Heterochromatin Protein 1 Is Required for the Normal Expression of Two Heterochromatin Genes in Drosophila

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ABSTRACT

The *Su(var)2-5* locus, an essential gene in Drosophila, encodes the heterochromatin-associated protein HP1. Here, we show that the *Su(var)2-5* lethal period is late third instar. Maternal HP1 is still detectable in first instar larvae, but disappears by third instar, suggesting that developmentally late lethality is probably the result of depletion of maternal protein. We demonstrate that heterochromatic silencing of a normally euchromatic reporter gene is completely lost by third instar in zygotically HP1 mutant larvae, implying a defect in heterochromatin-mediated transcriptional regulation in these larvae. However, expression of the essential heterochromatic genes *rolled* and *light* is reduced in *Su(var)2-5* mutant larvae, suggesting that reduced expression of essential heterochromatic genes could underlie the recessive lethality of *Su(var)2-5* mutations. These results also show that HP1, initially recognized as a transcriptional silencer, is required for the normal transcriptional activation of heterochromatic genes.

IN most cases, euchromatic genes that are moved into proximity of heterochromatin will be variably silenced, a phenomenon called position-effect variegation (PEV; reviewed in Spofford 1976; Reuter and Spierer 1992; Lu and Eissenberg 1998). There are, however, a number of loci (including essential genes) that normally reside in heterochromatin (reviewed in Hilliker *et al.* 1980), and proximity to major blocks of heterochromatin appears to be required for the normal expression of such genes (Wakimoto and Hearn 1990; Eberl *et al.* 1993; Howe *et al.* 1995; reviewed in Weiler and Wakimoto 1995). In both situations, the position effects are sensitive to the functional dosage of a number of genes known or believed to encode structural components of heterochromatin (Wallrath 1998); among these is the *Su(var)2-5* locus in Drosophila, which encodes the heterochromatin-associated protein HP1 (James *et al.* 1989; Eissenberg *et al.* 1990, 1992). Null/hypomorphic mutations at, and deficiencies for, this locus dominantly suppress the heterochromatic position-effect silencing of euchromatic genes mislocalized to heterochromatin (Wustmann *et al.* 1989). Interestingly, *Su(var)2-5* mutations dominantly enhance silencing of heterochromatin genes mislocalized to euchromatin (Hearn *et al.* 1991).

Su(var)2-5 is an essential gene. The lethality associated with *Su(var)2-5* mutations can be rescued with a heat-shock-driven HP1 cDNA transgene, even if heat-shock induction of the transgene is delayed until the third larval instar (Eissenberg and Hartnett 1993). A late larval lethal period for *Su(var)2-5* could be explained if there were a significant maternal contribution of HP1 activity; indeed, there is genetic evidence for a maternal effect of *Su(var)2-5* mutation (Grigliatti 1991) and biochemical evidence for substantial HP1 protein in the oocyte before fertilization (Eissenberg *et al.* 1994).

Here, we examine directly the development and lethality of *Su(var)2-5* mutant flies to look for specific defects that would suggest an essential function of HP1. We show that individuals heteroallelic for *Su(var)2-5* mutations survive to the third instar larval stage in expected Mendelian proportions. Using *Su(var)2-5* alleles that encode truncated HP1 protein, we show that maternally encoded HP1 protein is still present in significant quantity in first instar larvae, but becomes undetectable by the third larval instar. Silencing of a variegating euchromatic gene is completely lost in *Su(var)2-5* homozygous mutant third instar larvae. We show that expression of the essential heterochromatic genes *rolled* and *light* is significantly reduced in *Su(var)2-5* mutant larvae. Our results show that HP1 is required for normal transcriptional activity of heterochromatic genes.

MATERIALS AND METHODS

Fly stocks: All crosses were performed at room temperature using standard cornmeal-sucrose medium containing 0.04% methylparaben as a mold inhibitor. The *Su(var)2-5* alleles have been described previously (Sinclair *et al.* 1983; Wustmann *et al.* 1989; Eissenberg *et al.* 1990, 1992; Eissenberg and

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Hartnett 1993; Platero *et al.* 1995) except for *Su(var)2-5^{149}*. *Su(var)2-5^{149}* was isolated originally as an ethylnitrosourea-induced dominant suppressor of *rst^{3}* (S. Gorski and R. Cagan, unpublished results). This mutation failed to complement the recessive lethality of *Su(var)2-5^{02}*, *Su(var)2-5^{04}*, and *Su(var)2-5^{05}*, and the HP1 coding sequence contains a C-to-T transition mutation in the first position of codon 132, replacing a glutamine codon with a stop codon. This allele encodes a truncated HP1 protein detectable by Western blot. The protein is truncated just before the C-terminal chromo shadow domain (Aasland and Stewart 1995), deleting the previously mapped nuclear targeting domain and most of the overlapping C-terminal heterochromatin binding domain (Powers and Eissenberg 1993). Like the truncated protein encoded by the previously characterized *Su(var)2-5^{04}* allele, the *Su(var)2-5^{149}* gene product appears to be much less stable *in vivo* than the full-length protein (data not shown). This might either be due to misfolding or to the inability of these truncated proteins to enter the nucleus. Thus, the basis for both the *Su(var)2-5^{04}* and *Su(var)2-5^{149}* mutations is similar: synthesis of a truncated HP1 protein that is maintained at dramatically lower steady-state levels than wild-type protein.

Su(var)2-5 alleles were maintained over a *CyO* balancer marker with *y^{+}* (Indiana University Stock Center) in a background of *y^{1}* (from Pam Geyer) or *Df(1)w, y^{1} w^{67c23}*. *Tp(3; Y)BL2* is described in Lu *et al.* (1998). In measuring recovery of *Su(var)2-5* mutant larvae, larval collections were done from vials in which no pupae were present so that differences in the rate of pupariation could not influence the ratio of *yellow* to *yellow^{+}* larvae. Similarly, larvae for Western and Northern blots [heteroallelic *Su(var)2-5* mutants and their heterozygous sibs] were collected before any pupae were detected in the vial; under these conditions, all larvae of the same stage were of a similar size and appeared equally healthy.

The derivation and structures of translocation stocks showing *rolled* position effects are described in Eberl *et al.* (1993).

Western blot analysis: Larvae were homogenized in SDS-PAGE sample buffer with protease inhibitors, proteins were electrophoresed in a 12% SDS-polyacrylamide gel and transferred to nitrocellulose paper, and blots were probed with a polyclonal rabbit anti-HP1 serum (directed against a synthetic polypeptide representing amino acids 25–47 of *Drosophila melanogaster* HP1, a gift of S. C. R. Elgin) as described in Eissenberg *et al.* (1992) except that Western blot detection used the ECL chemiluminescent detection system (Amersham, Piscataway, NJ). For stage-specific blots 92–113 first instar larvae, 38–54 second instar larvae, or 9–13 third instar larvae were homogenized in 100 μl of SDS-PAGE sample buffer. To equalize loadings, soluble protein was determined for each of the homogenates as follows: 10 μl of homogenate was added to 5 μl H_2O; 10 μl 80% trichloroacetic acid was added to precipitate protein; precipitated protein was recovered by centrifugation; the pellet was washed once with chloroform:ether (1:1). The protein was redissolved in 10 μl 100 mm NaCl/50 mm Tris-Cl (7.0)/20 mm EDTA/0.01% SDS; 490 μl H_2O was then added; and the protein was quantitated using the Coomassie Plus protein assay reagent (Pierce Chemical, Rockford, IL) relative to a BSA standard curve. For all Western blots, a duplicate gel was run simultaneously with identical loadings and stained with Coomassie to check for protein degradation in specific samples; no evidence for such degradation was seen.

Imaginal disc staining: Larval tissues were fixed and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) as described (Lu *et al.* 1998).

Slot blot analysis: Total RNA was isolated from 200 48-hr-old (±15 min) flies of each genotype exactly as described by Jowett (1986). Poly(A^{+})RNA was isolated from total RNA using the mRNA QuickPrep Micro mRNA purification kit (Pharmacia, Piscataway, NJ). mRNA was quantitated by A_{260} and immediately analyzed by slot blotting. Poly(A^{+})RNA (2 mg) from each genotype was applied to Zeta-Probe nylon membrane (Bio-Rad, Richmond, CA) using a Minifold II slot blot system (Schleicher and Schuell, Keene, NH). The membrane was then prehybridized and hybridized in solutions containing 50% formamide, 2× SSC, 0.05 m $NaPO_4$ (pH 7.4), 0.2% SDS, 0.1 mg polyadenylate/ml, 0.25 mg yeast tRNA/ml, 0.02% polyvinylpyrrolidone/ficoll, 0.2 mg BSA/ml with or without the radiolabeled *rl* or *Actin 5C* cDNAs, respectively, at 42°. Radioactive probes for the *rolled* and *Actin 5C* coding regions were made using the Random Prime labeling kit (Boehringer Mannheim, Indianapolis) and [^{32}P]dCTP (ICN). Hybridization was first performed with the *rolled* probe and was visualized by autoradiography. To control for variation between samples, the same Zeta-Probe membranes were stripped and reprobed with the *Actin 5C* coding region. To strip the membrane of *rl* probe, the membranes were washed at 95° for 30 min in 0.1× SSC and 0.1% SDS with constant checking using a BICRON surveyor M. Each band on the *rl* cDNA autoradiogram was quantified with its corresponding band of the *Actin 5C* control autoradiogram using an Alpha Innotech IS-1000 densitometer. To compare test samples to their corresponding control, we employed the following calculation:

$$\frac{\text{Area of Control (}\textit{Actin 5C}\text{)}}{\text{Area of Experimental (}\textit{Actin 5C}\text{)}} \times \frac{\text{Area of Experimental (}\textit{rl}\text{)}}{\text{Area of Control (}\textit{rl}\text{)}},$$

where Area of Control (*Actin 5C*) = area of the control sample from the *Actin 5C* blot; Area of Experimental (*Actin 5C*) = area of the experimental sample from the *Actin 5C* blot; Area of Experimental (*rl*) = area of the experimental sample from the *rl* blot; and Area of Control (*rl*) = area of the control sample from the *rl* blot. Area refers to the area under a densitometric curve. Control RNA was prepared from an Oregon-R wild-type stock. Quantitative values for every sample expressed relative to the densitometric values obtained for each control in the respective experiment.

Malpighian tubule analysis: Malpighian tubules were dissected from third instar larvae, submerged in phosphate-buffered saline (PBS) containing 2.5 μg/ml of DAPI (Sigma, St. Louis) for 5 min, and washed several times with PBS. Tubules containing one or more cells with no or greatly diminished levels of autofluorescent granules relative to adjacent cells were scored as *light* variegating. Malpighian tubules were photographed using an Olympus AH-3 fluorescence microscope.

Northern blot analysis: Total nucleic acids were prepared from 10–15 third instar larvae essentially according to Meyerowitz and Hogness (1982). In this method, the DNA remains high in molecular weight and is separable from RNA upon electrophoresis. Most is trapped in the well, but a small amount sometimes enters and runs at the limit mobility with an apparent size of >15 kb. RNA was sized electrophoretically in a 1.2% agarose-formaldehyde gel and blotted to nitrocellulose paper. Cloned probes were labeled with [^{32}P]dCTP by random priming (Feinberg and Vogelstein 1984) and hybridized essentially according to Wahl *et al.* (1979), except that hybridization was performed at 65° without formamide. The *light* probe was the 3.0-kb *light* cDNA clone described in Warner *et al.* (1998). The *rolled* probe was the cDNA clone pNB40-17.4 (Biggs *et al.* 1994).

RESULTS

***Su(var)2-5* homozygotes die in late third instar:** A previous study suggested that *Su(var)2-5* homozygotes can

TABLE 1

Recovery of larvae heteroallelic (yellow mouth hooks) or heterozygous (black mouth hooks) for *Su(var)2-5* alleles

	First and second instar		Third instar	
♀♀ Cross ♂♂	Yellow	Black	Yellow	Black
$\frac{Su(var)2\text{-}5^{02}}{CyO,\ y^+} \times \frac{Su(var)2\text{-}5^{04}}{CyO,\ y^+}$	263	366	237	527
$\frac{Su(var)2\text{-}5^{04}}{CyO,\ y^+} \times \frac{Su(var)2\text{-}5^{205}}{CyO,\ y^+}$	240	532	403	657
$\frac{Su(var)2\text{-}5^{05}}{CyO,\ y^+} \times \frac{Su(var)2\text{-}5^{02}}{CyO,\ y^+}$	162	259	90	202
$\frac{Su(var)2\text{-}5^{205}}{CyO,\ y^+} \times \frac{Su(var)2\text{-}5^{05}}{CyO,\ y^+}$	128	229	137	258
$\frac{Su(var)2\text{-}5^{04}}{CyO,\ y^+} \times \frac{Su(var)2\text{-}5^{05}}{CyO,\ y^+}$	30	50	50	117
$\frac{Su(var)2\text{-}5^{04}}{CyO,\ y^+} \times \frac{Su(var)2\text{-}5^{149}}{CyO,\ y^+}$	168	342	71	208
Sum of all crosses:	991	1778	988	1969

survive to the third larval instar (Eissenberg and Hartnett 1993). However, this inference was based on the latest period at which induction of an HP1 cDNA transgene could still rescue mutant flies to adulthood and did not rule out some contribution from basal transgene expression. We determined the lethal period directly by using genetically marked larvae. To distinguish larvae heteroallelic for *Su(var)2-5* mutations from heterozygous sibs, an X chromosome marked with *y* and a *CyO* balancer chromosome marked with y^+ were used in all crosses; thus, mutant larvae are distinguished as having yellow mouth hooks, compared to the black mouth hooks of balancer-bearing larvae. Larvae from parents carrying distinct *Su(var)2-5* alleles were used to avoid effects due to second site lethals. Table 1 summarizes the results of crosses using six different heteroallelic combinations of *Su(var)2-5* alleles. In all cases, a ratio of $y{:}y^+$ of ~1:2 was observed through third instar (*CyO* homozygotes die in late embryogenesis), indicating no genotype-specific lethality throughout normal larval development.

Interestingly, while heterozygous *Su(var)2-5* larvae pupate normally, larvae heteroallelic for different *Su(var)2-5* mutations continue as third instar larvae for several days longer than their heterozygous sibs, eventually dying as third instar larvae or (in some allelic combinations) early pupae. Dissected heteroallelic third instar larvae revealed reduced optic lobes in the larval brain and reduced or missing imaginal discs for most alleleic combinations. The reduced brains could be explained by the recent report of extensive mitotic defects in heteroallelic larval neuroblasts (Fanti *et al.* 1998).

Maternal HP1 decays throughout larval development, and is undetectable by third instar: The survival of zygotically *Su(var)2-5* mutants to late third instar could be explained if significant levels of maternal HP1 were present throughout embryonic and early larval development. We exploited the fact that *Su(var)2-5^{04}/Su(var)2-5^{149}* larvae make no full-length HP1 protein of their own [see materials and methods for a description of the *Su(var)2-5^{149}* allele] to measure directly the level of maternally loaded full-length HP1 during larval development. As shown in Figure 1, full-length (maternal) HP1 protein is clearly detectable in first instar mutant larvae. We estimate conservatively that maternal HP1 is present in mutant first instar larvae at ~20% of levels seen in their heterozygous sibs. We found that maternal protein

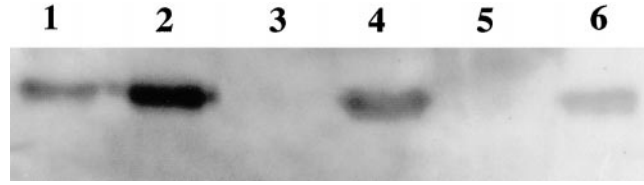

Figure 1.—Maternal HP1 in first, second, and third instar larvae. Larvae were genotyped by mouth hook pigmentation [yellow mouth hooks, *Su(var)2-5^{04}/Su(var)2-5^{149}*; black mouth hooks, *Su(var)2-5^{04}/CyO, y^+* or *Su(var)2-5^{149}/CyO, y^+*] and analyzed by Western blot as described (see materials and methods). Lanes 1, 3, and 5, *Su(var)2-5^{04}/Su(var)2-5^{149}*; lanes 2, 4, and 6, *Su(var)2-5^{04}/CyO, y^+* or *Su(var)2-5^{149}/CyO, y^+*. Lanes 1 and 2, first instar larvae; lanes 3 and 4, second instar larvae; lanes 5 and 6, third instar larvae. A total of 20 μg total soluble protein, determined by protein assay, was loaded in each lane; Coomassie-stained gels of the same samples run in parallel confirmed the equal loading and found no evidence for selective protein degradation in specific genotypes (not shown).

is often undetectable by second instar and consistently undetectable by third instar.

Total HP1 concentrations (zygotic plus maternal) decline during larval development in *Su(var)2-5*$^{+}$ flies (Figure 1 and our unpublished data). Based on quantitative Western blot analysis, using recombinant HP1 as a standard of comparison, we estimate that there is ~20 ng of HP1 in a wild-type third instar larva.

To estimate the amount of HP1 per nucleosome, we prepared DNA from 100 third instar larvae and find that there is ~2 μg of DNA per larvae. Assuming 200 nucleotide pairs per nucleosome, a nucleosome sequesters 132 kD of DNA, so there are ~15 pmol nucleosomes per third instar larva. Our estimate of ~20 ng of HP1 per third instar larva implies ~1 pmol HP1 per third instar larva, yielding an estimate of about one molecule of HP1 for 15 nucleosomes in a third instar larva. About 20% of the diploid Drosophila genome is heterochromatic. However, it is important to remember that much of the DNA in third instar larvae is found in polytene cells, where much of the heterochromatin is underrepresented. If we assume that heterochromatin DNA is, on average, ~10-fold underrepresented in third instar larvae, this would imply that 2% of total larval DNA is heterochromatin, giving ~0.3 pmol heterochromatic nucleosomes per third instar larva. This would yield an estimate of three molecules of HP1 per heterochromatic nucleosome.

Silencing is lost precociously in the undifferentiated imaginal tissue of *Su(var)2-5* mutant larvae: Next, we looked for evidence that transcriptional regulation is abnormal in *Su(var)2-5* mutants. *Su(var)2-5* was originally identified as a haploinsufficient suppressor of heterochromatic position-effect variegation (Sinclair *et al.* 1983; Wustmann *et al.* 1989), so we looked for evidence that suppression of heterochromatic silencing in *Su(var)2-5* mutant larvae differed from that observed in *Su(var)2-5* heterozygotes.

Since *Su(var)2-5* mutants die as third instar larvae, we used a larval marker for heterochromatic silencing, the variegation of *lacZ* in *Tp(3;Y)BL2* (Lu *et al.* 1996). In third instar eye imaginal discs, silencing of the *lacZ* reporter in the transgene is nearly complete in the undifferentiated cells ahead of the morphogenetic furrow, but silencing is dramatically relaxed in the differentiating cells behind the furrow in a pattern anticipating the variegation seen in adult eyes after pupal eclosion (Lu *et al.* 1998; Figure 2A). In flies heterozygous for a mutation in *Su(var)2-5*, the relaxation in differentiating cells behind the furrow is much more extensive, but the silencing in the undifferentiated cells ahead of the furrow is only slightly affected (Figure 2B). Thus, the dominant suppression of position-effect variegation caused by *Su(var)2-5* occurs primarily at the relaxation phase of heterochromatic silencing. In contrast, *Su(var)2-5* mutant larvae show complete loss of heterochromatic silencing in all disc cells, regardless of their differentiation state (Figure 2C). This result demonstrates a clear deficit in heterochromatic silencing in *Su(var)2-5* mutant larvae.

Expression of the heterochromatic *rolled* locus is reduced in *Su(var)2-5* mutant larvae: Silencing of normally euchromatic genes by heterochromatin requires a chromosome rearrangement that places the euchromatic gene next to a heterochromatic breakpoint. However, the expression of several normally heterochromatic genes is reduced when these genes are rearranged to lie next to a euchromatic breakpoint (Wakimoto and Hearn 1990; Hearn *et al.* 1991; Eberl *et al.* 1993; Howe *et al.* 1995). *rolled*, the Drosophila ERK-1/MAP kinase (Biggs *et al.* 1994), is located deep in the pericentric heterochromatin of the right arm of chromosome 2 (Hilliker and Holm 1975). Eberl *et al.* (1993) reported a series of rearrangements that left the *rolled* gene isolated in a small block of heterochromatin, distant from the centromere and from any large block of heterochromatin. These rearrangements, when heterozygous with the *rl* hypomorphic visible allele *rl*^{1}, exhibited a *rolled* visible phenotype of curved wings. That this is due to a position effect was demonstrated by the fact that rearranged *rolled* alleles could be reverted to an *rl*$^{+}$ phenotype at high frequency by further chromosomal rearrangements that brought the *rolled* gene near a large heterochromatic block.

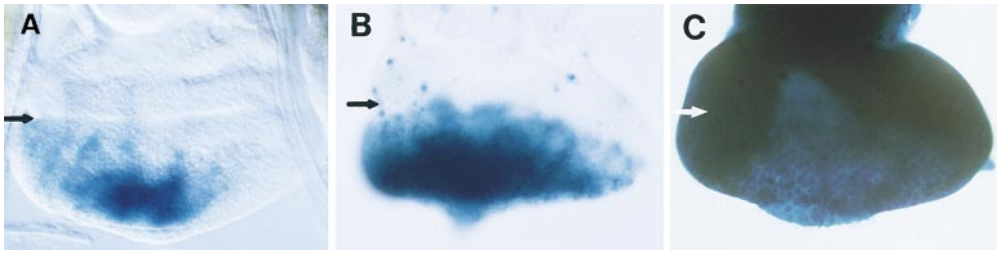

Figure 2.—Loss of heterochromatic silencing of a euchromatic gene in *Su(var)2-5* mutant larvae. Eye antennal discs were dissected from third instar larvae after heat shock and stained for β-galactosidase activity using X-gal. (A) Disc from a *Tp(3;Y)BL2* larva. (B) Disc from a *Su(var)2-5*/*CyO*; *Tp(3;Y)BL2* larva. (C) Disc from a *Su(var)2-5*^{02}/*Su(var)2-5*^{05}; *Tp(3;Y)BL2* larva. Arrows indicate the position of the morphogenetic furrow.

To test the role of HP1 in regulating heterochromatic gene expression, the effect of the *Su(var)2-5*^{205} allele on *rolled* position effects was tested using representative rearrangements. The severity of the *rolled* phenotype associated with *Su(var)2-5*^{205} *b lt rl*/*T(2;3) 33-6*, *Su(var)2-5*^{205} *b lt rl*/*T(2;3)127-3*, and *Su(var)2-5*^{205} *b lt rl*/*T(2;3) 76-7* is greatly enhanced relative to the respective controls, *b lt rl*/*T(2;3) 33-6*, *b lt rl*/*T(2;3)127-3*, and *b lt rl*/*T(2;3)76-7* from the aforementioned study (Eberl *et al.* 1993). In each case, the wings are much more curved and the eyes are much reduced in size, and there is a general reduction in viability as assayed by eclosion frequency (~50% at 25°). Clearly, *Su(var)2-5*^{205} is a dominant enhancer of *rolled* position effects.

To test whether HP1 regulates expression of a heterochromatic gene in its normal chromosomal position, we tested the ability of *Su(var)2-5*^{205} to enhance the semilethality and phenotypic defects in *rl*^{1} hemizygotes. Eclosion rates and phenotypes were scored for adults heterozygous for *rl*^{1} and the deficiency *Df(2R)PRF*, which is deleted for *rl* (Eberl *et al.* 1993). Eclosion rates of *rl*^{1} hemizygous flies are ~88% of their heterozygous *rl*^{1}/*rl*$^{+}$ sibs at 18°; the rate of eclosion, relative to heterozygous *rl*^{1}/*rl*$^{+}$ sibs, is nearly halved by the presence of *Su(var)2-5*^{205} (Table 2). Among surviving adults, *rl*-dependent eye size reduction and wing defects were enhanced by *Su(var)2-5*^{205} (Table 3). Thus, *Su(var)2-5*^{205} acts as an enhancer of *rl*. These results suggest that HP1 is required for the normal activity of *rl* in its normal chromosomal position in heterochromatin.

To test whether the effects of HP1 dosage on *rl* expression were the result of reduced *rl* transcription, we examined the effect of *Su(var)2-5*^{205} mutation on *rolled* transcript levels. Steady-state *rolled* mRNA levels were determined in young adults heterozygous for *rl*, with and without the *Su(var)2-5*^{205} allele using slot blot hybridization. Table 4 shows these values, corrected for an *Actin 5C* internal loading control and expressed as a fraction of wild-type *rolled* mRNA levels, for three separate experiments. These data show that *Su(var)2-5*^{205} acts dominantly to decrease *rolled* transcription.

Interestingly, the lethal phenotype of *rolled* mutants (late larval lethality with defective or missing discs) is similar to the lethal phenotype of *Su(var)2-5*. Furthermore, heteroallelic *Su(var)2-5* mutants rescued to adulthood by induction of an HP1 cDNA transgene beginning in late larval development have dramatically reduced eyes (Eissenberg and Hartnett 1993), suggesting that the effect of reduced *rolled* expression had begun to occur before transgene expression was induced. To test whether late larval lethality of heteroallelic *Su(var)2-5* mutants is associated with reduced larval *rolled* expression in larvae that are wild type for *rolled*, we examined *rolled* RNA levels in *Su(var)2-5* mutant larvae. *rolled* expresses two major transcripts that are normally detectable in most or all wild-type larval tissues (Bergh ella and Dimitri 1996). Northern blot analysis reveals

TABLE 2

***Su(var)2-5* enhances *rl*-dependent lethality**

	b lt rl ♂♂ × *Df(2R)PRF/SM1* ♀♀				*Su(var)2-5*^{205} *b lt rl*/*CyO* ♂♂ × *Df(2R)PRF/SM1* ♀♀			
			b lt rl/*Df(2R)PRF*			*Df(2R)PRF*/*CyO* or *Su(var)2-5*^{205} *b lt rl*/*SM1*	*Su(var)2-5*^{205} *b lt rl*/*Df(2R)PRF*	
Temperature	Total progeny	*b lt rl*/*SM1*	Observed	Expected	Total progeny		Observed	Expected
25°	420	361	59***	210	518	483	35***	173
18°	725	386	339 (NS)[a]	362	593	480	113***[a]	198

(NS) $P > 0.05$. *** $P < 0.001$.

[a] At 18°, the proportion of *rl* hemizygotes observed was 94% of expected, whereas the proportion of *Su(var)2-5*^{205}*lt* hemizygotes was 57% of expected, a highly significant difference ($P < 0.01$).

TABLE 3

***Su(var)2-5* enhances *rl*-dependent phenotypes**

	Eye size[a]	Vein interruptions[b,c]			Wing phenotype[c,d]
		L3	L4	L5	
25°					
b lt rl / Df(2R)PRF	⅓ to ½	Significant	Entire	Slight	Strongly rolled, blistered in some flies
Su(var)2-5^{205} b lt rl / Df(2R)PRF	⅓	Significant	Entire	Significant	Strongly rolled, blistered, not fully expanded in some flies
18°					
b lt rl / Df(2R)PRF	⅓ to ½	Slight in some flies	Significant to entire	None	Strongly rolled, no blisters, fully expanded
Su(var)2-5^{205} b lt rl / Df(2R)PRF	¼ to ⅓	Slight in most flies	Significant to entire	None	Strongly rolled, no blisters, fully expanded

Examination of the *rl* hemizygous flies reported in Table 1. Sample sizes for each row (in descending order): 59, 35, 339, and 113. All flies were examined at the same time. Each genotype was directly compared to the other and to their rl^+ sibs and to Oregon-R.

[a] Eye size was visually estimated relative to wild type. ⅓ to ½ means that eye size varied continuously and uniformly by inspection between ⅓ and ½. ⅓ means that eye size was uniformly ~⅓.

[b] Slight, <¼ of length of vein; significant, between ¼ and ¾ of length; entire, >¾ of vein length; significant to entire, flies vary from significant to entire within the genotype.

[c] Where there is no qualifier as "some" or "most," all flies of a genotype have the indicated phenotype.

[d] Strongly rolled, extreme wing curvature.

that *rolled* expression is significantly reduced (~40% of wild-type levels by PhosphorImager quantitation, normalizing to *rp49* hybridization signal) in such larvae relative to their heterozygous sibs or to wild-type larvae (Figure 3).

The heterochromatic *light* locus undergoes variegated silencing in *Su(var)2-5* mutant larvae: *light*, the Drosophila homolog of the yeast vacuolar sorting protein 41 (Warner *et al.* 1998), is located in the pericentric heterochromatin of the left arm of chromosome 2 near the euchromatic boundary. To determine whether HP1 is required for the activation of other heterochromatic genes, or is specific to *rolled*, we examined *light* expression in *Su(var)2-5* mutant larvae. As a phenotypic assay, *light* expression in third instar larval Malpighian tubules was scored using the appearance of *light*-dependent autofluorescent granules in the cytoplasm of tubule cells. To further enhance the sensitivity of this assay, we used the *Su(var)2-5^{205}* allele, which was induced on a *light* mutant chromosome. Including the *Su(var)2-5^{205}* chromosome in the zygotic background means that loss of expression of only one functional *light* allele is sufficient to render a cell phenotypically light$^-$ (*i.e.*, lacking autofluorescent granules). Furthermore, introducing the *Su(var)2-5^{205}* chromosome maternally reduces the *light* maternal effect on Malpighian tubule expression and further enhances the sensitivity of this assay.

Loss of zygotic *Su(var)2-5* function leads to significant variegation of *light*. An example of this variegation is shown in Figure 4 (B, arrows). Individual Malpighian tubule cells lacking most or all of the *light*-dependent autofluorescent granules can be seen in tissue from *Su(var)2-5* larvae. Table 5 summarizes the effect of *Su(var)2-5* mutation on *light* expression in third instar larval Malpighian tubules. In crosses in which the *Su(var)2-5^{205}* chromosome is maternal, the variegation is more pronounced than in the reciprocal cross (reflecting the *light* maternal effect; Nicklа 1972), but significant variegation was seen in *Su(var)2-5* mutant larvae in both crosses.

To confirm that *light* variegation in *Su(var)2-5* mutant larvae reflects reduced *light* transcription, steady-state

TABLE 4

Relative *rolled* mRNA concentrations in young adults

Genotype	Blot 1	Blot 2	Blot 3	Mean (SD)
+/+	(1.00)	(1.00)	(1.00)	(1.00)
+/*b lt rl*	0.93	0.94	0.83	0.90 (0.06)
+/*Su(var)2-5^{205} b lt rl*	0.67	0.77	0.56	0.67 (0.11)[a]

Each blot represents an independent experiment using separate mRNA preparations.

[a] Significantly different ($P < 0.5$) from +/+ and +/*Su(var)2-5^{205} b lt rl* based on a *t*-test of the arcsin-transformed data.

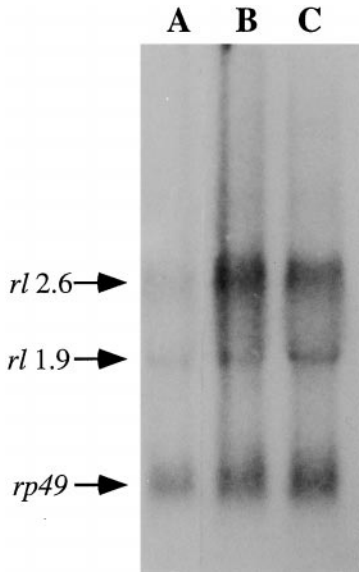

Figure 3.—*rolled* expression is reduced in *Su(var)2-5* mutant larvae. Northern blot analysis of total RNA from third instar larvae with different doses of *Su(var)2-5*. Lane A, *Su(var)2-5*^{04}/*Su(var)2-5*^{149}; lane B, *Su(var)2-5*^{04}/*CyO*; lane C, *v*^{36f}; *ry*^{506}. The blot was probed with cDNA clones for *rolled* and ribosomal protein 49 (*rp49*). The two *rolled* transcripts, 2.6 and 1.9 kb, previously reported in larvae (Berghella and Dimitri 1996) are indicated by arrows. The 0.65-kb *rp49* transcript (Wong *et al.* 1981) is the highest mobility RNA detected in this blot.

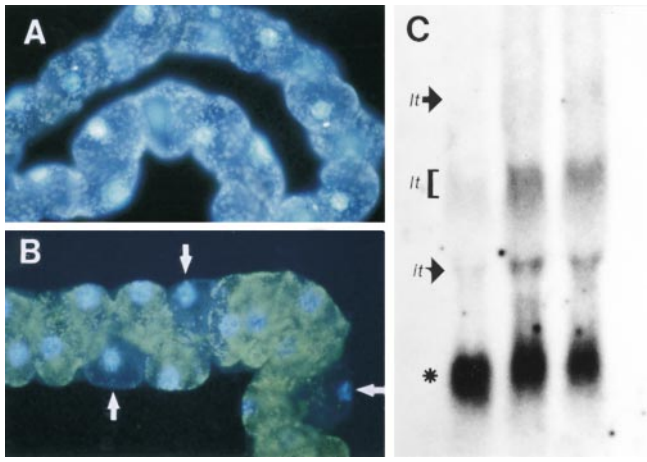

Figure 4.—*light* expression is reduced in *Su(var)2-5* mutant larvae. (A) Malpighian tubule from *Su(var)2-5*/*CyO y*$^{+}$ larva. (B) Malpighian tubule from *Su(var)2-5*^{205}/*Su(var)2-5*^{05} larva. Arrows point to cells with few or missing cytoplasmic fluorescent granules, indicating loss of *light* activity. Tissues were stained with DAPI to highlight nuclei. (C) Northern blot analysis of total RNA from third instar larvae with different doses of *Su(var)2-5*. Left lane, *Su(var)2-5*^{04}/*Su(var)2-5*^{149}; middle lane, *Su(var)2-5*^{04}/*CyO*; right lane, *v*^{36f}; *ry*^{506}. The blot was probed with cDNA clones for *light* and ribosomal protein 49 (*rp49*, Wong *et al.* 1981). Seven major *light* transcripts have been reported (Devlin *et al.* 1990) ranging in size from 1 to 13 kb; their relative positions are indicated by the labeled arrows and bracket. The 0.65-kb *rp49* transcript (asterisk) is the highest mobility RNA detected in this blot.

levels of *light* RNA were compared in larvae bearing zero, one, or two functional *Su(var)2-5* alleles. While *light* transcripts accumulate to comparable levels in wild-type and heterozygous *Su(var)2-5* larvae, *light* transcription is markedly reduced (to ~40% of wild-type levels by PhosphorImager quantitation, normalizing to *rp49* hybridization signal) in larvae heteroallelic for two mutant *Su(var)2-5* alleles (Figure 4C). Note that in this case both *light* alleles are wild type.

DISCUSSION

Genetic evidence implicates HP1 in the mechanism of euchromatic gene silencing by heterochromatin. The locus encoding HP1 in Drosophila, *Su(var)2-5*, was identified in screens for mutations that dominantly suppress the variegated silencing caused by heterochromatic position effects (Sinclair *et al.* 1983; Wustmann *et al.* 1989). HP1 homologs from yeast, mice, and humans have also been shown to promote silencing (Lorentz *et al.* 1992; Allshire *et al.* 1995; Le Douarin *et al.* 1996; Lehming *et al.* 1998; Seeler *et al.* 1998). Furthermore, HP1 shares significant structural homology with the *Polycomb* gene product, which is itself a silencer of homeotic genes (Paro and Hogness 1991). Thus, HP1 is widely considered to be a transcriptional repressor.

The role of HP1 in heterochromatic silencing could, in principle, be in setting the initial levels of variegation, the maintenance of silencing, or both. In a previous study, we showed that a *white-lacZ* reporter subject to PEV is silenced nearly completely in undifferentiated

TABLE 5

***light* variegation in *Su(var)2-5* mutant larvae**

Cross ♀♀	Cross ♂♂	*yellow* phenotype	Tubules showing *light* variegation	Tubules without *light* variegation	Fraction showing variegation
Su(var)2-5^{205} / *CyO, y*$^{+}$	*Su(var)2-5*^{05} / *CyO, y*$^{+}$	*yellow*	26	55	0.47
		yellow^{+}	2	93	0.02
Su(var)25^{05} / *CyO, y*$^{+}$	*Su(var)2-5*^{205} / *CyO, y*$^{+}$	*yellow*	7	53	0.13
		yellow^{+}	1	70	0.01

imaginal disc cells, but that silencing becomes dramatically relaxed as disc cells begin to differentiate (Lu *et al.* 1998). In the eye disc, the relaxation of silencing appears in a concerted fashion immediately after morphogenetic furrow passage. Here, we show that the dominant suppression of PEV imposed by *Su(var)2-5* mutation is primarily exerted in the differentiating cells behind the morphogenetic furrow, anticipating the suppression of PEV observed in the adult eye. Thus, while silencing is relaxed with the onset of differentiation, as previously reported, the haploinsufficient effect of *Su(var)2-5* is primarily manifested during this relaxation phase. This result demonstrates a role for HP1 in the maintenance of heterochromatic silencing during differentiation.

Surprisingly, heterochromatic silencing in the undifferentiated cells ahead of the furrow was insensitive to a 50% reduction in *Su(var)2-5* dosage. Silencing ahead of the furrow is lost, however, in discs from larvae lacking all functional zygotic HP1. This result shows that silencing in undifferentiated cells also requires HP1, since this silencing is lost in *Su(var)2-5* null flies. Thus, the silencing in differentiated and undifferentiated cells differs in extent and sensitivity to HP1 dosage, but the maintenance of silencing in both cell types has a common basis in a requirement for HP1.

Hearn *et al.* (1991) showed that one allele of *Su(var)2-5*, *Su(var)2-5*^{205}, enhances the repression of heterochromatic genes that have been displaced from their heterochromatic context by rearrangements. This finding suggested that HP1 could promote the normal expression of heterochromatic genes. Since several heterochromatic genes are essential, it also suggested that the recessive lethality of *Su(var)2-5* mutations could be a consequence of reduced expression of one or more such genes. More recently, Clegg *et al.* (1998) reported that flies doubly heterozygous for *Su(var)2-5*^{205} and any of three other *Su(var)*s had reduced eye pigmentation, suggesting reduced expression of *light* in its normal heterochromatic position. This suggests that HP1 cooperates with other *Su(var)* gene products in regulating normal *light* expression. In this study, however, individual *Su(var)*'s [including *Su(var)2-5*] had negligible effects.

Here, we have examined both the dominant and recessive phenotypes of mutations in the heterochromatin-associated protein HP1 to look for an essential requirement for HP1 in development. We propose that reduced expression of one or more essential heterochromatic genes results in the recessive late larval lethality of *Su(var)2-5*. In support of this hypothesis, we show that the essential heterochromatic genes *rolled* and *light* are misregulated in *Su(var)2-5* mutants.

rolled transcription at its normal chromosomal location is reduced in *Su(var)2-5* mutant flies. Since no maternal ROLLED protein is detectable in third instar larvae homozygous for *rolled* deficiencies (P. C. R. Emtage and A. J. Hilliker, unpublished results), the RNA levels we are detecting in mutant larvae and adults reflect zygotic gene expression. In the case of the heteroallelic mutant larvae, it should be emphasized that at the time the larvae were collected for Northern analysis, the *Su(var)2-5* larvae appeared healthy and would have lived on for several more days as third instar larvae before dying; indeed, we cannot rule out a further decline in *rolled* RNA preceding larval death. Thus, reduced expression of *rolled* could contribute to the defects associated with loss of HP1. Of course, reduced expression of other heterochromatic genes probably also contributes to lethality due to HP1 deficiency.

light also experiences variegated inactivation in *Su(var)2-5* larval Malpighian tubules, and *light* transcripts are dramatically reduced overall in *Su(var)2-5* mutant larvae. It is important to stress that the repressed *light* locus in these experiments is also in its normal chromosomal location. We conclude that silencing of *light* in these experiments is a direct consequence of HP1 depletion, depriving the *light* locus of the heterochromatin context required for its normal expression. Several other genes reside in heterochromatin, and it will be interesting to see whether dependence on HP1 is a general attribute of gene expression in heterochromatin.

Mutations in *rolled*, like *Su(var)2-5* mutations, lead to late larval or early pupal lethality with defective or missing imaginal discs (Hilliker 1976; Dimitri 1991). At the cytological level, *rolled* mutations cause defects in mitosis, including overcondensed and/or lagging anaphase chromosomes (Inoue and Glover 1998). Intriguingly, neuroblasts of larvae doubly mutant for hypomorphic alleles of *rl* and *abnormal spindles* (encodes a microtubule-associated protein; Saunders *et al.* 1997) show telomeric stickiness and increased frequency of aneuploid mitotic figures (Inoue and Glover 1998). These phenotypes were also seen in neuroblasts of larvae heteroallelic for *Su(var)2-5* mutations (Fanti *et al.* 1998); indeed, the highest frequency of defects occurs in larvae heteroallelic for the *Su(var)2-5*^{205} allele, which is carried on a chromosome marked with a hypomorphic *rl* allele. Reduced expression of *rolled* caused by loss of HP1, then, could contribute to mitotic defects in HP1 mutant larval brains.

How can HP1 be required both for activation of heterochromatic genes and silencing of euchromatic genes? Wakimoto and Hearn (1990) proposed that certain heterochromatin-associated proteins function to support normal transcription of heterochromatic genes when those genes are at their normal chromosomal sites and that position effects result when heterochromatic genes are deprived of such essential heterochromatic proteins by displacement away from heterochromatin "compartments" where such proteins are in high concentration. Such context-dependent regulatory activity has also been described for yeast RAP1 (repressor/activator protein 1); RAP1 is required for high-level expres-

sion of many ribosomal protein and glycolytic enzyme genes, but it promotes position-effect silencing at the *HM* silent mating type cassettes and telomeres (reviewed in Shore 1994). Genetic evidence suggests that RAP1 has distinct activator and silencing domains that could recruit or stabilize distinct chromosomal complexes at distinct chromosomal sites (Shore 1994). Similarly, HP1 could interact with different proteins or protein complexes to promote silencing or activation in different chromosomal contexts. Another possibility is that HP1 may contribute to the formation of a particular chromatin structure that interferes with activation of euchromatic genes but to which heterochromatic genes have become adapted and dependent. Loss of HP1 would deplete the nucleus of this particular chromatin conformation, releasing silenced genes from repression while simultaneously depriving the resident heterochromatin genes of their functional context.

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