

A Role for the *Drosophila* SU(VAR)3-9 Protein in Chromatin Organization at the Histone Gene Cluster and in Suppression of Position-Effect Variegation

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ABSTRACT

Mutations in the gene for *Su(var)3-9* are dominant suppressors of position-effect variegation (PEV). We show that SU(VAR)3-9 is a chromatin-associated protein and identify the large multicopy histone gene cluster (HIS-C) as one of its target loci. The organization of nucleosomes over the entire HIS-C region is altered in *Su(var)3-9* mutants and there is a concomitant increase in expression of the histone genes. SU(VAR)3-9 is a histone H3 methyltransferase and, using chromatin immunoprecipitation, we show that SU(VAR)3-9 is present at the HIS-C locus and that the histone H3 at the HIS-C locus is methylated. We propose that SU(VAR)3-9 is involved in packaging HIS-C into a distinct chromatin domain that has some of the characteristics of β -heterochromatin. We suggest that methylation of histone H3 is important for the chromatin structure at HIS-C. The chromosomal deficiency for the HIS-C is also a suppressor of PEV. In contrast to what might be expected, we show that hemizyosity for the HIS-C locus leads to a substantial increase in the histone transcripts.

HETEROCHROMATIC regions are highly condensed segments of DNA that are replicated late in the cell cycle and contain relatively few genes (GATTI and PIMPINELLI 1992; LOHE and HILLIKER 1995). Euchromatic genes juxtaposed to heterochromatin are silenced in a fraction of the cells in which they are normally expressed, leading to a variegated phenotype. This phenomenon is termed position-effect variegation (PEV; LEWIS 1950; GRIGLIATTI 1991; WEILER and WAKIMOTO 1995), and its molecular basis has remained an enigma since Muller first described it in 1930 (MULLER 1930). The silencing of the variegating gene is due to spread of heterochromatin-like structures into the euchromatic gene (GRIGLIATTI 1991; WALLRATH and ELGIN 1995; BOIVIN and DURA 1998) and/or to its physical relocation into new compartments in the nucleus that lack the appropriate gene-specific transcription factors (SCHLOSSHERR *et al.* 1994; DERNBURG *et al.* 1996). Over 35 dominant suppressors of PEV, called *Su(var)* loci, have been isolated on the basis of the hypothesis that such mutations would identify genes that participate in the formation and regulation of chromatin/heterochromatin and/or attachment of chromatin to the nuclear matrix (REUTER and WOLFF 1981; SINCLAIR *et al.* 1981; GRIGLIATTI 1991; HENIKOFF 1996).

Su(var)3-9 is one such locus and mutations in the gene are dominant suppressors of PEV. In addition, extra copies of the wild-type *Su(var)3-9* gene enhance PEV (TSCHERSCH *et al.* 1994), suggesting that the gene

product is required in stoichiometric amounts relative to other proteins. SU(VAR)3-9 contains a chromo and a SET domain that are present in known chromatin proteins (TSCHERSCH *et al.* 1994; PLATERO *et al.* 1995). The chromodomain appears to promote associations with chromatin structures (PLATERO *et al.* 1995) and the SET domain is part of the catalytic domain of histone H3-specific methyltransferase (HMTase; REA *et al.* 2000). The dominant suppression of PEV in *Su(var)3-9* mutants, the dosage sensitivity of the *Su(var)3-9* locus, and the presence of the conserved chromo and SET domains suggest that SU(VAR)3-9 is a component of chromatin and that it may regulate chromatin structure at specific domains within the genome.

To date only seven to eight *Su(var)*s have been characterized at the molecular level. The majority encode chromatin-associated proteins that are located at many euchromatic and heterochromatic sites. Their widespread distribution in the genome makes it difficult to determine what effect mutations in the *Su(var)* genes have on chromatin structure and/or on gene expression at specific loci. Hence our goal has been to identify a SU(VAR) that associates with a well-defined target locus.

In this article we show that SU(VAR)3-9, a modifier of PEV, is a component of the histone gene complex (HIS-C). SU(VAR)3-9, like SUV39H1, has HMTase activity. Mutations in *Su(var)3-9* cause dramatic alterations in packaging of HIS-C, suggesting that one function of SU(VAR)3-9 is to generate the unique chromatin structure encompassing the large HIS-C region. In addition, *Su(var)3-9* mutants show an increase in the steady-state level of histone transcripts, presumably as a consequence of the altered chromatin structure. Chromatin

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immunoprecipitation experiments confirm the presence of SU(VAR)3-9 at the HIS-C locus and the methylation of histone H3 at the HIS-C locus. The deregulation of histone gene transcription in *Su(var)3-9* mutant strains leads to changes in histone protein levels. Thus the *Su(var)3-9* locus is a *trans*-regulator of histone gene expression. Unexpectedly, hemizyosity for the HIS-C region also shows an increase in histone transcript levels, and combining a dominant *Su(var)3-9* mutation with the HIS-C deficiency causes a further increase in histone transcript levels. Hence, there is a correlation between increases in histone gene expression and suppression of PEV, regardless of whether the increase in *his* transcripts is caused by mutations in *Su(var)3-9* or by hemizyosity for the HIS-C locus, but the magnitude of the suppression differs. Whether the changes in availability of normal and specifically modified forms of histones and their influence on chromatin structure is a direct or an indirect cause of the silencing of the *white*⁺ gene in the variegating strain *In(1)white^{md}* remains to be determined.

MATERIALS AND METHODS

Drosophila strains: All fly strains were grown on glucose/yeast/cornmeal medium under standard conditions. Oregon-R was used as the wild-type strain. The EMS-induced *Su(var)3-9* mutants 311, 319, and 330 were previously isolated in a genetic screen (SINCLAIR *et al.* 1983) and the HIS-C deficiency strains DS5 and DS6 are described in MOORE *et al.* (1979, 1983). The *P*-element insertion lines P17 and P25 were isolated as suppressors of PEV during a *P*-element mobilization screen (R. C. MOTTUS and T. A. GRIGLIATTI, unpublished data). P17^{r10} and P25^{r2} are complete revertants of P17 and P25 and were generated as part of this study. P17^{r12} is a partial revertant of P17, confirmed by PCR and DNA sequence analysis. The genomic region spanning the coding region of *Su(var)3-9* was sequenced in the mutants 319 and 330. Both have a single nucleotide change that results in a single amino acid substitution in the SET domain of SU(VAR)3-9. The 319 mutation causes a SER-to-LEU substitution at amino acid 616 and the 330 mutation causes an ASP-to-ASN substitution at amino acid 536.

Crosses and pigment assays for analysis of variegation: For variegating eye pigment genes, +/Y; *Su(var)^{mutant}/Balancer*, +/Y; *Df(2R)DS5*, or *DS6/Balancer* males were mated to *w^{md}/w^{md}*, +/+ virgin females. Or, *w^{md}/Y*; *Su(var)^{mutant}/Balancer*, *w^{md}/Y*; *Df(2R)DS5*, or *DS6/Balancer* males were mated to *w^{md}/w^{md}*, +/+ virgin females and the amount of eye pigment in the appropriate progeny classes was determined. Individuals derived from analogous crosses between *w^{md}/Y*; +/+ males and *w^{md}/w^{md}*; +/+ virgin females served as controls. A minimum of 150 adults of each genotype, aged 5–7 days posteclosion, were frozen in a dry-ice ethanol and decapitated by agitation. Five heads of each genotype were suspended in 25 μ l of 0.25 M β -mercaptoethanol in a 1% aqueous NH₄OH and sonicated. A minimum of 25 samples for each genotype were centrifuged for 2 min at 12,000 \times g and a 5- μ l aliquot was removed from each supernatant and spotted onto a strip of chromatography paper (Whatman no. 4) attached to a microscope slide. The amount of pigment was determined fluorometrically using a MPS-1 Zeiss microscope. In each case the amount of pigment was standardized against a wild type (Oregon-R) and fully mutant (*w⁻* males

and females) and expressed relative to the amount of pigment in wild type (Oregon-R).

Antibody source: A rabbit polyclonal antibody, α -3-9^{chr}, was raised against the chromodomain region of SU(VAR)3-9 (amino acids 187–330). The polyclonal antibody was fractionated and purified by affinity chromatography using Protein A agarose beads and chromodomain-polypeptide-coupled sepharose beads (HARLOWE and LANE 1988). The antibody detects a protein of 78 kD but it does not cross-react with other chromodomain-containing proteins, including HP1 and Polycomb (data not shown). The 78-kD protein is enriched in nuclear fractions from both *Drosophila* embryos and Kc12 tissue culture cells. The 78-kD protein product is reduced in embryo extracts prepared from a homozygous viable *Su(var)3-9* mutant 330 and, in addition, α -3-9^{chr} serum, depleted by preabsorption with a SU(VAR)3-9-chromodomain peptide column, failed to detect the 78-kD cross-reacting species from *Drosophila* embryo extracts and failed to detect purified SU(VAR)3-9 protein. Antibodies against HP1 (C1A9) and histone H3 were provided by S. Elgin (JAMES *et al.* 1989) and M. Roberge (SAUVE *et al.* 1999), respectively. An antibody to histone H1 was obtained from J. Kadonaga (University of California at San Diego). Antidimethyl-lysine 9 histone H3 was purchased from Upstate Biotechnology (Lake Placid, NY).

In situ hybridization and immunostaining of larval salivary glands: *In situ* hybridization of salivary glands was performed as described (CRYDERMAN *et al.* 1999). A 140-kb *Drosophila* genomic DNA fragment from the 39D region containing repeats of the histone genes (BACR05D8, Berkeley *Drosophila* Genome Project) served as the template for the biotinylated probe prepared by nick translation (SAMBROOK *et al.* 1989). After hybridization the glands were washed at 37° for 45 min with three changes of 50% formamide in 2 \times SSC followed by three 5-min washes in 2 \times SSC and two rinses in PBT (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM Na₂H₂PO₄, 0.1% Triton X-100). The glands were blocked in PBTB (PBT, 1% BSA) and then incubated in PBTB with α -3-9^{chr} for 1 hr. The glands were washed for 15 min in PBT and then incubated with an avidin-alexa-594 conjugate to detect the biotinylated probe and with α -rabbit-alexa-488 (Molecular Bioprobes) to detect α -3-9^{chr}. After two washes of 10 min each in PBT the glands were treated with RNase A (1 μ g/ml in PBT) for 20 min, and then incubated with a DNA-specific fluorescent dye TO-PRO-3 (0.1 μ g/ml). The glands were mounted on slides in glycerol containing 2% 1,4-diazabicyclo[2.2.2]octane. Samples were analyzed and images collected on a Bio-Rad (Richmond, CA) MRC 600 scanning laser confocal microscope equipped with a krypton/argon laser. TO-PRO-3 has a broad emission spectra and can be detected as a faint background signal when observing the fluorescence arising from the alexa-594 fluor.

Northern analysis: Total RNA was isolated from ~50 female flies using the TRIZOL reagent (GIBCO BRL, Gaithersburg, MD) and resuspended in diethyl-pyrocyanate-treated water. Preparation of formaldehyde-agarose gels, electrophoresis, and subsequent manipulations of gels was as described (SAMBROOK *et al.* 1989). Radiolabeled probes were prepared by random primer extension of coding regions of the histone genes, HIS1, HIS2B, HIS3, and HIS4, or of the ribosomal protein gene, RP49. Autoradiographs were scanned and the intensity of the bands quantified using the National Institutes of Health Image software.

Purification of nuclei from Drosophila and micrococcal nuclease analysis: Nuclei were isolated from *Drosophila* flies from the following strains: Oregon-R: *Su(var)3-9^{P17}/TM3*, which contains a *P*-element insertion in the *Su(var)3-9* locus; *Su(var)3-9³¹⁹* and *Su(var)3-9³³⁰*, two homozygous viable, EMS-induced mutants of *Su(var)3-9* that contain missense mutations in the SET domain. Flies (~2 g) from each strain were

processed as described (WU *et al.* 1979). The nuclei were resuspended [60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl (pH 7.4), 0.5 mM dithiothreitol, 0.25 M sucrose] and stored at -70° or adjusted to 0.1 mM CaCl_2 and 3 mM MgCl_2 for MNase I digestion at 26° . MNase I was added to a final concentration of 10 units/20 μg of DNA. Half the reaction mixture was removed after 60 sec and the remainder after 2 or 5 min, and the reaction was terminated by addition of EDTA to 12.5 mM. RNase A was added to a final concentration of 20 $\mu\text{g}/\text{ml}$ and incubated at 37° for 30 min. The mixture was adjusted to 0.5% sodium dodecylsulfate and Proteinase K (500 $\mu\text{g}/\text{ml}$) and incubated at 37° overnight. The DNA fragments were isolated by ethanol precipitation following a phenol:chloroform extraction, resuspended in 25 μl of TE buffer, and subjected to a *Bgl*II restriction digest that cuts at a unique site in HIS-C and establishes a fixed end on nuclease-digested fragments. The DNA was reisolated by ethanol precipitation, separated by electrophoresis on 1.25% agarose gels, and then transferred to a nylon membrane (SAMBROOK *et al.* 1989). The DNA fragments were detected by indirect end-labeling using a probe for the histone H1 coding region.

***In vitro* HMTase I assay:** *In vitro* HMTase I reactions were performed as described by REA *et al.* (2000).

Chromatin immunoprecipitation protocol: Formaldehyde crosslinked chromatin fragments were prepared by sonication from Kc1 tissue culture cells as described by STRUTT *et al.* (1997) and crosslinked nucleoprotein complexes were purified by immunoprecipitation using antibodies against the chromodomain region of SU(VAR)3-9 and the dimethyl-lysine 9 histone H3 peptide. Two control reactions were included in our immunoprecipitation experiments: the nonspecific antibody that recognizes the T7-Tag (Novagen) and Protein A sepharose beads alone. The DNA-protein complexes were reverse crosslinked for 5 hr at 65° . DNA was purified by phenol-chloroform extraction and recovered by ethanol precipitation. Precipitated DNA was analyzed by PCR using primers specific to three intergenic regions of the *his* unit (X14215; MATSUO and YAMAZAKI 1989). Two pairs of primers corresponding to the 5S rDNA cluster (accession no. X06938; SAMSON and WEGNEZ 1988) and to the Stellate cluster (accession no. X15899.1; LIVAK 1990), two other reiterated loci, were used as controls to ascertain specific immunoprecipitation of histone sequences. The sequences of the primers are: iH2Af (85-105), 5'-CCGGAGCAAACGGTGAATACG-3'; iH2Br (506-26), 5'-GATGGCATAGCTCTCCTTCCCT-3'; iH3f (3720-40), 5'-GCGTGGCGCCTTTCCACCAAGT-3'; iH4r (4104-24), 5'-GACGCTTGGCGCCACCTTTC-3'; iH4f (4353-74), 5'-CCGCACCCTCTACCGATTTGG-3'; iH2Ar (4852-72), 5'-CCGAGAAGAAGGCCTAAA CGT-3'; 5Sf, 5'-TGGCTACAAACAGAATGAAAAC-3'; 5Sr, 5'-AACTAAGAAGGCAGCAGGCAGC-3'; Stef, 5'-GGCCATCGAGT CCTCAGCCGA-3'; Ster, 5'-GATCCCCGAGGAACCAATCGAT-3'.

For DNA amplification the input DNA and the immunoprecipitated DNA, diluted 1:100-, 1:25-, and 1.10-fold, were used in the reaction. The PCR conditions used for amplification were denaturation at 92° for 50 sec, annealing at 56° for 1 min, and extension at 72° for 2 min 30 sec. This cycle was repeated 25 times.

RESULTS

SU(VAR)3-9 is associated with the histone cluster: We raised an antibody against an *Escherichia coli* expressed polypeptide representing one-third of SU(VAR)3-9 that includes the chromodomain region. The antibody appeared to detect SU(VAR)3-9 localized to the 39D-E region as well as to many other sites in the euchromatin.

The localization pattern of SU(VAR)3-9 was recently presented by SCHOTTA *et al.* (2002) and, in our experience, the immunolocalization of SU(VAR)3-9 to the 39D-E region is especially strong (data not shown). Since this is the site of the large multicopy HIS-C, it raised the possibility that SU(VAR)3-9 is associated with this cluster of tandemly reiterated genes. HIS-C includes ~ 0.5 Mb of DNA and contains 110 copies of the five histone genes (SAMAL *et al.* 1981), and it occupies bands 39D_{2,3}-39E_{1,2}. To demonstrate unambiguously that the major site of SU(VAR)3-9 localization corresponds to HIS-C, we performed a combined *in situ* hybridization and immunolocalization experiment. Salivary glands were hybridized with a biotinylated probe, prepared from a 155-kb DNA clone containing the histone repeats, followed by antibody staining using α -3-9^{chr}. Figure 1 (a-c) shows that SU(VAR)3-9 (green) colocalizes with the histone cluster sequences (intense red) and therefore we conclude that the most prominent site of SU(VAR)3-9 localization is HIS-C. Since the HIS-C genomic region and its chromatin structure are well defined, and its product is very abundant as well as essential, this locus provides an almost ideal site for examining the effect, if any, of *Su(var)3-9* mutations on chromatin structure and gene expression.

The chromatin structure of HIS-C is altered in *Su(var)3-9* mutants: First we asked whether SU(VAR)3-9 plays a substantive role in organizing and modulating chromatin structure at the HIS-C locus. The five histone genes are organized on a 5- or a 4.8-kb stretch of DNA, present at a ratio of 4:1, respectively, as depicted in Figure 2. The repeat unit is reiterated ~ 110 times to form HIS-C and spans >500 kb DNA (SAIGO *et al.* 1981). The positions of DNase I and MNase I hypersensitive sites in HIS-C have been determined (SAMAL *et al.* 1981) and reveal an extensive pattern of nuclease cleavage sites in the intergenic regions, indicating a unique nucleosomal arrangement. This nucleosomal organization is repeated over the entire cluster. The regularity of the histone gene repeats and the uniformity of the pattern of hypersensitive sites over the HIS-C region provides an excellent platform for examining chromatin structure.

We examined MNase I hypersensitive sites in wild-type (Oregon-R) and two mutant homozygous *Su(var)3-9* strains, 330 and 319. There is one *Bgl*II site in the histone gene unit, located just downstream of the HIS1 coding region, and it conveniently liberates the histone gene repeat unit. The DNA recovered after the MNase I digest was restricted with *Bgl*II. Thus, the fragments generated by the micrococcal nuclease digest have one end defined by the *Bgl*II restriction site. After electrophoresis and transfer to a nylon membrane, the fragments containing histone sequences were detected with a probe to the H1 coding region (Figure 2a). Under limiting MNase I digest conditions the pattern of cleavage sites in Oregon-R nuclei is the same as reported previously (SAMAL *et al.* 1981); however, the pattern in

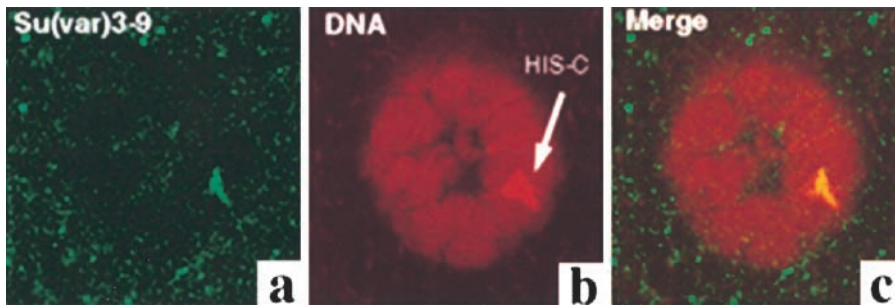


FIGURE 1.—Detection of SU(VAR)3-9 in *Drosophila* salivary gland nuclei to the HIS-C locus. A combined *in situ* hybridization and immunostaining experiment was performed to detect SU(VAR)3-9 with DNA sequences corresponding to the histone genes. (a–c) A single salivary gland nucleus. SU(VAR)3-9, detected with α -3-9^{chr} (a), is shown in green and HIS-C DNA (b) is the intense red signal. The colocalization of the signals is shown in the merged image (c).

the two homozygous *Su(var)3-9* mutant lines is vastly different (Figure 2a, lanes 2 and 3 *vs.* lanes 1 or 4). The extensive set of hypersensitive sites that are present over the intergenic region between HIS1 and HIS3 in Oregon-R is absent in *Su(var)3-9* mutant lines and is replaced with a single site located downstream of the HIS3 coding region. The pattern of hypersensitive sites over other intergenic regions is also strikingly altered. The MNase I sites located in the HIS3–HIS4 intergenic region of wild-type flies are completely absent in the 319 mutant line, and in the 330 mutant line they are replaced with a single site, which is positioned upstream of the HIS3 coding region. The cleavage sites at the HIS4–HIS2A and HIS2A–HIS2B intergenic sequences are also changed to single regions of hypersensitivity (Figure 2a). Clearly, the MNase I analysis indicates that the pattern of cleavage sites is dramatically altered at all intergenic regions in the *Su(var)3-9* mutants, indicating that changes occur at each of the five histone genes and are not restricted to just one gene. In addition, these changes must occur at all, or virtually all, 110 copies of the histone intergenic regions since we observed a uniform pattern of cut sites in the two *Su(var)3-9* lines, and no residual bands correspond to the wild-type pattern. A more extensive MNase I digest was performed to determine whether the histone repeats in the *Su(var)3-9* mutant lines still retain the regular arrangement of nucleosomes that is observed in Oregon-R (SAMAL *et al.* 1981). Again, the DNA fragments produced by MNase I digest have one end defined by the *Bgl*III restriction site. After electrophoresis and transfer to the nylon filter the histone sequence-containing fragments were detected using a probe for the HIS1 coding region. In wild-type flies a regular organization of the nucleosomes over the HIS-C is observed and this is retained in the *Su(var)3-9* mutant lines (Figure 2b). The nucleosomal ladder is homogeneous and has no smear, indicating that each nucleosome is positioned relatively precisely, and therefore each histone repeat must have a nucleosome at the equivalent DNA site. There is no wholesale uncoupling of the chromatin organization over the entire histone cluster in the *Su(var)3-9* mutant strains. Thus, the altered patterns of the nuclease hypersensitivity sites observed in the *Su(var)3-9* mutants are most probably the result

of local reorganization of nucleosomes in each *his* gene unit.

We conclude from these analyses that the chromatin structure of the entire HIS-C is altered in the *Su(var)3-9* mutants. We suggest that SU(VAR)3-9 is involved in the process of positioning and maintaining the nucleosome structure over HIS-C and functions to package HIS-C into a distinct chromatin domain.

Expression of the histone genes is altered in *Su(var)3-9* mutants: Next we asked whether the alteration in the chromatin structure of HIS-C has any effect on the transcription of the histone genes. We have isolated >30 different mutant alleles of *Su(var)3-9*, generated by chemical or transposable element mutagenesis and identified on the basis that they all strongly suppress PEV. We analyzed the steady-state level of histone transcripts in a subset of these mutant strains, including: (1) a *P*-element insertion in the 5' untranslated region of *Su(var)3-9* (P25); (2) strain P25^{r2}, which is a complete revertant of P25; and (3) strain 330, which is an EMS-induced missense mutation in *Su(var)3-9* that causes an ASP-to-ASN substitution in the SET domain of SU(VAR)3-9 (see MATERIALS AND METHODS). Prior to isolating total RNA from these *Su(var)3-9* alleles, we established lines with the same genetic backgrounds to minimize any variations introduced by balancer chromosomes or other factors. The isolation of total mRNA and Northern blot analysis were performed on six independent isolates to quantify the change in histone transcript levels. We examined the transcript levels of all five histone genes, but focus on HIS4 as a representative of the core histone genes and the linker HIS1 gene. After electrophoretic separation of mRNA and transfer to nylon membranes, the filters were probed for HIS1 and HIS4 transcripts (Figure 3a). A ribosomal protein transcript, RP49, was used as the loading control. The *P*-element insertion *Su(var)3-9*^{P25/+} (P25/+) exhibited 2.3- and 2.4-fold increases in HIS1 and HIS4 transcript levels, respectively (Figure 3a, lane 3 *vs.* 1, and Figure 3b). Strain P25^{r2}, which is a complete revertant of the *P* insert and reverts all of the *Su(var)3-9* visible phenotypes, has HIS1 and HIS4 levels that are similar to wild type (Figure 3a, lanes 6 and 1, and Figure 3b). Very similar results were obtained with a second *P*-insert mutation, *Su(var)3-9*^{P17}, and its complete revertant, P17^{r10}

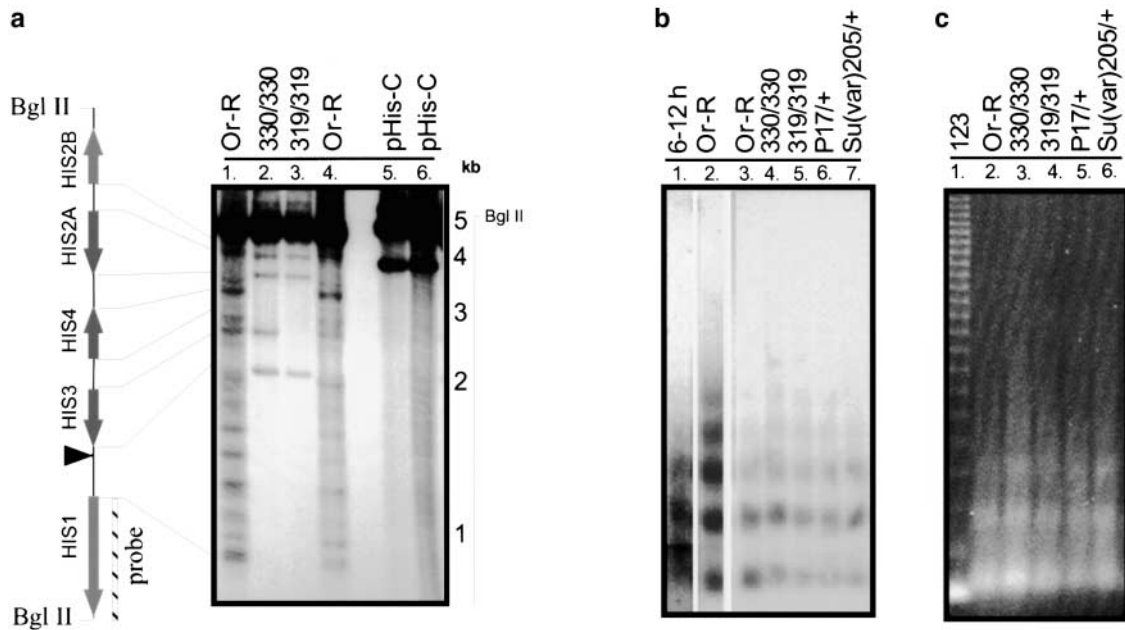


FIGURE 2.—Micrococcal nuclease I hypersensitive sites at HIS-C in *Su(var)3-9* mutant lines. (a) Nuclei were digested in the presence of 0.2 units/ μ l of MNase I. DNA fragments were isolated, digested with *Bgl*II, separated electrophoretically, and transferred to a nylon membrane. Sequences from the histone cluster were detected by indirect end-labeling using a probe to the HIS1 coding region. Lane 1, nuclei from Oregon-R digested for 90 sec; lanes 2 and 3, nuclei from homozygous *Su(var)3-9* mutant lines 330 and 319, respectively; lane 4, nuclei from Oregon-R digested for 60 sec; lane 5, pBac, the 155-kb DNA clone containing histone gene repeats digested with *Bgl*II; lane 6, pBac clone containing the histone repeats digested with MNase I and treated exactly as the nuclei isolated from *Su(var)* lines. The schematic shows the organization of the 5-kb histone gene unit and the flanking *Bgl*II sites. The approximate position of the 200-bp deletion that distinguishes the 4.8-kb histone repeat unit is indicated by an arrowhead. The hatched line indicates the region of HIS1 to which a probe was made. (b) Histone cluster retains a regular nucleosomal organization in *Su(var)3-9* mutant lines. Nuclei were digested with micrococcal nuclease I for 5 min, and DNA was isolated, separated on agarose by electrophoresis, and transferred to a nylon membrane. Sequences in HIS-C were detected by hybridization using a histone H1 probe. Lane 1, nuclei isolated from 6- to 18-hr-old embryos. Lanes 2 and 3, nuclei from adult Oregon-R (long and short exposure). Lane 4, nuclei from the *Su(var)3-9* mutant line 330. Lane 5, nuclei from the *Su(var)3-9* mutant line 319. Lane 6, nuclei from the heterozygous *P*-insert line P17. Lane 7, nuclei from the *Su(var)2-5* mutant line for HP1. (c) Ethidium-bromide-stained agarose gel prior to transfer of DNA onto nylon membrane used in hybridization experiment in b to indicate equal loading of DNA in each lane. Lane 1 is a 123-bp DNA ladder and lanes 2-6 correspond to lanes 3-7 in b.

(data not shown). A partial remobilization strain P17^{r12}, which retains a part of the *P*-element insert in *Su(var)3-9*, continues to show elevated levels of HIS1 and HIS4, similar to the parental strain P17. These data suggest that the elevation in histone transcripts is due to alteration of the *Su(var)3-9* gene. However, it could be argued that the *P*-element inserts themselves lead to overexpression of *Su(var)3-9*. Hence, we also examined an EMS-induced missense mutation of *Su(var)3-9*, 330, which carries a mutation in the SET domain and displays no detectable HMTase activity. The *Su(var)3-9³³⁰/Su(var)3-9³³⁰* mutant exhibited a 1.9- and 1.8-fold increase in HIS1 and HIS4 transcript levels respectively, relative to wild type (Figure 3a, lanes 8 and 1, and Figure 3b). Finally, Northern blot analyses of the HIS2A and HIS3 mRNA levels show a similar increase in the steady-state levels of these histone gene products in all of the mutant *Su(var)3-9* lines (data not shown).

In summary, our Northern analyses show an increase in the steady-state level of histone transcripts in both the

P-element insertion lines and the EMS-induced *Su(var)* mutants. The histone mRNA levels are restored to near wild-type amounts in lines that have the *P*-element completely excised. We conclude from the observed increase in histone transcript in the *Su(var)3-9* mutants, relative to Oregon-R, that SU(VAR)3-9 protein is involved in the regulation of histone gene expression.

Expression of the histone genes is altered in a HIS-C deficiency strain: Twenty years ago, MOORE *et al.* (1979) demonstrated that hemizyosity for the HIS-C region suppressed PEV, whereas deficiencies of equal or larger size adjacent to, but not including any of the HIS-C, had no effect on PEV. While the histone levels in these HIS-C deletion strains were never measured, the inference was that a 50% reduction in the number of histone genes would lead to a substantial reduction in the amount of histones available to the cell at the time of DNA replication and that this would alter chromatin packaging and, hence, PEV. In contrast, we observe that mutations in *Su(var)3-9*, all of which suppress PEV,

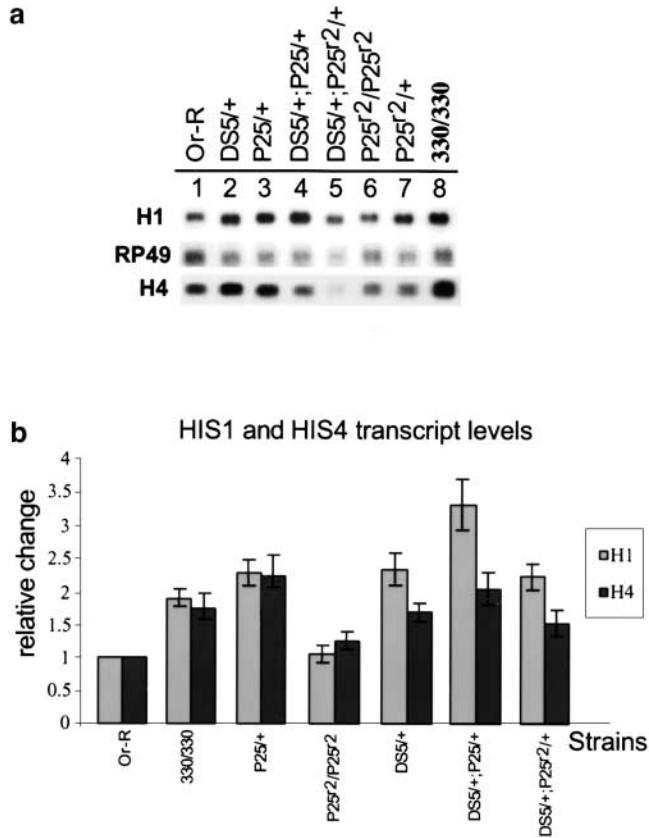


FIGURE 3.—Detection of histone transcripts by Northern analysis. Total mRNA was isolated from female flies, separated by electrophoresis, and transferred to nylon membranes. (a) Lane 1, RNA from Oregon-R. Lane 2, RNA from HIS-C deficiency *Df(2L)DS5/+*. Lane 3, RNA from a hemizygous line carrying a *P*-element insert P25 in *Su(var)3-9* (P25/+). Lane 4, RNA from the double-mutant strain *Df(2L)DS5/+* (complete hemizygosity for HIS-C) and P25/+ [*P* insert in *Su(var)3-9*]. Lane 5, RNA from *Df(2L)DS5/+* and a complete revertant of the P25 insert in *Su(var)3-9* (DS5/+; P25^{r2}/+). Lane 6, RNA from a strain homozygous for the complete revertant of the P25 insert in *Su(var)3-9* (P25^{r2}/P25^{r2}). Lane 7, RNA from a strain heterozygous for the complete revertant of the P25 insert in *Su(var)3-9* (P25^{r2}/+). Lane 8, RNA from a homozygous viable EMS-induced *Su(var)3-9* strain (330/330). Each lane is loaded with 10 μ g of RNA. Multiple Northern blots were prepared and sequentially probed to detect HIS1, RP49, and HIS4. The values for HIS1 and HIS4 transcripts were normalized relative to Oregon-R. (b) A summary of the quantification of Northern blot analyses. The data are presented as a bar chart for HIS1 (light shading) and HIS4 (dark shading) and are from six and five different blots, respectively. The strain designations are as indicated in a. The error bars reflect the standard error of the mean.

increase the amounts of the five histone transcripts by about twofold. To reconcile these apparently disparate results, we examined the histone transcript levels in *Df(2L)DS5*. Like *Df(2L)65* and *Df(2L)161* strains, *Df(2L)DS5* (DS5) carries a chromosomal deficiency that removes the HIS-C locus, but DS5 is considerably smaller than deletions 65 or 161 (MOORE *et al.* 1983). We measured the amount of histone mRNA produced in *Df(2L)DS5/+*

TABLE 1

Relative amount of mRNA in wild type *vs.* *Su(var)3-9* mutations and wild-type *vs.* histone deletions

Genetic strain	Per HIS-C template		
	HIS1	HIS4	Ratio H1:H4
Ore-R	1	1	1
P25/+	2.28	2.38	0.96:1
P25 ^{r2} /P25 ^{r2}	1.05	1.24	0.85:1
330/330	1.9	1.76	1.08:1
DS5/+	4.68	3.37	1.39:1
DS5/+; P25/+	6.62	4.10	1.62:1
DS5/+; P25 ^{r2} /+	4.46	3.04	1.47:1

The relative amounts of HIS1 and HIS4 transcript levels were determined from Northern blot analyses based on six independent experiments (see Figure 3 for details). Note that while there is about a twofold increase in expression of HIS1 and HIS4 levels in the *Su(var)3-9* mutations the stoichiometry remains the same. However, the HIS1 and HIS4 stoichiometry is altered in all lines carrying the HIS-C deletions. P25 is an insertion into the first intron of *Su(var)3-9*, and P25^{r2} is P25 with the *P* element completely excised. 330/330 is a homozygous line carrying an EMS-induced point substitution in *Su(var)3-9*. DS5/+ [*Df(2L)DS5*] is the chromosomal deficiency strain that removes one chromosomal copy of HIS-C. This chromosomal deficiency was previously shown to suppress PEV (MOORE *et al.* 1979).

(one copy of the HIS-C) relative to Oregon-R (diploid). Rather unexpectedly, the HIS-C deficiency strain also displayed a dramatic increase in its histone H1 and H4 transcript levels relative to Oregon-R (Figure 3a, lane 2), suggesting an upregulation of HIS-C on the wild-type autosome. The steady-state H1 and H4 mRNA levels in the *Df(2L)DS5/+* strain are 2.34- and 1.68-fold higher than those in wild-type, respectively. But since the *DS5/+* strain has half the number of histone genes as the wild-type strain, then, on a per-template basis, the expression of HIS1 and HIS4 mRNA in the DS5 strain is 4.7 and 3.4 times higher than that in the wild-type strain, respectively (Table 1). Clearly, these results were unexpected and are counterintuitive. We also examined the effects of combining the histone deletion with a single dominant allele of *Su(var)3-9* (P25, Figure 3a, lane 3) and with a complete revertant of this mutation (P25^{r2}, Figure 3a, lane 5). The double-mutant strain [dominant *Su(var)3-9* mutation and the HIS-C deletion] had a 6.6- and 4.1-fold increase in histone HIS1 and HIS4 mRNA levels/template, respectively, and the HIS1 and HIS4 mRNA levels in the DS5/+; P25^{r2}/+ strain were essentially indistinguishable from those in the DS5/+ strain (Table 1). Thus, the *Su(var)3-9* mutation and the HIS-C deletion appear to work additively, suggesting that the further increase in HIS1 and HIS4 transcript levels observed in the double mutant, DS5/+; P25/+ , compared with the deficiency (strain DS5/+), must be due to the alteration in *Su(var)3-9* expression. A similar increase

in HIS2B levels was also observed (data not shown). A visual representation of the quantitative changes in HIS1 and HIS4 levels in the various mutant and revertant strains relative to Oregon-R is shown in Figure 3b along with SEM. We note that the relative stoichiometry of HIS1 and HIS4 mRNA levels is not maintained in the HIS-C deletion strains.

In summary, our Northern analyses show that hemizygosity for the HIS-C region, like the mutations in *Su(var)3-9*, leads to an increase in histone transcript levels. This suggests that suppression of PEV observed in the HIS-C deficiency lines (MOORE *et al.* 1979, 1983) is associated with increases, not decreases, in histone transcript levels. The relative increase in steady-state *his* transcript levels is similar to that observed for both the *P*-element insertion lines and the EMS-induced *Su(var)* mutants, although on a per-template basis the HIS-C deletion shows a much higher increase. The double-mutant strain that combined the histone deletion with an allele of *Su(var)3-9* displayed an additive increase in histone transcript levels, which may suggest that the regulatory mechanisms that control histone gene expression in the HIS-C deficiency strain and the *Su(var)3-9* mutants differ. Interestingly, the deletion of one copy of the HIS-C leads to a decoupling of histone transcript levels, leading to the possibility that both the amount and the stoichiometry of the histone proteins is altered in the deficiency line.

***Su(var)3-9* mutants are stronger suppressors of PEV than is the HIS-C deficiency:** MOORE *et al.* (1979) showed that hemizygosity for the HIS-C region was a moderate suppressor of PEV (MOORE *et al.* 1979, 1983). For example, the eye pigment levels in *In(1)w^{md}/Y; Df(2L)DS5/+* and *In(1)w^{md}/Y; Df(2L)DS6/+* males were 24–38% of wild type, respectively, compared to pigment levels of 4–7% found in *In(1)w^{md}/Y; +/+* males. In contrast, *Su(var)3-9* mutants appear to be strong suppressors of PEV. Having observed that the histone transcript levels in the HIS-C deficiency strain and the *Su(var)3-9* mutants are elevated to approximately similar levels within the cell, we revisited the issue of whether dominant *Su(var)3-9* mutations and hemizygosity for HIS-C differed in their ability to suppress PEV. We performed a standard eye pigment assay and determined the amount of drosoprotein in the eyes of the *Su(var)3-9* mutant and the histone deficiency lines carrying the variegating rearrangement *In(1)white^{md}* (Table 2). For this analysis we used the *Su(var)3-9* mutants 319 and 330, previously used in Northern and nuclease hypersensitivity experiments, and EMS-induced mutant 311, selected as a random representative of other *Su(var)3-9* mutations, and two HIS-C deletions, *Df(2L)DS5* and *DS6*. The *white⁺* gene in *In(1)w^{md}/Y; Su(var)3-9/+* mutants is expressed to 90–100% of the wild-type level. However, in the HIS-C-deficiency-bearing strain the *white⁺* gene in the variegating strain *In(1)white^{md}* is expressed to only 25–40% of the wild-type level (Table 2). Similar differences in the levels of suppression of PEV

TABLE 2

Mean percentage of the wild-type amount of drosoprotein in the eyes of *In(1)white^{md}*; deficiency of HIS-C/+ and *In(1)white^{md}*; *Su(var)3-9/+* flies

Genetic strain	% <i>white</i> drosoprotein	
	Male	Female
<i>In(1)white^{md}/Y; +/+</i>	7 ± 1	11 ± 2
<i>In(1)white^{md}/Y; Su(var)3-9³¹¹/+</i>	100 ± 4	100 ± 11
<i>In(1)white^{md}/Y; Su(var)3-9³¹⁹/+</i>	87 ± 3	90 ± 9
<i>In(1)white^{md}/Y; Su(var)3-9³³⁰/+</i>	100 ± 7	100 ± 7
<i>In(1)white^{md}/Y; DS6/+</i>	39 ± 8	37 ± 7
<i>In(1)white^{md}/Y; DS5/+</i>	27 ± 5	26 ± 6

The amount of drosoprotein pigment was measured in the eyes of flies with indicated genotypes using a fluorometric assay (MOORE *et al.* 1979). The numbers are expressed as the mean percentage of pigment in wild-type fly eyes ± SEM. Three *Su(var)3-9* mutations, 311, 319 and 330, and the two deficiencies, DS5 [*Df(2L)DS5*] and DS6 [*Df(2L)DS5*], which remove the entire HIS-C region, were examined.

were observed when *In(2LR)bw^{idw2}* or *T(2;3)Sb^V* was used as the variegating reporter gene (data not shown). We conclude from this analysis that the *Su(var)3-9* mutants are stronger suppressors of PEV than is the HIS-C deficiency. These data also confirm that both *Su(var)3-9* mutants and hemizygosity of the HIS-C locus are general suppressors of PEV.

SU(VAR)3-9 is a histone H3 methyltransferase: Why are the *Su(var)3-9* mutants strong suppressors of PEV whereas the HIS-C deletion is a moderate suppressor, even though they both elevate the histone transcript levels to the same extent? One possibility is that the suppression of PEV associated with *Su(var)3-9* mutants is due to a combination of factors, including a lack of the wild-type SU(VAR)3-9 function elsewhere in the genome. SUV39H1, which is the human homolog of *Drosophila* SU(VAR)3-9, is a methyltransferase, and it specifically methylates LYS-9 of histone H3 (REA *et al.* 2000). Recently, it has been shown that methylated histone H3 is enriched in heterochromatic regions (JACOBS *et al.* 2001; NAKAYAMA *et al.* 2001) and is present at loci that are subject to gene silencing (NAKAYAMA *et al.* 2001). Therefore, one possibility is that the silencing of the *white⁺* gene in the variegating rearrangement *In(1)white^{md}*, as well as down-modulation of the histone genes, requires methylated histone H3. We asked if SU(VAR)3-9 has a methyltransferase activity. Indeed, similar to the observations of SCHOTTA *et al.* (2002), we were able to show that *Drosophila* SU(VAR)3-9 is a methyltransferase (data not shown). The reaction is specific for histone H3.

Histone protein levels are altered in *Su(var)3-9* mutant strains: As outlined earlier, *Su(var)3-9* mutants and the HIS-C deletion strain show approximately twofold increases in histone mRNA levels. We were curious about

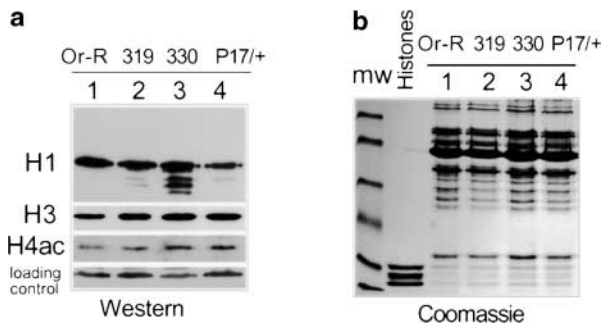


FIGURE 4.—Detection of histone H1 isoforms in *Su(var)3-9* mutant lines. (a) Histone H1 was detected using an antibody against the *Drosophila* H1 protein. Four isoforms detected in line 330/330 (lane 3) are present in much-reduced amounts in line 319/319 (lane 2) and P17/+ (lane 4) while absent in Oregon-R (lane 1). The same membrane was probed with antibodies to acetylated histone H4 (H4ac) and histone H3 (H3). A moderate but notable increase in the amount of acetylated H4 and H3 was consistently detected in the *Su(var)3-9* lines (lanes 2–4) relative to Oregon-R (lane 1). A lamin antibody cross-reacting polypeptide was used as a loading control. (b) A Coomassie profile of a duplicate gel used in the Western analysis. Approximately 75 μ g of total nuclear protein was loaded in each lane.

whether the twofold increase in histone gene transcripts led to any detectable increase in histone proteins associated with total chromatin in *Drosophila* nuclei. Hence, we examined the histone protein amounts in nuclei isolated from *Su(var)3-9* mutant and wild-type strains. Clearly, we did not expect a twofold increase in the histones incorporated into chromatin. Doubling the number of nucleosomes per unit length of DNA is probably impossible, but an increase of 10–20% would probably have a significant impact on the expression of many genes in the genome, perhaps including the packaging of loci that are subject to PEV in variegating strains.

We focused our attention on the level of histone H1 protein. Since it binds the internucleosomal linker DNA sequences (THOMAS 1999) and is involved in the higher-order packaging of chromatin (RAMAKRISHNAN 1997), an increase in H1 availability could have significant and dramatic effects on chromatin structure. H1 was examined in nuclei isolated from wild-type and *Su(var)3-9* mutant adult flies by Western blot analyses using an antibody that specifically recognizes the fly H1 (CROSTON *et al.* 1991). The mature *Drosophila* H1 protein migrates as a single band of \sim 34 kD (CROSTON *et al.* 1991; NER and TRAVERS 1994). The relative amount of this 34-kD product is similar in all lines, including Oregon-R (Figure 4a). In addition to the expected 34-kD H1 product, the nuclei from the *Su(var)3-9* mutants also contain faster migrating species, which are not observed in wild-type strains. Nuclei from the homozygous EMS-induced mutant *Su(var)3-9* strain 330 showed the most dramatic change. In addition to the 34-kD product, four new isoforms were observed, the fastest of which migrated at

\sim 26 kD (Figure 4a, lane 3). Two other *Su(var)3-9* mutants, 319 homozygotes, and P17/+ heterozygotes produce these same novel bands but in lesser amounts (Figure 4a, lanes 2 and 4). While the exact nature of the faster-migrating bands, which cross-react with the H1 antibody, is unknown, they probably correspond either to isoforms of H1 or to specific degradation products, which might be expected if the total level of H1 within the nucleus was increased as a result of overexpression. Clearly, *Su(var)3-9* mutants show an overall change in the total amount of H1 protein, and all mutants examined produce H1 isoforms not detected in nuclei isolated from wild-type strains. The most dramatic increase in total H1 protein is in the 330 homozygous strain. The heterozygous *P*-insert line, P17/+, shows a slight decrease in the 34-kD H1, but it still displays a faster-migrating isoform. Since these H1 profiles are from isolated nuclei, both the altered amount and the novel isoforms of H1 are most probably associated with chromatin.

We also examined the amount of core histone levels in isolated nuclei in the *Su(var)3-9* mutants. Under normal conditions equal amounts of the four core histones are incorporated into chromatin. Therefore we chose to examine only two of the core histones. We examined the total amount of histone H3 as a representative of unmodified histones, and we examined acetylated histone H4 as a representative of modified histones. The proteins were detected by Western blots using antibodies specific for these proteins (Figure 4a) and three independent blots were analyzed. In each case the total H3 and acetylated H4 levels were 20–25% higher in the *Su(var)3-9* mutants than in wild type, and the homozygous line, 330/330, always showed the greatest difference from wild type (Figure 4a, compare lane 1 with lane 3). While the increase in protein levels is not in the same range as the twofold increase observed for the histone transcripts, the 20–25% increase could have significant influence on packaging of many loci, including the variegating loci. We conclude from the Western analysis of nuclei from *Su(var)3-9* mutants that a twofold change in histone transcript levels results in an increase in the total histone proteins produced in the cytoplasm and that a significant proportion of these are transported to the nucleus and incorporated into chromatin.

SU(VAR)3-9 is physically associated with HIS-C and histone H3 at the HIS-C locus is methylated: Our data show that SU(VAR)3-9 is a *trans*-acting regulator of the HIS-C locus; mutants in *Su(var)3-9* alter both the chromatin structure of the HIS-C locus and the amount of transcripts it produces. *Su(var)3-9* could regulate the HIS-C packaging, and thus its expression, either directly or indirectly. Indeed, since SU(VAR)3-9 has an HMTase domain, its action may be transient. We used chromatin immunoprecipitation (ChIP) analyses to determine whether the histone H3 of the HIS-C locus was methylated and, if so, whether SU(VAR)3-9 was physically associated with the HIS-C locus. We used a commercially

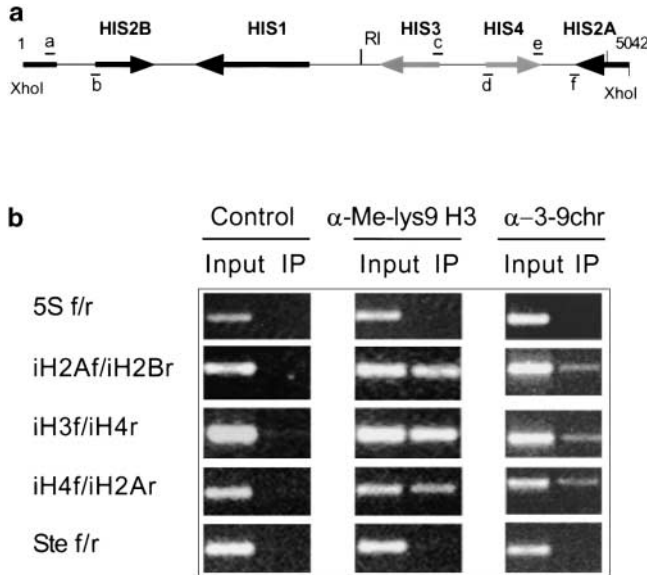


FIGURE 5.—HIS-C sequences are immunoprecipitated with α -Me-Lys9 H3 and α -3-9^{chr} antibodies. (a) A schematic of the 5-kb *his* repeat unit as an isolated *XhoI* fragment (LIFTON *et al.* 1978; SAMAL *et al.* 1981). The arrows indicate the five histone gene transcription units. The lines above and below the unit (labeled a–f) indicate the forward (above) and reverse (below) oligonucleotide primers used in the polymerase chain amplification reactions to detect intergenic DNA sequences in immunoprecipitated chromatin. (b) The primer pairs were used to amplify DNA immunoprecipitated from chromatin with a control antibody, α -Me-lys9 H3, and α -3-9^{chr}. Intergenic sequences were selectively amplified from chromatin immunoprecipitated with α -Me-lys9 H3 and α -3-9^{chr} antibodies. 5S rDNA or Stellate DNA sequences were not detected in immunoprecipitated chromatin when primer pairs 5Sf/5Sr and Stef/Ster were used in the amplification reactions.

available antibody that selectively recognizes H3 methylated at LYS9 (NOMA *et al.* 2001) and our α -3-9^{chr} antibody to immunoprecipitate nucleoprotein complexes from crosslinked chromatin prepared from Kc1 tissue culture cells. Any enrichment of DNA fragments corresponding to HIS-C was detected by PCR. We present the PCR data for three pairs of oligonucleotide primers corresponding to intergenic regions between H3 and H4, between H4 and H2A, and between H2A and H2B (Figure 5a) in the 5-kb *his* repeat (a more detailed ChIP analysis of the HIS-C locus will be presented elsewhere). The ChIP analysis shows that sequences corresponding to the intergenic region of the histone genes are enriched with the α -Me-Lys9 H3 and α -3-9^{chr} antibodies (Figure 5b). The enrichment is specific to these antibodies since the control α -T7 antibody (Figure 5b) and the Protein A sepharose alone (data not shown) failed to immunoprecipitate HIS-C sequences. We also examined the 5S rDNA and Stellate genes as examples of tandemly reiterated sequences located elsewhere in the genome. We were unable to detect 5S rDNA sequences or Stellate sequences in ChIP experiments with either the α -Me-Lys9 H3 or the α -3-9^{chr} antibody, indicating that SU(VAR)3-9

and methylated LYS9 H3 are not present at these reiterated loci. The fact that we detected SU(VAR)3-9 at the HIS-C locus in ChIP analyses but failed to detect it at two other tandemly reiterated loci leads us to conclude that SU(VAR)3-9 is physically associated with HIS-C sequences. The HIS-C locus also contains methylated histone H3, which strongly supports a role for SU(VAR)3-9 in methylation of H3 LYS9.

DISCUSSION

We have examined the expression and chromatin structure of the *his* genes in *Su(var)3-9* mutants. Our data show that mutations in a *Su(var)3-9* alter chromatin structure and concomitantly alter gene expression. This is the first demonstration that mutations in a *Su(var)* gene actually alter chromatin structure and that this alteration in structure is associated with an alteration in gene expression. Even though the *Drosophila* SU(VAR)3-9 protein is 223 amino acids larger than its mammalian counterpart, this study shows that *Drosophila* SU(VAR)3-9 also has a histone H3 methyltransferase. Finally, the ChIP data indicate that a significant proportion of the H3 histones associated with the HIS-C locus are methylated and that the *Su(var)3-9* protein associates with the HIS-C locus. Collectively, these data suggest that SU(VAR)3-9 is a *trans*-regulator of histone gene expression and that it regulates histone gene packaging and expression by forming part of the HIS-C chromatin rather than by transiently associating with the region.

Our data highlight an intriguing discrepancy in the localization of SU(VAR)3-9 protein. Reuter and colleagues have shown that SU(VAR)3-9 is localized to many euchromatic sites and to the chromocenter of polytene chromosomes (SCHOTTA *et al.* 2002). In addition, the pattern of localization of methylated histone H3 is similar to the pattern observed for SU(VAR)3-9 (JACOBS *et al.* 2001), a modification that is carried out by SU(VAR)3-9. Our antibody behaves differently and does not detect SU(VAR)3-9 at the chromocenter (data not shown). One clear possibility is that the epitope of SU(VAR)3-9 to which the antibody was raised is hidden at many binding sites, including the chromocenter. Indeed this is exactly the situation that occurs with human HP1 gamma. Antibodies that recognized the amino terminal half of the protein failed to detect HP1 gamma at centric regions of chromosomes, while an antibody that recognized the carboxy end of the protein did detect HP1 gamma in centric regions of chromosomes (MINC *et al.* 2000). We are addressing this issue by preparing antibodies to other regions of SU(VAR)3-9.

***Su(var)3-9* mutations alter histone gene expression and chromatin structure at HIS-C:** Our data show that the pattern of hypersensitive sites over the histone genes is dramatically altered in *Su(var)3-9* mutants relative to Oregon-R. For example, both the number and the relative position of the hypersensitive sites in the noncoding

region between HIS1 and HIS3 genes are drastically reduced, from 10 primary sites in Oregon-R to 1 in the *Su(var)3-9* mutants (Figure 2). The absence of smearing in the nuclease digestion pattern suggests that the change in chromatin structure observed in *Su(var)3-9* mutants is homogeneous, occurring at each of the 110 copies of the histone repeat unit. While there is a dramatic alteration in nuclease hypersensitive sites, we observed no loss of nucleosomes in *Su(var)3-9* mutants *vs.* wild type. We interpret this to mean that the structure of the HIS-C region is altered in *Su(var)3-9* mutants but the reorganized chromatin is ordered and uniform over each of the 110 *his* gene units. Since the number of nuclease hypersensitive sites is reduced, especially in the intergenic regions, in virtually all of the *his* repeat units in *Su(var)3-9* strains *vs.* wild type we infer that *Su(var)3-9* mutants provide a more "open" chromatin configuration. Indeed, our data show that *P*-element insertions and EMS-induced mutations in *Su(var)3-9* cause an increase in the steady-state levels of the histone transcripts. The increase in histone mRNA levels varies somewhat from one mutant genotype to another, as expected, but generally averages about twofold in the various *Su(var)3-9* mutants examined. The strains in which the *P* element is precisely excised show that a return of histone transcript amounts to wild-type levels. This is the first time it has been shown that a protein modifying histone gene expression is a component of the histone chromatin domain. This is also the first demonstration that alterations in SU(VAR)3-9 protein are associated with alterations in both chromatin structure and gene expression at one of its target loci.

Finally, since histone gene transcription is tightly regulated and coordinated with DNA replication, one presumes that the rate of histone gene transcription must be modulated from one tissue type or developmental stage to another commensurate with the changes in the length of S-phase. There are two ways in which this modulation in histone gene transcription could be accomplished. Either the transcription rates of all 110 copies of the *his* unit are modulated similarly or, alternatively, the number of *his* units that are actively transcribed varies with the length of the S-phase. While the *Su(var)3-9* mutants cause a dramatic change in the number of nuclease hypersensitive sites, especially in the intergenic regions, the histone units were uniformly cut (no smearing). This uniformity in packaging of the histone repeat units infers that all 110 copies of the *his* repeats are packaged and transcribed similarly; that is, the HIS-C is modulated as a cluster. The only other hypothesis consistent with these observations is that silent *his* units completely lack hypersensitive sites while active *his* units have hypersensitive sites. But even under this scenario the *Su(var)3-9* mutants must alter the hypersensitive site pattern of the actively transcribed *his* units. The active *his* units could be distinguished from the inactive units through a replication-independent

deposition of the variant histone H3.3 into the nucleosomes encompassing the active units. Such a situation is observed with the regulation of transcription of the rDNA arrays (AHMAD and HENIKOFF 2002). Methylated-lysine 9 histone H3 is absent in transcriptionally active loci but is present in the inactive loci.

Reduction in number of histone templates, like mutations in *Su(var)3-9*, also causes an increase in histone gene expression: Histones not only are essential in all eukaryotic cells, but also must be made in the correct stoichiometric ratios to serve as the basic substrates of DNA packaging. Indeed, H2A-H2B and H3-H4 genes are generally organized as pairs and often clustered in many organisms (OSLEY 1991). The conservation of histone gene organization may reflect the need for, and maintenance of, this balanced expression. Here we show that strains heterozygous for a deletion of the HIS-C region, *Df(2L)DS5/+*, result in a three- to fivefold increase in the histone mRNA production/template. Unlike the mutations in the *Su(var)3-9* gene, hemizyosity for the HIS-C region appears to uncouple the stoichiometry in histone production. Therefore, while mutations in *Su(var)3-9* and hemizyosity for the HIS-C region both result in an increase in histone gene expression, the mechanisms by which this increase occurs may differ. Consistent with this hypothesis, the effect of combining *Su(var)3-9* mutants with the HIS-C deletion appears to be additive, rather than epistatic or synergistic. We do not know why hemizyosity for the HIS-C region leads to an increase in histone transcripts. We point out that in response to deletion of one copy of HIS-C, the HIS-C region on the normal homolog is capable of undergoing magnification (CHERNYSHEV *et al.* 1980). Magnification, which requires four to five generations, is not the basis of the increase in transcript levels that is reported in our study since we observed the increase in transcript levels among the F₁ of outcrossed strains. We hypothesize that the gene regulation of the HIS-C locus may be pairing sensitive and that the deletion of one copy of HIS-C disrupts pairing and leads to overexpression of the histone genes from the remaining copy. Consistent with this hypothesis, in *Drosophila* embryos the histone locus was found to pair more frequently and more rapidly than other euchromatic loci (FUNG *et al.* 1998). We are currently testing the HIS-C deletion stocks and mutations in genes that influence transvection to see if any of these alter the chromatin structure and/or the expression of the HIS-C locus.

Alteration in histone gene expression influences the expression of many loci, including those involved in telomeric position-effect variegation (TPEV): DNA microarray studies in *Saccharomyces cerevisiae* have shown that when the histone stoichiometry is altered, for example, by deletion of the yeast histone gene pair HTA2-HTB2 encoding H2A and H2B or by reduction of histone H4 levels, the expression profiles of ~10–15% of the genetic loci are altered (NORRIS *et al.* 1988; WYRICK

et al. 1999). We examined nuclei isolated from *Su(var)3-9* mutants and wild-type strains to determine whether the twofold increase in histone gene transcripts led to a detectable increase in the amount of histones associated with total chromatin. We did not expect, nor did we detect, a doubling of the amount of histone proteins associated with chromatin. However, we did detect an increase of ~20–25% in histone H3, acetylated H4, and H1. Since all three histone types, which represented core, modified, and linker histones, respectively, were increased to similar levels, we believe that the alteration in HIS-C expression caused by *Su(var)3-9* mutations alters chromatin packaging at many areas of the genome. The 20% increase in the amount of nuclear histones that we detected in *Su(var)3-9* mutants correlates reasonably well with the observation that the expression of 10–15% of the yeast genome is altered when histone levels are altered in yeast strains (NORRIS *et al.* 1988; WYRICK *et al.* 1999). Studies in yeast have also shown that telomeric silencing, a gene-silencing phenomenon closely resembling PEV, is strongly reduced when one of two H3 and H4 gene pairs is deleted (NORRIS *et al.* 1988; KAUFMAN *et al.* 1998). Furthermore, mutations in HIR1 and SPT21 genes of yeast, which cause a misregulation of histone gene transcription in opposite directions, change the histone stoichiometry and result in suppression and enhancement of telomeric-position effect, respectively (KAUFMAN *et al.* 1998). While all these studies link alterations in histone amounts to altered gene expression at many loci in the genome, and in particular, to alterations in the gene silencing associated with TPEV, no mechanism has been proposed to explain these effects.

Finally, the doubling in histone mRNA that we detected in *Su(var)3-9* mutant strains does not result in a twofold increase in the amount of nucleosomes or histones associated with chromatin in these strains. Clearly, doubling the number of histones/unit of DNA is probably impossible and, if attempted, would no doubt result in lethality early in development. Since we detected no significant reduction in viability among *Su(var)3-9* homozygotes, HIS-C hemizygotes, or double-mutant strains, we assume that there is a mechanism that prevents excess formation and/or transport of the histones to the nucleus.

SU(VAR)3-9 and PEV: While we have no evidence that directly links alterations in histone levels to PEV, the histones are key packaging components and we find it curious that both hemizygosity for HIS-C and mutations of *Su(var)3-9* increase histone transcript levels by about twofold. This implies that alteration in histone transcript levels can suppress PEV either directly or indirectly. But clearly the *Su(var)3-9* mutants are very strong dominant suppressors of PEV, whereas HIS-C deletion lines are only moderate suppressors of PEV (MOORE *et al.* 1979, 1983). However, the level to which the histone transcripts are elevated in the *Su(var)3-9* mutants and in the HIS-C deletion lines is similar. This indicates that

enlarging the pool of histones available to the cell at the time of DNA replication cannot be the only factor influencing the packaging and/or expression of the genes subjected to PEV. Since methylated-lysine 9 H3 is associated with heterochromatin and silenced genes (JACOBS *et al.* 2001; NAKAYAMA *et al.* 2001), the HMTase activity of SU(VAR)3-9 could play a significant role in determining directly or indirectly the transcriptional competency of a variegating locus. Perhaps the decrease in methylated histone H3 gives a dramatic advantage to the formation of euchromatin, which replicates early, and concomitantly impairs the formation of heterochromatin, perhaps through the extended presence and use of the variant histone H3.3 (AHMAD and HENIKOFF 2002). Equally, the lack of methylation may tend to position the variegating segment of the genome in a compartment of the nucleus that is more favorable to its transcription. We are in the process of investigating the role of SU(VAR)3-9 in PEV and will refrain from further speculations until our data are complete.

In summary, we believe that the HIS-C locus provides a good target locus for dissecting the role of various chromatin-associated proteins in both chromatin architecture and gene expression. Our data show that the HIS-C region in *Drosophila*, and perhaps also in other organisms, is an excellent domain for examining proteins involved in establishing chromatin-domain structure and in regulating gene expression. Our future experiments are directed at examining how the entire HIS-C region is packaged into chromatin with the aim of determining the status of other histone modifications and the involvement of *trans*-regulators of histone gene expression.

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