Dosage Compensation of the *Drosophila white* Gene Requires Both the X Chromosome Environment and Multiple Intragenic Elements

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ABSTRACT

The Xlinked *white* gene when transposed to autosomes retains only partial dosage compensation. One copy of the gene in males expresses more than one copy but less than two copies in females. When inserted in ectopic X chromosome sites, the *mini-white* gene of the *CaspeR* vector can be fully dosage compensated and can even achieve hyperdosage compensation, meaning that one copy in males gives more expression than two copies in females. As sequences are removed gradually from the 5' end of the gene, we observe a progressive transition from hyperdosage compensation to full dosage compensation to partial dosage compensation. When the deletion reaches -17, the gene can no longer dosage compensate fully even on the X chromosome. A deletion reaching +173, 4 bp preceeding the AUG initiation codon, further reduces dosage compensation both on the X chromosome and on autosomes. This truncated gene can still partially dosage compensate on autosomes, indicating the presence of dosage compensation determinants in the protein coding region. We conclude that full dosage compensation requires an X chromosome environment and that the *white* gene contains multiple dosage-compensation determinants, some near the promoter and some in the coding region.

IN *Drosophila*, X chromosome dosage compensation refers to the mechanism by which one copy of the X-linked genes in males achieves the same level of expression as two copies of the same genes in females. Biochemical and genetic evidence indicates that dosage compensation is the result of hyperactivation of the X-linked genes in males (reviewed by LUCCHESI and MANNING 1987). In salivary gland polytene nuclei, the male X chromosome is distinct from autosomes and the female X chromosome in both appearance and chromatin structure. With only half as many chromonemes as the paired chromosomes, it looks just as thick in polytene chromosome spreads as the female X or the autosomes and has a puffer, less condensed appearance. Large segments of the X chromosome genetically translocated onto autosomes retain their decondensed appearance and continue to dosage compensate (SEECOF *et al.* 1969; TOBLER *et al.* 1971; HOLMQUIST 1972). Conversely, autosomal sections translocated onto the X chromosome retain their condensed morphology and do not dosage compensate (LAKHOTIA 1970; ROEHRDANZ *et al.* 1977). Genetic studies indicate that the four male-lethal genes *mle*, *msl1*, 2 and 3 are important regulators of dosage compensation: mutations in these

genes lead to loss of dosage compensation and to a concomitant increase in condensation of the male X chromosome, which becomes half as thick as the paired autosomes (BELOTE and LUCCHESI 1980a,b; LUCCHESI *et al.* 1982). Recently, it was found that the products of the *mle* and *msl1* and *msl3* genes bind preferentially to the male X chromosome, possibly in a heteromultimeric complex (KURODA *et al.* 1991; GORMAN *et al.* 1993; PALMER *et al.* 1993, 1994). Moreover, an isoform of the histone H4, which is acetylated on lysine 16, is also specifically enriched on the male X chromosome (TURNER *et al.* 1992), and this association depends on activities of all the *msl* genes (BONE *et al.* 1994). Together with the genetic data, the unique association of the *mle* class proteins and especially the modified histone H4 with the male X chromosome point to a direct linkage between chromatin structure and dosage compensation.

Notwithstanding the rapid progress made in the molecular identification of *trans*-acting regulators, a critical missing link in current understanding of dosage compensation is the nature of the *cis*-acting sequences called dosage compensation determinants. There must be sequence features distinguishing the X chromosome from autosomes and responsible for the specific binding of the *mle* class of proteins and the modified histone H4. The dosage compensation determinants might be simple oligonucleotide repeats scattered throughout the X chromosome that are recognized and bound by the *mle* regulators. In one scenario, this would lead to modifi-

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cation of the histones and therefore to relaxed nucleosome and chromatin packaging. In this view, the hypertranscription would be a result of relaxed chromatin structure. In another scenario, these determinants would be associated closely with and act to stimulate the transcription of individual X-linked genes. In this second view, it is the initiation and elongation of transcription from the X-linked genes that matters and the relaxed conformation of the male X chromosome would result from the hyperactivation of the constituent genes. The most widely held view leans more toward the second extreme: dosage-compensation determinants are thought to be rather closely associated with individual genes and function like enhancer elements to upregulate transcription of X-linked genes in males, although these elements also might affect chromatin structure over some distance. The reality could lie somewhere in between: dosage compensation might result from the regional effects on chromatin structure due to the presence of elements somewhere in that region as well as from elements closely linked to individual genes.

Some evidence suggests that the X chromosome itself has unique structural features that might be related to dosage compensation. Dinucleotide (CA/GT) $_n$, (CT/GA) $_n$ and mononucleotide (C/G) $_n$ repeats have been reported to be twice as frequent on the X chromosome as on autosomes. It has been proposed that these repeats induce a less compact non-B conformation of DNA that might help decondense the chromosome and contribute to dosage compensation under the control of some male-specific function (LOWENHAUPT *et al.* 1989). However, other than their enrichment on the X chromosome, there is as yet no evidence that they are involved in dosage compensation. Autosomal genes introduced into the X chromosome through *P*-element-mediated transposition frequently acquire partial dosage compensation (SCHOLNICK *et al.* 1983; SPRADLING and RUBIN 1983; MARSH *et al.* 1985). This suggests that dosage compensation could result from relaxed regional chromatin structure. On the other hand, many authors report that X-linked transgenes even if including only ≤ 10 kb of X chromosome sequence, frequently retain dosage compensation at autosomal locations, indicating that dosage compensation determinants are linked closely to individual genes and that the relaxed structure of the X chromosome might result from decondensation of the hypertranscribed regions along the entire chromosome.

Whatever the mechanism of action of the dosage-compensation determinants might be, the foremost task is to locate such elements. Serious search began with the analysis of genes transposed from the X to autosomes. Dosage compensation of four X-linked genes, *white*, *sgs4*, *Pgd* and the *hsp82* of the *Drosophila*

pseudoobscura has been examined using transposons inserted on autosomes (HAZELRIGG *et al.* 1984; KRUMM *et al.* 1985; LEVIS *et al.* 1985; PIRROTTA *et al.* 1985; JAFFE and LAIRD 1986; HOFMANN *et al.* 1987; GUTIERREZ *et al.* 1989; SASS and MESELSON 1991). Earlier reports generally concluded that these genes retained full dosage compensation when transposed onto autosomes, and it appeared straightforward to define the sequences responsible for dosage compensation by deletional and mutational analysis of these transgenes. However, this has not turned out to be the case. Some uncertainties concerning the degree of dosage compensation were noted early on. For example, significant variation in male/female ratio of autosomal expression was observed by KRUMM *et al.* (1985) and HOFMANN *et al.* (1987) with a *sgs4* transgene containing 2.5-kb 5' sequence and by McNABB and BECKENDORF (1986) with a *sgs4* transgene containing 0.84-kb 5' sequence, making the level of *sgs4* dosage compensation difficult to evaluate. Various transposons containing *white* gene sequences, ranging from the intact gene with 3.0 kb of both 5' and 3' sequences to a modified version with only 200 bp of upstream sequence, seemed to retain full dosage compensation at autosomal locations because males heterozygous for the transposon produce two to three times as much pigment as heterozygous females. However, despite the apparently full or even hyperdosage compensation in the heterozygous state, females with two copies of the transposon have a two- to three-fold higher level of pigmentation than males with one copy (HAZELRIGG *et al.* 1984; LEVIS *et al.* 1985; PIRROTTA *et al.* 1985). This conflict with the apparently full or hyperdosage compensation indicated by the comparison of heterozygotes suggested that the pigment response to gene dosage might be nonlinear and that the dosage compensation observed might in fact be incomplete.

Recently, more reports have shown that the transposons containing X chromosome genes do not achieve full dosage compensation at autosomal sites. The most dramatic case concerns the *hsp82* of the *D. pseudoobscura*. *hsp82* is X-linked and dosage compensated in *D. pseudoobscura*. When the *pseudoobscura hsp82* gene was inserted into autosomes of *D. melanogaster*, it initially was found to retain full dosage compensation (cited in JAFFE and LAIRD 1986). However, a reexamination of the same transgenic lines by another group failed to detect any autosomal dosage compensation (SASS and MESELSON 1991). In a similar reexamination of the autosomal *Pgd* transgene, the same lines previously reported to show full autosomal dosage compensation now display only partial dosage compensation, with male to female expression ratios ranging from 1.3 to 1.5 (GUTIERREZ *et al.* 1989; SCOTT and LUCCHESI 1991). Finally, KELLUM and SCHEDL (1991) noticed that the

mini-white gene present in the *CaspeR* vector dosage compensates less on autosomes than on the X chromosome. All the studies, with the exception of those with *white*, used northern blots or RNase protection assays to quantitate the male/female expression ratio. The discrepancies in the literature are at least partly attributable to the technical difficulties associated with quantitating effects of less than a factor of two by these methods as required to assess the significance of partial dosage compensation.

In this report, we investigated the quantitative aspects of the partial dosage compensation shown by many *white* gene constructs, taking into account the nonlinearity of the pigmentation response to gene dosage. This nonlinearity enhances differences in pigmentation resulting from fractional degrees of dosage compensation and allows the detection of effects that would be very difficult to determine by quantitation of mRNA levels. We provide evidence that both the wild-type *white* gene and the *mini-white* gene of the *CaspeR* vector are in fact only partially dosage compensating on autosomes. In contrast, the same *mini-white* gene of the *CaSpeR* vector retains full dosage compensation when inserted at ectopic X chromosome sites. Further, at X locations, the *mini-white* transposon frequently displays hyperdosage compensation, meaning that one copy of the gene in males expresses more than two copies in females. When sequences from the 5' end of the *mini-white* gene are gradually removed, we observe a clear cut, progressive decline in dosage compensation even at X chromosome sites. The trend of progressive decline also holds on autosomes although it is less obvious than on the X. These results indicate that *white* dosage compensation determinants are an integral part of the gene; that being on the X chromosome is necessary but not sufficient for full dosage compensation and that multiple dosage compensation determinants are present both in upstream and in coding regions of the *white* gene.

MATERIALS AND METHODS

Transposon constructs: The structures of the four *mini-white* transgenes are depicted in Figure 1. Construction of the $(-113)miniW$ and $BgB(+173)miniW$ was detailed by QIAN *et al.* (1992). $z4(-17)miniW$ was made by attaching to 5' end of the $(-17)miniW$ gene a 40-bp synthetic oligonucleotide containing four *zeste* protein binding sites. The *CaspeR4* and $(+173)miniW$ constructs were injected into embryos of *Df(1)w^{67c23(2)}* and transgenic lines were obtained by germ-line transformation (SPRADLING and RUBIN 1982). Five out of eight X-linked lines of the $(-113)miniW$ and seven out of nine X-linked lines of the $(-17)miniW$ also were derived from embryonic germ-line transformation. Three more X-linked $(-113)miniW$ lines and two more X-linked $(-17)miniW$ lines were obtained for the purpose of increasing sample size by mobilizing the autosomal inserts onto the X chromosome using the D2-3 genomic transposase (ROBERTSON *et al.* 1988). Transformant lines suspected of containing more than one

FIGURE 1.—*mini-white* gene constructs analysed for dosage compensation. Four *mini-white* gene constructs with 5' end points at -305, -113, -17 and +174 are illustrated. These four transgenes revealed presence of dosage-compensation determinants in the interval of -305 and +173, as well as contributions from sequences downstream of +173. The (-17) and $(+173)miniW$ genes do not express by themselves in the eye. The eye expression of the $(-17)miniW$ construct was restored by the addition of a 40-bp synthetic oligonucleotide containing four *zeste* protein binding sites; and the eye expression of the $(+173)miniW$ gene was obtained by placing at its 5' end the eye enhancer of the *white* gene, which is normally located 1.2 kb upstream (QIAN *et al.* 1992). The wild-type version of the *white* gene is given at the top for comparison.

insert were back crossed to the recipient *w^{67c23}* flies to separate the inserts. In cases when two inserts were on the same chromosome, they were recombined out by crossing transformant females to *w^{67c23}* males. As a result, the lines analyzed all contained single inserts unless they have tandem insertions on the same chromosome that are less than one recombination unit apart.

Pigment assays: The degree of dosage compensation of transformant lines first was assessed by visually comparing the eye colors of the male and female flies. The X-linked lines of the four constructs with varying 5' sequences were further analysed by a quantitative pigment assay (HAZELRIGG *et al.* 1984). The impression obtained from visual inspection agrees well with the results of pigment assays. An important issue is that of the nonlinearity of the pigment response to transposon dosage. To exclude the possibility that pairing of two transposon copies might enhance their expression, we tested a number of pairs of transposons each inserted at a different site. Although we did not measure the pigment levels, the eye color resulting from the presence of two transposons at non-homologous sites showed the same nonlinear increase as that observed when either of the two transposons was homozygous. In addition, quantitation of *white* mRNA does not reveal any detectable departure from additivity. We conclude therefore that it is the deposition of pigment that depends in a nonlinear way on the amount of *white* gene product.

A major source of variation in the pigment assay is the flies'

body size, which depends on the crowdedness of the culture condition. To reduce this variation, care was taken to ensure that the heterozygous male (M1), female (F1) and the homozygous female (F2) flies from each line were cultured in parallel and the flies were collected at about the same time. Six pairs of flies were allowed to mate and lay eggs for 4 days in 75 × 20 mm vials. These flies then were removed and the progeny allowed to develop at room temperature. Flies to be assayed were collected 2–3 hr after eclosure and aged at 25° for 5 days. Each pigment assay used 15 fly heads. For each line, the pigments of the F2, M1, F1 flies were assayed three times and the mean of three measurements were used to calculate the F2/M1 and F1/M1 ratios. The mean ± SD and the two ratios were tabulated in Table 2. The OD480 reading of w^{67c23} controls is 0.002 for both male and female samples, which was not subtracted from pigment value of the transformant lines. The overall trends are identical if the 0.002 is taken into account. In a few cases, pigments were assayed more than three times and the numbers of measurements are indicated in parentheses.

Statistical analysis: To determine whether the degrees of dosage compensation displayed by lines transformed with the different constructs are significantly different, we subjected the four sets of F2/M1 values and of F1/M1 values reported in Table 2 to the analysis of variance (ANOVA) and Duncan-Kramer tests (MILTON and TSOKOS 1983). The ANOVA test asks whether the variance in the four sets of values is greater than that expected in the null hypothesis that they are no different than random variation would predict. The test shows that both the F1/M1 and the F2/M1 ratios are significantly different ($P < 0.01$) but does not specify which set is different from which other set. We then applied the Duncan-Kramer test, which compares the different sets pairwise. The results of this test are as follows. For the F2/M1 ratios: (–305)*miniW* does not differ from (–113)*miniW*, $P > 0.05$; (–305)*miniW* differs from (–17)*miniW*, $P < 0.01$; (–305)*miniW* differs from (+173)*miniW*, $P < 0.01$; (–113)*miniW* differs from (–17)*miniW*, $0.01 < P < 0.05$; (–113)*miniW* differs from (+173)*miniW*, $P < 0.01$; (–17)*miniW* differs from (+173)*miniW*, $P < 0.01$. For the F1/M1 ratios: (–305)*miniW* differs from (–113)*miniW*, $0.01 < P < 0.05$; (–305)*miniW* differs from (–17)*miniW*, $P < 0.01$; (–305)*miniW* differs from (+173)*miniW*, $P < 0.01$; (–113)*miniW* differs from (–17)*miniW*, $0.01 < P < 0.05$; (–113)*miniW* differs from (+173)*miniW*, $P < 0.01$; (–17)*miniW* differs from (+173)*miniW*, $P < 0.01$.

Taken together, these results indicate that the differences between each construct and the others are statistically significant and imply that the four *mini-white* genes dosage compensate to different degrees. The interesting exception is the difference between the F2/M1 ratios of the (–305) and (–113) constructs. The fact that these two constructs do not appear to be statistically significant is probably due to the fact that the (–305) construct is the only one that gave hypercompensating lines, thus introducing a large variance in the F2/M1 values for that set.

RESULTS AND DISCUSSION

The *mini-white* gene is fully compensated on the X but only partially on autosomes: The marker gene *mini-white* of the *P*-transformation vector *CaspeR* is an abbreviated version of the *white* gene. This gene contains 305-bp upstream sequence and 500-bp 3' sequence with much of its 3.0-kb first intron removed (PIRROTTA

TABLE 1
Summary of hyper-, fully and partially compensating lines of each end point

End points	No. of lines	M1 > F2	M1 = F2	M1 < F2
–305				
<i>CaspeR4</i>				
Autosomal	18	0	2	16
X	10	5*	4	1
–113				
(–113) <i>miniW</i>				
Autosomal	14	0	0	14
X	8	0	7	1
–17				
<i>z(4)(–17)miniW</i>				
Autosomal	7	0	0	7
X	9	0	1	8
+173				
<i>BgB(+173)miniW</i>				
Autosomal	20	0	0	20
X	9	0	0	9

Transformant lines are categorized by visually comparing the eye colors of males carrying one copy of the transposons (M1) with those of homozygous females (F2). For the X-linked lines, quantitative pigment data are presented in Table 2. The results of visual inspection agree well with the pigment assay data. The lines with F2/M1 ratios of <1.00 correspond to the M1 > F2 lines; those with F2/M1 ratios of 1.13–1.63 correspond to the M1 = F2 lines; and those with F2/M1 ratios of >1.70 correspond to the M1 < F2 lines. The reason that M1 = F2 lines have F2/M1 ratios >1.0 is because female eyes are significantly bigger than male eyes. Although lines of the M1 < F1 category do not dosage compensate fully, all of them still dosage compensate partially, which can be readily seen in the heterozygous comparison: one copy of the transgene expresses more in males than in females. The asterisk indicates that one of the M1 > F2 *CaspeR4* lines was poorly viable and was lost before the pigment assay was done. As a result, the pigment data are available for four instead of five of the M1 > F2 X-linked *CaspeR4* lines (Table 2).

1988). In most transformed lines, one copy of the gene confers yellow to orange-red eye color and therefore behaves very much like a hypomorphic *white* allele. This low level of expression makes the *mini-white* gene well suited for dosage-compensation analysis, recalling that dosage compensation was first observed by MULLER (1932) with a hypomorphic *white* allele, $w^{apricot}$ (w^a). In numerous experiments involving *CaspeR* transformant flies, we noticed that, like other *white* transgenes, one copy of the gene inserted at autosomal sites in males expresses more than one copy in females but still expresses less than two copies in females (Table 1, Figures 2 and 3). In contrast, at X chromosome sites, one copy in males equals or surpasses the expression of two copies in females, in most cases (9 out of 10 lines, Table 1 and Figure 2). When quantitated by pigment assays, the X chromosome *CaspeR* lines give a hemizygous male to

FIGURE 2.—Dosage-compensation capacity of the mini-*white* genes as percentage of fully compensating lines. Two points are apparent here: X-linked lines compensate much better than the autosomal lines and the dosage compensation ability of the *white* gene decreases as 5' sequences are removed progressively. The numbers are derived from Table 1. Although the difference between the (−17) and the (+173) constructs is dependent on a single X-linked line of (−17) that dosage compensates fully, the quantitative analysis shows that these two constructs differ significantly in their residual degree of dosage compensation (Table 2 and Figure 3, A and B).

heterozygous female ratio of 3.5:10 (Table 2). Previously reported male to female pigment ratios for heterozygous autosomal *white* transgenes, including the full-length *white* gene and one that is very similar to the *CaspeR mini-white* gene, are only two- to threefold (HAZELRIGG *et al.* 1984; LEVIS *et al.* 1985; PIRROTTA *et al.* 1985). The equality of male expression with that of homozygous females at ectopic X chromosomal sites and the 3.5:10 heterozygous male/female ratio on the X compared with 2:3 ratio on autosomes indicate that the transgene is fully dosage compensated only on the X chromosome and that the 2:3 male/female ratio for autosomal *white* transgenes observed previously represents in fact partial dosage compensation.

The more than twofold increase in pigment level in response to the twofold increase in gene dosage has been noted before (LEVIS *et al.* 1985; PIRROTTA *et al.* 1985) and most likely reflects a nonlinear increase of pigment deposition relative to the increase in *white* transcription. Such a nonlinear response is not due to some synaptic effect on *white* transcription in flies homozygous for a transposon because a similar nonlinear increase in pigmentation is observed when two independent transgenes located on separate chromosomes are combined into a single fly. In contrast, mRNA levels appear to be additive, within the limits of the assay

(VARJAVAND 1993). The nonlinear response of pigmentation to gene dosage accounts for the apparent paradox that one copy of the gene in males gives two to three times more pigment than one copy in females but still less than two copies in females. *white* encodes a membrane receptor-like molecule thought to be necessary for the transport of pigment precursors into the pigment granules of cells in the ommatidia (DRESEN *et al.* 1988; PEPLING and MOUNT 1990) rather than being directly involved in the pigment synthetic pathway. The nonlinear response of pigmentation to *white* transcription suggests that a small increase in *white* product can lead to a disproportionate increase in pigment transport. Curiously, the nonlinear response of pigmentation to gene dosage has not been observed for the endogenous *white* gene. The pigment level simply doubles when flies heterozygous for the hypomorphic alleles *w^a* or *w^{posin}* (*w*) are compared with homozygous flies. It is possible that the expression level of these weak alleles happens to fall within the linear part of the dose-response curve whereas the higher expression levels of the *CaspeR mini-white* and other *white* transgenes are in a highly nonlinear range.

Somewhat surprisingly, ~50% of the X-linked *CaspeR* lines display hyperdosage compensation, meaning that one copy in males gives more expression than two copies in females (Tables 1 and 2). The likely explanation for this is that the X chromosomal flanking sequences in those lines enhance the dosage compensation of the *mini-white* transgene. Interestingly, hyperdosage compensation has been observed for the *w^b* alleles of the native *white* gene (GREEN 1959). *w^b* mutations abolish the activity of the eye enhancer of the gene (DAVISON *et al.* 1985; QIAN *et al.* 1992) and as a result the dosage compensation determinants of neighboring genes may influence positively the dosage compensation of the less active *white* gene. In addition, *w^a* is also slightly hypercompensating (HIEBERT and BIRCHLER 1992).

The difference between the X-linked and autosomal-linked mini-*white* transgene in dosage compensation is obvious and clear-cut. Reasoning that the more condensed state of autosomes could compromise the dosage-compensation ability of transposed X genes, we attempted to insulate the *CaspeR mini-white* gene with the *su(Hw)* protein target sequence from the gypsy transposon, which was reported to reduce positional effects on transgenes (ROSEMAN *et al.* 1993). Flies containing a construct in which the *mini-white* gene is flanked on both sides by the *su(Hw)* target sequence may show some increase in dosage compensation at autosomal sites but most lines still lack full compensation. A difference in the degree of dosage compensation between autosomal and X chromosomal mini-*white* transgenes also was observed by KELLUM and SCHEDL (1991) in their analysis of the *scs* insulating element. However,

FIGURE 3.—Typical phenotypes of X lines of the (-305), (-17), ($+173$)*mini-white* constructs and of autosomal lines of (-305), ($+173$)*mini-white* constructs. In all cases, the female is on the left, the male on the right. (A) An X-linked *CaspeR* line showing hyper dosage compensation (female T/T, male T). (B–D) An autosomal *CaspeR* line illustrating partial dosage compensation. (B) Flies homozygous for the transposon (male and female T/T). (C) Homozygous female of the same line compared to heterozygous male (female T/T, male T). (D) Heterozygous female compared with heterozygous male of the same line (female T, male T). Partial dosage compensation of a (-17) line (E) and of a ($+173$)*miniW* line (F) inserted at X chromosomal sites, indicating that these two genes lack sequences required for full dosage compensation and that the X chromosome environment alone is insufficient for complete dosage compensation. In both cases the female is T/T, the male T. (G) An autosomal ($+173$)*miniW* line that still dosage compensates, indicating the presence of dosage-compensation determinants downstream of the translational start site (female T/T, male T/T).

recent results suggest that, in the presence of additional X chromosome sequences, *su(Hw)* target sequences can help to insulate the *mini-white* gene against the adverse effects of an autosomal environment and permit increased dosage compensation (R. ROSEMAN and P. GEYER, personal communication). We conclude therefore that the autosomal environment has a negative influence that normally interferes with the establishment of full dosage compensation by X chromosome genes unless additional dosage-compensation determinants are included in the transposon or unless the X chromosome gene is insulated against the effects of the autosomal environment. In light of our observation that the *white* gene retains only partial dosage compensation on autosomes, it seems likely that other X-linked genes also dosage compensate but do so only partially on au-

tosomes. The conflicting results reported in the literature are most likely due to the technical difficulties associated with quantitating small differences of <2.0 -fold, as we have found when measuring the mRNA levels of the autosomal *CaspeR mini-white* transgene (VARJAVAND 1993). There is a remote possibility that the transposed genes were indeed fully dosage compensating when initially assayed but the level of dosage compensation declined in the course of several years, perhaps because of sequence modifications of the transgenes. This possibility could be easily tested by generating a few new autosomal lines of these transposons to determine if they are fully dosage compensating.

Dosage compensation of the *white* gene is conferred by multiple elements: The fact that autosomal *white* transgenes dosage compensate only partially suggests

TABLE 2
Pigment Assay of X-linked lines of four deletion constructs

End points	F2	M1	F1	F2/M1	F1/M1
<i>-305, CaspeR4</i>					
10	0.116 ± 0.001	0.059 ± 0.001	0.016 ± 0.005 (7)	1.97	0.27
11	0.034 ± 0.001	0.023 ± 0.001	0.007 ± 0.001	1.48	0.30
4	0.426 ± 0.008	0.299 ± 0.003 (4)	0.045 ± 0.004	1.43	0.15
11b	0.447 ± 0.008	0.324 ± 0.012	0.041 ± 0.002	1.38	0.13
21a	0.085 ± 0.008	0.075 ± 0.004	0.019 ± 0.001	1.13	0.25
15	0.204 ± 0.002	0.289 ± 0.003	0.028 ± 0.001	0.71	0.10
1b	0.040 ± 0.001	0.060 ± 0.003	0.012 ± 0.001	0.67	0.20
5b	0.286 ± 0.031 (8)	0.451 ± 0.018	0.045 ± 0.006 (8)	0.63	0.10
1a	0.227 ± 0.010	0.371 ± 0.011 (5)	0.037 ± 0.005 (5)	0.61	0.10
<i>-113, (-113)miniW</i>					
1	0.059 ± 0.002	0.026 ± 0.001	0.012 ± 0.002	2.27	0.46
17	0.075 ± 0.004	0.046 ± 0.001	0.012 ± 0.001	1.63	0.26
7a	0.085 ± 0.003	0.053 ± 0.002	0.013 ± 0.001	1.60	0.25
5e	0.042 ± 0.002	0.027 ± 0.001	0.009 ± 0.001 (5)	1.56	0.33
12a	0.063 ± 0.002	0.041 ± 0.003	0.014 ± 0.001	1.54	0.34
13d	0.085 ± 0.001	0.060 ± 0.002	0.013 ± 0.001	1.42	0.22
5	0.057 ± 0.002	0.041 ± 0.002	0.012 ± 0.001	1.39	0.29
3a	0.072 ± 0.002	0.058 ± 0.003	0.014 ± 0.003	1.24	0.24
<i>-17, z(4)(-17)miniW</i>					
11	0.402 ± 0.018 (6)	0.111 ± 0.005 (5)	0.023 ± 0.005 (6)	3.62	0.21
7	0.045 ± 0.018	0.018 ± 0.002	0.006 ± 0.001	3.62	0.21
9b	0.058 ± 0.004	0.025 ± 0.003	0.017 ± 0.003	2.32	0.68
9a	0.043 ± 0.002	0.021 ± 0.001	0.006 ± 0.001	2.05	0.29
11a	0.054 ± 0.003	0.027 ± 0.003 (4)	0.013 ± 0.001	2.00	0.48
3a	0.039 ± 0.003	0.020 ± 0.002	0.009 ± 0.001	1.95	0.45
1	0.018 ± 0.003 (7)	0.010 ± 0.001 (6)	0.003 ± 0.001	1.80	0.30
2	0.068 ± 0.006	0.038 ± 0.000	0.020 ± 0.002	1.79	0.53
19	0.020 ± 0.001	0.014 ± 0.002	0.007 ± 0.001	1.43	0.50
<i>+173, BgB(+173)miniW</i>					
19	0.085 ± 0.001	0.020 ± 0.000	0.013 ± 0.001	4.25	0.65
1	0.087 ± 0.002	0.026 ± 0.001	0.017 ± 0.002	3.35	0.65
11c	0.050 ± 0.004	0.016 ± 0.001	0.011 ± 0.003	3.13	0.69
1d	0.109 ± 0.002	0.035 ± 0.001	0.025 ± 0.003	3.11	0.71
18	0.093 ± 0.002	0.033 ± 0.001	0.022 ± 0.002	2.82	0.67
2	0.107 ± 0.008	0.040 ± 0.001	0.023 ± 0.002	2.68	0.58
22a	0.113 ± 0.004	0.046 ± 0.002	0.028 ± 0.002	2.46	0.61
16	0.108 ± 0.006	0.047 ± 0.002	0.031 ± 0.002	2.30	0.66
17	0.117 ± 0.003	0.052 ± 0.003	0.032 ± 0.002	2.25	0.62

For each line, the pigment of the F2, M1, F1 flies were assayed three times and the mean ($\pm$ SD) of three measurements were used to calculate the F2/M1 and F1/M1 ratios. In a few cases, pigments were assayed more than three times and the numbers of measurements are indicated in parentheses. The ANOVA statistical test shows that the F2/M1 ratios and F1/M1 ratios of the four end points differ from each other statistically as a group ($P < 0.01$) and a follow-up Duncan-Kramer test confirms that each end point differs from any of other end-points in all but one case (see MATERIALS AND METHODS for statistical analysis). One of the X-linked *CaspeR4* lines was poorly viable and was lost before the pigment assay. Therefore, only 9 lines were assayed instead of 10 lines listed in Table 1. The lost line hyperdosage compensates ($F2 < M1$).

either that autosomes exert some negative effects on dosage compensation or that some feature of the X chromatin environment is important for full dosage compensation. These possibilities make the task of identifying dosage-compensation determinants on autosomes much more difficult than originally thought. While analyzing the *zeste-white* interaction (QIAN *et al.* 1992), we noticed that dosage compensation of the *white* gene

could be better studied on the X chromosome. A set of four deletion constructs removing increasing amounts of 5' sequence from the *CaspeR mini-white* gene displayed a progressive loss of dosage compensation at X chromosome sites. The 5' end points of the four transgenes are at -305, -113, -17 and +174 (Figure 1). The (-17) and (+173)miniW genes by themselves have no activity in the eye. The (-17)miniW construct

acquires ability to express in the eye by the addition of a 40-bp synthetic oligonucleotide containing four *zeste* protein binding sites. The 173 end point is 4 bp before the initiation codon and therefore lacks the promoter altogether. Eye expression of the (+173)*miniW* gene was obtained by placing at its 5' end the *white* gene eye enhancer, which is normally located 1.2 kb further upstream (QIAN *et al.* 1992). Transcription of this gene initiates in the sequences flanking the insertion site, presumably driven by an extraneous promoter. The last two constructs, therefore, differ by more than just their breakpoints. However, essentially similar results were obtained when the *zeste* target sequences were added to the (−113)*miniW* gene or when the *hsp70* promoter is used to drive the expression of the (+173)*miniW* construct instead of the eye enhancer. These changes did not affect the degree of dosage compensation of the deletion endpoint, supporting our conclusion that the four breakpoints retained progressively decreasing dosage compensation determinants.

All four constructs retain some degree of dosage compensation, in all cases greater in X chromosome sites than in autosomal sites. The steady decline in dosage compensation resulting from progressive loss of 5' sequences is obvious in the heterozygous state (or in males carrying the transposon on the X) but most evident when heterozygous males are compared with homozygous females. Table 1 summarizes the dosage-compensation results obtained by visually comparing the eye color of males with one copy of the gene (M1) with that of females with two copies of the gene (F2). Transformant lines of the four *mini-white* genes are classified into three easily recognizable groups: hyperdosage compensating (M1 > F2); fully dosage compensating (M1 = F2) and partially dosage compensating (M1 < F2). The difference between the X-linked and autosomal lines is obvious. For example, the (−305) *mini-white* gives full dosage compensation only rarely on autosomes but very frequently on the X chromosome: only 2 out of 18 autosomal lines compensate fully in contrast to 9 out of 10 X chromosomal lines which either hyper- or fully dosage compensate. Similarly, none of the autosomal lines containing the (−113) or (−17) *mini-white* genes compensate fully while many of the X-linked lines of these two constructs achieve full dosage compensation (Figure 2). These results are consistent with the conclusion that an X-chromosome environment is necessary for full dosage compensation. Figure 2 displays in graphic form the difference between X and autosomal lines and the progressive decline of dosage compensation as sequences are deleted from −305 to +173. As the 5' sequence from −305 gradually is deleted, the percentage of fully compensating lines declines steadily, indicating that the gene becomes progressively less capable of dosage compensation. When the deletion

reaches −17, most of the lines can no longer dosage compensate fully even on the X chromosome; when the 5' endpoint reaches +173, dosage compensation is further reduced (see below for quantitative data).

The decrease in dosage compensation resulting from removal of 5' sequences is detectable also in autosomal lines. For example, a small percentage of autosomal −305 lines can dosage compensate fully whereas none of the autosomal lines of other three constructs can. Nevertheless, it is the X-linked lines that are most illuminating. The quantitative analysis of dosage compensation in these X-linked lines as determined by pigment assay is reported in Table 2 and illustrated in Figure 4. All four constructs display considerable variation in dosage compensation among different lines, attributable to position effects. Taken as a whole, however, the differences between the groups become very clear. The four groups differ statistically as a group for both the F2/M1 ratios and F1/M1 ratios (ANOVA test, $P < 0.01$) and from each other when considered pairwise (see MATERIALS AND METHODS for details). Figure 4 shows that the (+173)*miniW* gene has the highest F2/M1 and highest F1/M1 pigment ratios, indicating the lowest degree of dosage compensation; the (−305)*miniW* (*CaspeR4*) has the lowest F2/M1 and lowest F1/M1 pigment ratio, indicating the highest level of dosage compensation; the other two endpoints lie between the (+173) and (−305) endpoints. Despite the variation among the lines of each construct, the difference between groups is striking. For example, the best compensating line of (173)*miniW* (F2/M1 = 2.25) is worse than the least compensating line of (−305)*miniW* (F2/M1 = 1.97). The same conclusion holds when the F1/M1 ratio is examined. The changes in the F2/M1 ratio and F1/M1 ratio from the (−305) endpoint to the (+173) endpoint are even larger than the factor of two that would be expected for a decrease from full-to-no dosage compensation. This amplified effect is an unexpected tribute to the nonlinear response of pigment level to gene dosage and is a critical factor facilitating the distinction of dosage compensation levels among the four endpoints. We expect that it would be technically very difficult to identify the differences in dosage compensation between any two neighboring endpoints by quantitating northern or RNase protection assays. The data indicate a direct correlation between the amount of 5' sequence in the construct and its ability to dosage compensate.

The pigment data in Table 2 shows that, despite the loss, all the (+173)*miniW* lines are still able to dosage compensate, though at much reduced level. The F1/M1 ratios of the lines of this construct are fairly constant, ranging from 0.61 to 0.71, demonstrating less expression in females than in males (the F1/M1 ratio would be >1.0 in the absence of dosage compensation). Considering that female eye is bigger than male eye, if cor-

FIGURE 4.—Comparison of dosage compensation of the four mini-*white* genes by F2/M1 (A) and F1/M1 (B) ratios. The F2/M1 or F1/M1 ratios of the different transformant lines of each construct (from Table 2) are arranged from high to low and connected by a line as a group. Considerable variation exists among the lines of each construct. Taken as a whole, however, differences among the groups are very clear (see MATERIALS AND METHODS for a statistical analysis). Both the F2/M1 and F1/M1 ratios indicate a gradual decline of dosage compensation from the -305 endpoint to the $+173$ endpoint.

rected for the difference between the eye sizes, the ratio should be even lower. Moreover, visual inspection indicates that all the autosomal, $(+173)$ miniW lines ($n = 20$) dosage compensate partially. A similar partial dosage compensation at autosomal sites was observed when the $(+173)$ miniW is driven by the promoter of *hsp70*, an autosomal gene (B. VARJAVAND and V. PIRROTTA, unpublished results). This indicates that the residual dosage compensation is not due to the 780-bp eye enhancer and that sequences downstream of $+173$, in the *white* protein coding region, also contribute to dosage compensation. The phenotypes of typical X lines of the (-305) , (-17) , $(+173)$ mini-*white* constructs and of autosomal lines of (-305) , $(+173)$ miniW constructs are shown in Figure 3. No deletion beyond $+173$ was attempted since damaging the protein coding region would defeat the sensitive pigment assay.

Our data indicate that all three subsegments in the interval between -305 and $+173$ contribute to dosage compensation to a comparable degree. Additional sequences both 5' and 3' of this 478-bp interval most likely also contribute to dosage compensation of the *white* gene. Recall that although the (-305) miniW gene contains sufficient information to dosage compensate fully on the X, it can only do so partially on autosomes. This could be attributed either to a negative effect of autosomes on the transgene due to a less permissive autosomal chromatin environment or to the lack of addi-

tional compensation determinants present in the native *white* gene. The fact that the *white* gene contained within the large TE89 element dosage compensates fully on the third chromosome (HIEBERT and BIRCHLER 1992) is consistent with either interpretation. The existence of additional dosage compensation elements upstream of -305 is possible but, more significantly, the partial compensation of the $(+173)$ miniW gene on autosomes indicates the presence of determinants also in the protein coding region. Three conclusions can be reached from these observations: first, the dosage compensation determinants of the *white* gene are closely associated with the gene; second, they consist of multiple elements, some of which are distributed along the promoter region and at least some are in the protein-coding or intronic regions; third, as was previously concluded from the behavior of autosomal genes inserted on the X, being on the X chromosome is necessary but not sufficient for full dosage compensation. We can argue that in the case of the *white* gene, both the X chromatin environment and the integral sequence motifs of the gene are important to achieve complete dosage compensation. Whether the essential X environment results solely from the dosage-compensation determinants closely associated with individual genes or whether additional intergenic elements exist remains to be determined. Nor can we assess, from the present results, the degree to which an autosomal environment

might interfere with the functioning of dosage compensation mechanisms.

What might be the mechanism of action of dosage-compensation determinants? The importance of sequences surrounding the promoter points to initiation as a crucial target of dosage compensation. This would conform to an enhancer-like mechanism for dosage compensation. The current view of enhancers is that they are discrete elements serving to facilitate the assembly and delivery of *trans*-acting factors to the promoter, stimulating transcription initiation in temporally and tissue-specific fashion. The DNA segments that we identified as contributing to dosage compensation of the *white* gene might function to assemble *trans*-acting regulators like enhancer elements. An attractive alternative, in view of the possible involvement of histone acetylation (TURNER *et al.* 1992), is that they may mediate a looser chromatin structure that could both favor the interaction of the promoter with transcription stimulating factors and facilitate the elongation of the transcript. The finding that dosage compensation determinants are multiple and additive and that they reside within the promoter and coding regions, both upstream and downstream of the initiation site would be consistent with such an interpretation. These determinants would be recognized and bound by the *trans*-acting dosage compensation regulators such as the products of the *male lethal* genes. These might recruit a specific enzyme that acetylates the lys16 of histone H4, resulting in a looser or more easily displaced nucleosome structure. It is known that histone acetylation can lead to a more relaxed nucleosome structure and higher transcription activity (TURNER 1991). In a series of experiments examining how RNA polymerase transcribes through histone octamers, STUDISKY *et al.* (1994) found that, during transcription, the nucleosome core is not dissociated from DNA but is transferred behind the transcribing RNA polymerase. These authors propose that as RNA polymerase encounters the nucleosome during transcription, it pulls DNA away from the histone octamer without completely stripping it off; the histone octamer then contacts and transfers onto the DNA behind the polymerase, which proceeds to the next nucleosome. In light of this transfer mechanism during transcription, it is easy to envision that a looser contact between DNA and the nucleosome core might permit a more efficient transfer and therefore faster transcription, leading to dosage compensation. The existence of upstream dosage-compensation determinants suggests that the access of polymerase or transcription factors to the promoter also contributes to dosage compensation in addition to the elongation process. Another possibility consistent with our data is that dosage compensation determinants facilitate DNA and/or DNA-RNA duplex unwinding during initiation and

elongation, further enhancing the effect of H4 acetylation. We note that one of the dosage compensation regulators, *mle*, contains a sequence motif found in RNA helicases (KURODA *et al.* 1991) and that other transcription enhancing factors such as *brahma* or SNF2/SWI2 have helicase structural motifs (LAURENT *et al.* 1992; PETERSON and HERSKOWITZ 1992; TAMKUN *et al.* 1992).

The rationale for examining dosage compensation of X-linked genes on autosomes is that if a gene retains a high degree of dosage compensation on autosomes, then dosage compensation determinants must be contained within the transgene and be identifiable by deletion or mutational analysis. Once such sequence motifs are found, their function can be verified by testing whether they can confer dosage compensation to a non-compensating gene at autosomal sites, independently of the global effect of the X chromosome. However, if an X chromosome environment makes an important contribution to the degree of dosage compensation of a gene and if multiple determinants both upstream and downstream of the promoter are involved, the feasibility of this approach becomes doubtful. The twofold effect on gene expression expected under full dosage compensation is already small enough to impose a technical challenge for accurate measurements by northern blot or RNase protection assays, the quantitation methods utilized in most dosage compensation studies. Were the effect further reduced to 1.5-fold or less, as observed in the case of our *mini-white* constructs or of *Pgd*, it would be difficult to ensure the accuracy of quantitation by these two assays. Furthermore, if dosage compensation involves transcript elongation and is mediated by sequences distributed throughout the transcription unit, it would not be possible to confer robust dosage compensation to a noncompensating gene by simply attaching to it an X chromosome sequence, even if this sequence contributes to dosage compensation of its native gene, as elongation through the coding region would become rate limiting.

Now the challenge is to identify the sequence features that constitute the dosage compensation motifs. We have not spotted easily recognizable sequences that are common to all three subsegments of the -305 to +173 interval, or for that matter in the sequence downstream of +173. It is possible that there is no unified primary sequence common for all dosage-compensation determinants of all X-linked genes. Multiple sequences might be required to recruit the products of male lethal genes in a cooperative way so that each contributing sequence might not have sufficiently distinctive features. To resolve this problem and understand the nature of dosage-compensation determinants, more sequences that contribute to dosage compensation need to be identified. A search for such elements by examining transgenes on autosomes poses severe difficulties but, as

more *trans*-acting dosage-compensation regulators are cloned, it may become possible to identify dosage-compensation determinants through protein-DNA interaction assays. However, a functional assay will still be needed to verify the activity of the sequences identified through protein-DNA interactions. The hyperdosage compensation phenotype of the *(-305)miniW* at ectopic X sites suggests that the *mini-white* gene can respond to additional dosage-compensation determinants in its vicinity. Because dosage compensation of the *(-17)miniW* gene is severely attenuated (Table 2 and Figure 4) it should be possible to use this gene as a reporter to identify candidate dosage-compensation elements of other X-linked genes. The activity of a candidate element would be verified if it could increase the dosage compensation of this reporter to the level of the *(-305)miniW* gene.

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