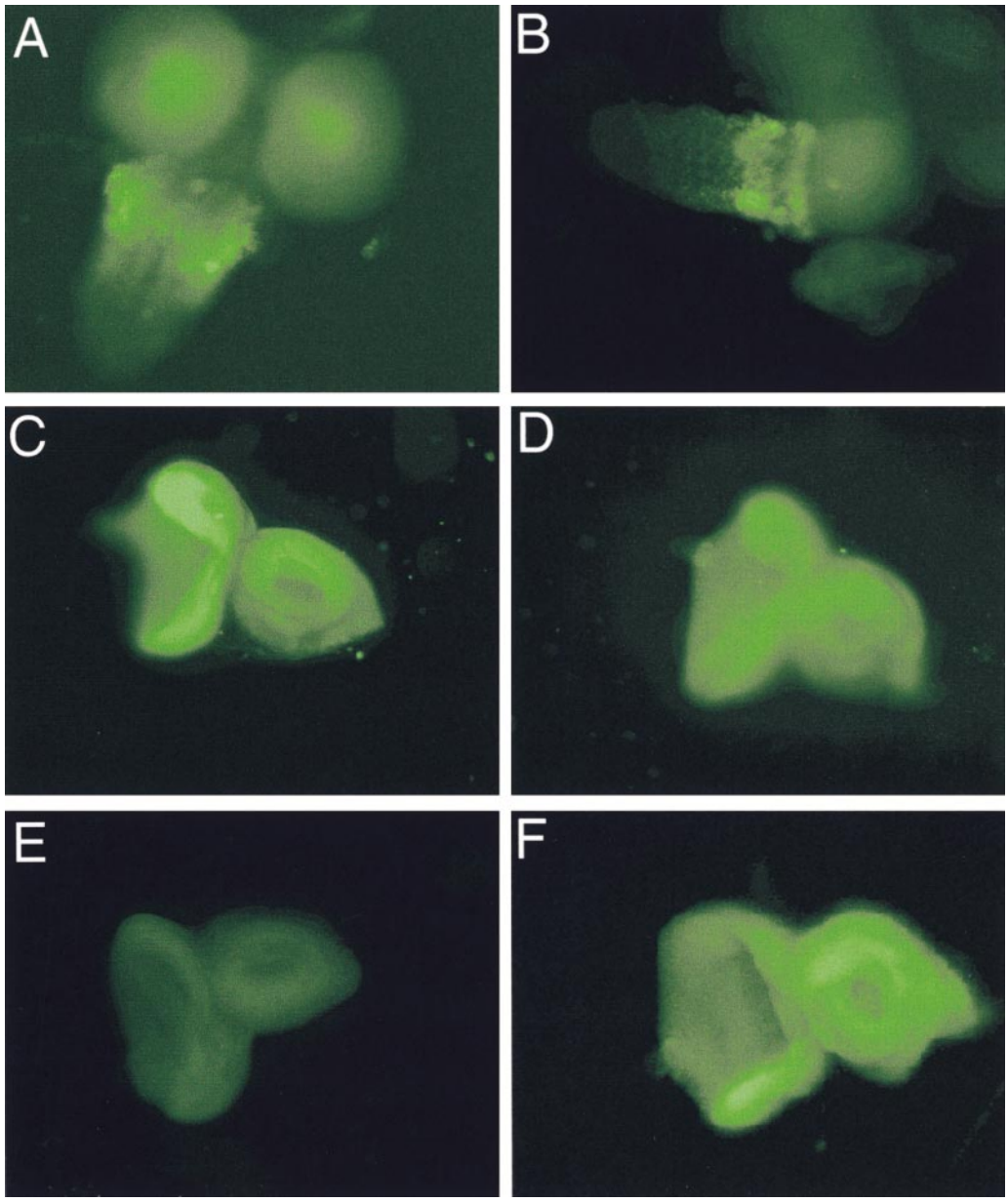

Figure 4.—Expression of homeotic proteins in wild-type and *lawc* mutant larvae. Expression of Ubx in the CNS of wild-type (A) and $lawc^{P1}$/*Df(1)RA2* (B) larvae. Expression of Labial in the eye-antenna imaginal disc of wild-type (C) and $lawc^{P1}$/*Df(1)RA2* (D) larvae. Expression of Antp in the eye-antenna disc of wild-type (E) and $lawc^{P1}$/*Df(1)RA2* (F) larvae.

homeotic products is seen with other members of the *trx*-G (LaJeunesse and Shearn 1995) and confirms the suggestion that *lawc* is a *trx*-G gene.

***lawc*^{P1} enhances position-effect variegation:** Mutations in the *trithorax-like (Trl)* and *mod(mdg4)* genes, both members of the *trx*-G, enhance position effect variegation (PEV; Dorn *et al.* 1993; Farkas *et al.* 1994; Gerasimova *et al.* 1995). To determine whether *lawc* may also be capable of enhancing PEV we made a recombinant between *In(1)*w^{m4} and $lawc^{P1}$. *In(1)*w^{m4} is an inversion on the X chromosome that juxtaposes the *white* gene next to the centromeric heterochromatin and results in a phenotype characterized by orange/brown dots against a red background (Figure 5A). $lawc^{P1}/lawc^{P1}$ females were crossed to *In(1)*w^{m4} males and the F_1 females were crossed back to $lawc^{P1}$ males. The subsequent F_2 progeny were scored for both w^{m4} and $lawc^{P1}$ phenotypes. A total of 8754 flies were scored before a recombinant was found due to the need to recover a double crossover event in an F_1 female. Flies of the genotype *In(1)*w^{m4}, $lawc^{P1}$ have eyes with large white/yellow patches against an orange background, *i.e.*, there is less *w* expression and thus an enhancement of PEV (Figure 5A). The same effect of the $lawc^{P1}$ mutation on PEV was observed for other variegating mutations such as *brown-Dominant* (bw^{D}) and *yellow-v2* (y^{v2}) (data not shown). This enhancement of PEV by *lawc* mutations can be rescued by a transgene containing the complete *lawc* gene (I. Zorin and V. Corces, unpublished results), suggesting that the effect is not due to second site mutations present elsewhere in the genome. The observed involvement of *lawc* in PEV suggests that the lawc protein might function at the level of chromatin organization.

Effects of *lawc* on the *gypsy* insulator: The *mod(mdg4)* product is a component of the *gypsy* insulator and has been shown to enhance PEV and to be a member of

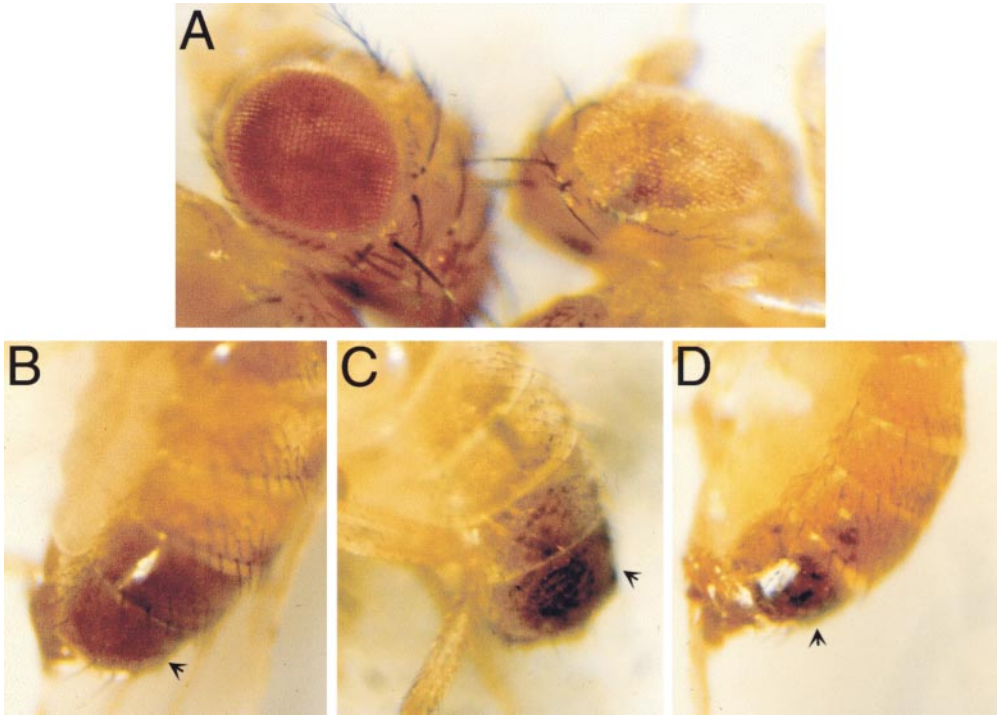

Figure 5.—Effects of *lawc*^{P1} on position-effect variegation and interactions with *mod(mdg4)*. (A) *In(1)w*^{m4} (left) and *In(1)w*^{m4}, *lawc*^{P1} (right) males. (B) Abdominal pigmentation in *y*^{2} males. (C) Pigmentation of abdominal segments in *y*^{2}; *mod(mdg4)*^{u1} males. (D) Pigmentation of the abdomen in *y*^{2} *lawc*^{P1}; *mod(mdg4)*^{u1} males.

the *trx*-G of genes (Dorn *et al.* 1993; Gerasimova *et al.* 1995; Gerasimova and Corces 1998). In the case of the *gypsy*-induced *yellow*^{2} (*y*^{2}) mutation, the abdominal pigment of male flies is lighter than usual in color, but uniformly distributed in the last two abdominal segments (Figure 5B). *y*^{2}; *mod(mdg4)*^{u1} males have variegated abdominal pigmentation with individual spots of dark wild-type pigment against a lighter pigmented background in the last two abdominal segments (Figure 5C). Gerasimova and Corces (1998) showed that some *trx*-G mutations, in heterozygous combinations with *y*^{2}; *mod(mdg4)*^{u1}, result in an increase in the number of cells with lighter pigmentation relative to the darkly pigmented regions, *i.e.*, an enhancement of the variegated phenotype. To test whether this is also the case with the *lawc* mutation, we analyzed the phenotype of *y*^{2} *lawc*^{P1}; *mod(mdg4)*^{u1} males. In the same manner as for other *trx*-G mutants, the variegated phenotype is enhanced in *y*^{2} *lawc*^{P1}; *mod(mdg4)*^{u1} males in that the areas of lighter pigmentation increase at the expense of the darkly pigmented regions (Figure 5D). *lawc*^{P1} therefore suppresses the *mod(mdg4)*^{u1} variegated phenotype, because the phenotype of *y*^{2} *lawc*^{P1}; *mod(mdg4)*^{u1} flies is closer to *y*^{2} than to *y*^{2}; *mod(mdg4)*^{u1}. This result suggests that in the presence of a *lawc*^{P1} mutation, the functionality of the insulator, which is impaired by mutations in *mod(mdg4)*, is partially restored. In addition, the *y*^{2} *lawc*^{P1}; *mod(mdg4)*^{u1} males also have strong transformation of the aristae to legs and ectopic bristles on the scutellum (Figure 2E), indicating that *mod(mdg4)* enhances the *lawc* phenotype. The ability of *lawc* to partially restore the functionality of the gypsy insulator in the background of a mutation in *mod(mdg4)* was also tested in the gypsy-induced *omb*^{D11} mutation (Tsai *et al.* 1997). As in the case of *y*^{2}, the *lawc*^{P1} mutation partially suppresses the phenotype of *mod(mdg4)*^{u1} (data not shown). These results suggest that the lawc protein is either a component of the *gypsy* insulator or it functions at the level of chromatin organization to perturb the effect of the su(Hw) insulator, just as it has been suggested for other *trx*-G products (Gerasimova and Corces 1998).

Mutations in the *lawc* gene affect the nuclear distribution of mod(mdg4) protein: The su(Hw) and mod(mdg4) proteins are located in several hundred sites in polytene chromosomes, but both proteins are distributed in a punctated pattern in the nuclei of diploid cells during interphase. Around 20–30 dots are observed in a typical nucleus, suggesting that many chromosomal sites come together in specific regions of the nucleus. We have proposed a model suggesting that the su(Hw)/mod(mdg4) proteins attach the chromatin fiber to a specific nuclear structure, and Pc-G/trx-G proteins are essential in maintaining this organization. Mutations in both *Pc*-G and *trx*-G genes cause a disorganization of the punctated pattern, supporting their role in the maintenance of this nuclear architecture (Gerasimova and Corces 1998). To test whether the lawc protein is also involved in the maintenance of the nuclear arrangement of the chromatin fiber in a manner that would explain the observed genetic interactions with *mod(mdg4)*, we analyzed the effect of *lawc* mutations on the nuclear distribution of mod(mdg4) protein. Figure 6 shows the results of this experiment. In *mod(m-*

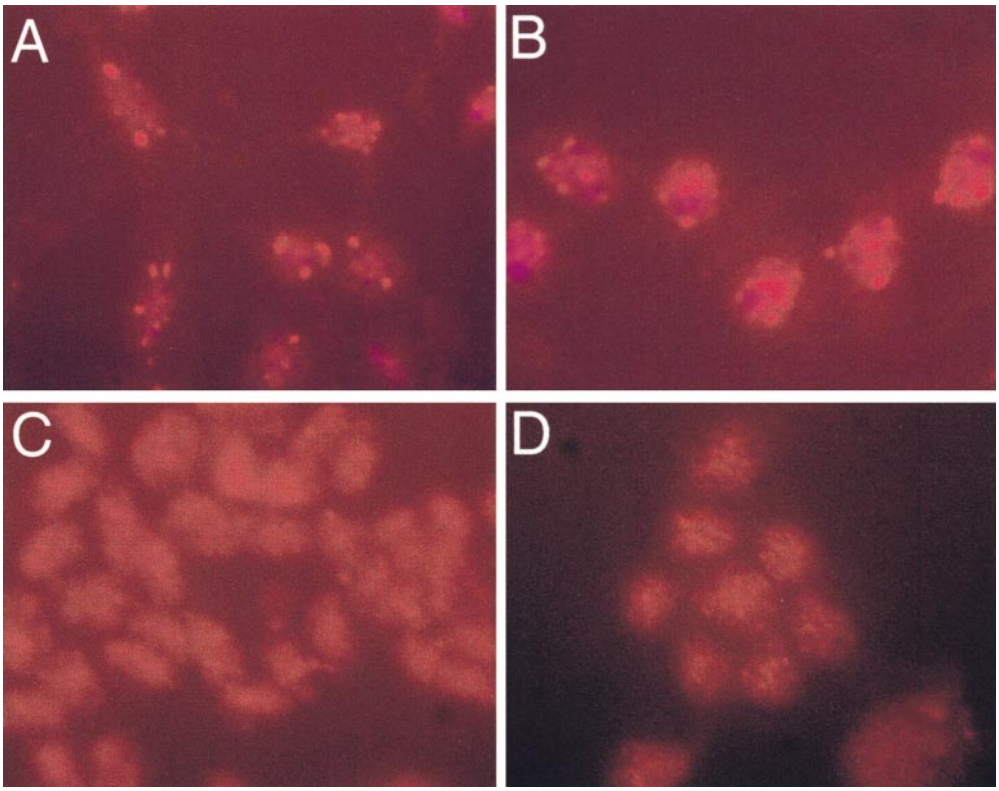

Figure 6.—Nuclear distribution of mod(mdg4) protein in various genetic backgrounds. Imaginal disc cells from third instar larvae were stained with antibodies against mod(mdg4) protein; red represents mod(mdg4) and blue is DNA stained with DAPI. (A) Nuclei from $y^2/y\ w^{67};\ mod(mdg4)^{u1}/+$ larvae. (B) Nuclei from $lawc^{P1}/lawc^{EF520}$ larvae. (C) Nuclei from $lawc^{P1}/lawc^{P1};\ mod(mdg4)^{u1}/+$ larvae. (D) Nuclei from $lawc^{P1}/lawc^{EF520};\ mod(mdg4)^{u1}/+$ larvae.

$dg4)^{u1}/+$ flies, the nuclear distribution of mod(mdg4) is normal, with several dots visible mostly around the nuclear periphery (Figure 6A). Flies carrying the $lawc^{P1}$ mutation show a similar distribution pattern of mod(mdg4) protein, suggesting that a hypomorphic mutation in the *lawc* gene is not sufficient to disrupt this pattern (Figure 6B). But flies of the genotypes $lawc^{P1}/lawc^{P1};\ mod(mdg4)^{u1}/+$ or $lawc^{P1}/lawc^{EF520};\ mod(mdg4)^{u1}/+$ show a dramatic alteration in the distribution of mod(mdg4) protein. The perinuclear localization of mod(mdg4) protein is lost in these flies, and instead the protein appears to be distributed throughout the nucleus, with a few small dots visible in the central region of some nuclei (Figure 6, C and D). These results suggest that *lawc*, as with other *trx*-G and *Pc*-G genes, is involved in the maintenance of the nuclear arrangement of the chromatin fiber imposed by the protein components of the *gypsy* insulator.

DISCUSSION

We have shown that *lawc* is a member of the *trx*-G and regulates homeotic gene expression. The *lawc* mutant phenotype results in homeotic transformations as well as ectopic bristles and wing margin incisions, indicating that *lawc* probably has functions in the regulation of genes other than homeotic selector genes. Like *lawc*, mutations in *ash-2*, another member of the *trx*-G, also result in the formation of ectopic bristles on the scutellum and thorax. Whereas $lawc^{P1}$ mutants have incisions in the tip and posterior wing margin, *ash-2* mutants have campaniform sensilla transformed to bristles on their wings. *ash-2* is thought to play an additional role in determining external sensory organs (Adamson and Shearn 1996). Mutations in *little imaginal discs* (*lid*) cause duplicated thoracic macrochaete; *lid* is a new *trx*-G gene and the Drosophila homologue to human RBP2 (J. J. Gildea and A. Shearn, personal communication). *brahma* mutant clones often have duplication of bristles and wing defects (Elfring *et al.* 1998). Both *trx* and *ash-2* mutations result in antenna-to-leg transformations in a manner similar to *lawc* (Ingham 1985; Adamson and Shearn 1996). *lawc* therefore has a pleiotropic phenotype, some aspects of which are shared with other members of the *trx*-G; in addition, *lawc* might also play a role in sensory cell development.

It has been hypothesized that *trx*-G proteins form a complex in the cell nucleus (reviewed in Paro and Harte 1996) and that there is no hierarchy between them so that mutations in any one member lead to a homeotic mutant phenotype. As has been pointed out by Shearn (1989), the penetrance of various transformation phenotypes depends on the allele used and the extent of the maternal contribution. Most experiments designed to demonstrate the identity of various *trx*-G members have made use of recessive null alleles; for example, double heterozygous combinations of null alleles of *ash-1*, *trx*, and / or *ash-2* lead to homeotic transformations (Shearn 1989). When $lawc^{P1}$ was combined with either *ash-1*, *ash-2*, *trx*, or *brm* individually, no en-

hancement of the homeotic phenotype was observed. A clear effect was seen when *lawc*^{P1} was used in combination with mutations in two *trx*-G genes, showing that *lawc*^{P1} enhanced the *trx*-G mutant phenotypes and vice versa. A deficiency for *trx* as well as null alleles of *ash-1* and *ash-2* can suppress the dominant *Pc* phenotype as heterozygotes (Capdevila and Garcia-Bellido 1981; Shearn 1989). Similarly, *lawc*^{P1} hemizygotes are able to suppress the phenotype of *Pc*, even though *lawc*^{P1} is not a null allele.

As expected from its properties as a *trx*-G gene, mutations in *lawc* affect the expression of homeotic genes. The observation that the *lawc*^{P1}/*Df(1)RA2* background leads to the reduction of some homeotic gene products (Ubx, Lab, Dfd) and not others (Scr, Antp) is not exceptional. In the case of *ash-2*, the level of Antp is not reduced in the first leg disc, and there is no change in the level of Ubx expression in the CNS, although there is patterned loss in the haltere and third leg disc. *ash-2* also causes a reduction of Scr in the first leg disc. *ash-1* mutations lead to a reduction of Antp in the first leg disc and lower levels of Ubx in the CNS, but only variable loss of Scr in the first leg disc (LaJeunnesse and Shearn 1995). Because the *trx*-G gene products appear to form a complex, it is possible that different *trx*-G gene proteins interact with different homeotic genes. In forming this complex, some *trx*-G products such as *Trl* might bind to DNA, whereas others bind to each other. *trx*-G members are diverse and range from transcription factors such as the Trl GAGA factor to putative nucleosome displacement factors such as brahma. *trx*-G proteins could exert their effects on gene expression at various levels in the process of regulating transcription. Some *trx*-G products must have a general role in transcription because they bind to many sites on polytene chromosomes, other than the sites of homeotic genes (Tsukiyama *et al.* 1994; Gerasimova and Corces 1998).

Because the *trx*-G products maintain preestablished patterns of gene expression through multiple cell divisions, it has been assumed that they function at the level of chromatin (Orlando and Paro 1993; Gindhart and Kaufman 1995). The fact that *lawc*^{P1} enhances PEV supports the idea that the lawc protein might play a role at the level of chromatin in the same manner as other *trx*-G products. The observed genetic interactions between *lawc* and *mod(mdg4)* suggest the possibility that lawc could be a component of the *gypsy* insulator. It is likely that lawc regulates insulator effects but it is not a structural component *per se*. Other *trx*-G proteins have been shown to participate in insulator function indirectly by contributing to the maintenance of a specific arrangement of the chromatin fiber within the nucleus (Gerasimova and Corces 1998) and results presented here support a similar function for lawc. Molecular characterization of the *lawc* gene is in progress to determine its pattern of nuclear localization and its function in the context of other *trx*-G proteins.

We thank Drs. Allen Shearn, Norbert Perrimon, and Jim Kennison for providing various strains used in the studies described here. We also thank Drs. Matt Scott, Juan Botas, Javier Lopez, and Bill McGinnis for supplying antibodies. Work reported here was supported by U.S. Public Health Award GM35463 from the National Institutes of Health.

LITERATURE CITED

Adamson, A. L., and A. Shearn, 1996 Molecular genetic analysis of *Drosophila ash-2*, a member of the trithorax Group required for imaginal disc formation. Genetics **144:** 621–633.

Breen, T. R., and P. J. Harte, 1993 *trithorax* regulates multiple homeotic genes in the bithorax and Antennapedia complexes and exerts different tissue-specific, parasegment-specific and promoter-specific effects on each. Development **117:** 119–134.

Capdevila, M. P., and A. Garcia-Bellido, 1981 Genes involved in the activation of the bithorax complex of *Drosophila*. Wilhelm Roux's Arch. **190:** 339–350.

Capdevila, M. P., J. Botas and A. Garcia-Bellido, 1986 Genetic interactions between the *Polycomb* locus and the Antennapedia and bithorax complexes of *Drosophila*. Roux's Arch. Dev. Biol. **195:** 417–432.

Casperson, T., and J. Schultz, 1938 Nucleic acid metabolism of the chromosomes in relation to gene reproduction. Nature **142:** 294–295.

Chung, J. H., A. C. Bell and G. Felsenfeld, 1997 Characterization of the chicken β-globin insulator. Proc. Natl. Acad. Sci. USA **94:** 575–580.

Dorn, R., V. Krauss, G. Reuter and H. Saumweber, 1993 The enhancer of position-effect variegation of *Drosophila E(var)3-93D* codes for a chromatin protein containing a conserved domain common to several transcriptional regulators. Proc. Natl. Acad. Sci. USA **90:** 11376–11380.

Duncan, I. M., 1982 *Polycomblike*: a gene that appears to be required for the normal expression of the bithorax and Antennapedia gene complexes of *Drosophila melanogaster*. Genetics **102:** 49–70.

Dura, J. M., and H. W. Brock, 1985 *Polyhomeotic*: a gene of *Drosophila melanogaster* required for correct expression of segmental identity. Mol. Gen. Genet. **198:** 213–220.

Eissenberg, J. C., T. C. James, D. M. Foster-Hartnett, V. Ngan and S. C. Elgin, 1990 Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **87:** 9923–9927.

Elfring, L. K., C. Daniel, O. Papoulas, R. Deuring, M. Sarte *et al.*, 1998 Genetic analysis of *brahma*: the *Drosophila* homolog of the yeast chromatin remodeling factor SW12/SNF2. Genetics **148:** 251–265.

Farkas, G., J. Gausz, M. Galloni, G. Reuter, H. Gyurkovics *et al.*, 1994 The *Trithorax-like* gene encodes the *Drosophila* GAGA factor. Nature **371:** 806–808.

Gdula, D. A., T. I. Gerasimova and V. G. Corces, 1996 Genetic and molecular analysis of the *gypsy* chromatin insulator of *Drosophila*. Proc. Natl. Acad. Sci. USA **93:** 9378–9383.

Gerasimova, T. I., and V. G. Corces, 1996 Boundary and insulator elements in chromosomes. Curr. Opin. Genet. Dev. **6:** 185–192.

Gerasimova, T. I., and V. G. Corces, 1998 Polycomb and trithorax group proteins mediate the function of a chromatin insulator. Cell **92:** 511–521.

Gerasimova, T. I., D. A. Gdula, D. V. Gerasimov, O.B. Simonova and V. G. Corces, 1995 A *Drosophila* protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegation. Cell **82:** 587–597.

Geyer, P. K., and V. G. Corces, 1992 DNA position-specific repression of transcription by a *Drosophila* Zn finger protein. Genes Dev. **6:** 1865–1873.

Gindhart, J. G., and T. C. Kaufman, 1995 Identification of *Polycomb* and *trithorax* Group responsive elements in the regulatory region of the *Drosophila* homeotic gene *Sex combs reduced*. Genetics **139:** 797–814.

Hagstrom, K., M. Muller and P. Schedl, 1996 Fab-7 functions as a chromatin domain boundary to ensure proper segment specifi-

cation by the *Drosophila bithorax* complex. Genes Dev. **10:** 3202–3215.

Ingham, P. W., 1983 Differential expression of bithorax complex genes in the absence of the *extra sex combs* and *trithorax* genes. Nature **306:** 591–593.

Ingham, P. W., 1985 A clonal analysis of the requirements for the *trithorax* gene in the diversification of segments in *Drosophila*. J. Embryol. Exp. Morphol. **89:** 349–365.

Ingham, P. W., and A. Martinez-Arias, 1986 The correct activation of the Antennapedia and bithorax complex genes requires the *fuzhi tarazu* gene. Nature **324:** 592–597.

Jack, J., D. Dorsett, Y. Delotto and S. Liu, 1991 Expression of the *cut* locus in the *Drosophila* wing margin is required for cell type specification and is regulated by a distal enhancer. Development **113:** 735–747.

James, T. C., and S. C. Elgin, 1986 Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila melanogaster* and its gene. Mol. Cell. Biol. **6:** 3862–3872.

Kalos, M., and R. E. K. Fournier, 1995 Position-independent transgene expression mediated by boundary elements from the apolipoprotein B chromatin domain. Mol. Cell. Biol. **15:** 198–207.

Kaufman, T. C., R. Lewis and B. Wakimoto, 1980 Genetic analysis of chromosome 3 in *Drosophila melanogaster*: the homeotic gene complex in polytene chromosome interval 84A-B. Genetics **94:** 115–133.

Kellum, R., and P. Schedl, 1991 A position-effect assay for boundaries of higher order chromosomal domains. Cell **64:** 941–950.

Kellum, R., and P. Schedl, 1992 A group of scs elements function as domain boundaries in an enhancer-blocking assay. Mol. Cell. Biol. **12:** 2424–2431.

Kennison, J. A., 1995 The polycomb and trithorax group proteins of *Drosophila*: trans-regulators of homeotic gene function. Annu. Rev. Genet. **29:** 289–303.

Kennison, J. A., and J. W. Tamkun, 1988 Dosage-dependent modifiers of *Polycomb* and *Antennapedia* mutations in *Drosophila*. Proc. Natl. Acad. Sci. USA **85:** 8136–8140.

Kuziora, M. A., and W. McGinnis, 1988 Different transcripts of the *Drosophila AbdB* gene correlate with distinct genetic sub-functions. EMBO J. **7:** 3233–3244.

LaJeunesse, D., and A. Shearn, 1995 Trans-regulation of thoracic homeotic selector genes of the Antennapedia and bithorax complexes by the trithorax group genes: *absent, small and homeotic discs 1* and *2*. Mech. Dev. **53:** 123–139.

Levefre, G., 1976 *The Genetics and Biology of Drosophila*, Vol. 1a, edited by M. Ashburner and E. Novitski, Academic Press, New York, pp. 31–66.

Lewis, E. B., 1978 A gene complex controlling segmentation in *Drosophila*. Nature **276:** 565–570.

Mihaly, J., I. Hogga, J. Gausz, H. Gyurkovics and F. Karch, 1997 In situ dissection of the Fab-7 region of the *bithorax* complex into a chromatin domain boundary and a Polycomb-response element. Development **124:** 1809–1820.

Orlando, V., and R. Paro, 1993 Mapping Polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. Cell **75:** 1187–1198.

Paro, R., and P. J. Harte, 1996 The role of Polycomb group and trithorax group chromatin complexes in the maintenance of determined cell states, pp. 507–528 in *Epigenetic Mechanisms of Gene Regulation*, edited by V. E. A. Russo, R. A. Martienssen and A. D. Riggs. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Roseman, R. R., V. Pirrotta and P. K. Geyer, 1993 The *su(Hw)* protein insulates expression of the *Drosophila melanogaster white* gene from chromosomal position-effects. EMBO J. **12:** 435–442.

Shearn, A., 1989 The *ash-1*, *ash-2* and *trithorax* genes of *Drosophila melanogaster* are functionally related. Genetics **121:** 517–525.

Simonova, O. B., B. A. Kuzin, P. G. Georgiev and T. I. Gerasimova, 1992 Novel regulatory mutation of *Drosophila melanogaster*. Genetika (Russ.) **28:** 164–167.

Struhl, G., 1981 A gene product required for correct initiation of segmental determination in *Drosophila*. Nature **293:** 36–41.

Tsai, S. F, C. C. Jang, G. G. Prikhod'ko, D. A. Bessarab, C. Y. Tang *et al.*, 1997 Gypsy retrotransposon as a tool for the in vivo analysis of the regulatory region of the *optomotor-blind* gene in *Drosophila*. Proc. Natl. Acad. Sci. USA **94:** 3837–3841.

Tsukiyama, T., P. B. Becker and C. Wu, 1994 ATP-dependent nucleosome disruption at a heat shock promoter mediated by binding of GAGA transcription factor. Nature **367:** 525–532.

Zhou, J., S. Barolo, P. Szymanski and M. Levine, 1996 The Fab-7 element of the *bithorax* complex attenuates enhancer-promoter interactions in the *Drosophila* embryo. Genes Dev. **10:** 3195–3201.

Communicating editor: J. A. Birchler