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Separable *cis*-acting control elements for expression of the *white* gene of *Drosophila*

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The *white* gene of *Drosophila* is required for the pigmentation of the eyes, ocelli, Malpighian tubules and testis sheath. We have examined the regulation of *white* expression in germ line transformants that carry the mRNA-coding sequences of *white* flanked by varying amounts of the sequences normally located adjacent to them. A segment with as little as 1.9 kb of 5′- and 2.1 kb of 3′-flanking DNA was expressed to give essentially wild-type pigmentation in all of these tissues and interacted in *trans* with the *zeste-1* allele. In contrast, transformants having segments with 1.1 kb of 5′-flanking DNA had reduced levels of pigment in their eyes, no detectable pigment in their testes and did not interact with *zeste-1*. The eyes, Malpighian tubules and ocelli of transformants with as little as 0.4 kb of 5′-flanking DNA were pigmented and the eye pigmentation exhibited dosage compensation. We conclude that there are sequence elements present in the region from 1.1 to 1.9 kb upstream from the 5′ end of the *white* transcript which are required for expression in the testes and interaction of *white* and *zeste-1*. The same or other elements in this region appear to augment the pigmentation of the eye.

Key words: gene regulation/gene transfer/dosage compensation/tissue specificity/*Drosophila*

Introduction

We have begun an analysis of the *cis*-acting sequences involved in the regulation of the *white* (*w*) gene of *Drosophila*. This gene shows tissue and temporal specificity in its expression and is dosage compensated. The *white* gene also interacts in *trans* with the products of mutant alleles of the *zeste* (*z*) locus. Cloned copies of the *white* gene, when reintroduced into the germ line of *Drosophila* by P-element-mediated DNA transformation, are properly regulated in these various aspects (Hazelrigg *et al.*, 1984; Gehring *et al.*, 1984). In this report we examine the regulation of transduced copies of the *white* gene from which varying lengths of the normal flanking sequences have been deleted. A central question which we wish to address is whether independent regulatory elements exist for expression in different tissues, for dosage compensation and for *zeste* interaction. Germ line transformation in *Drosophila* offers a number of advantages over assays of gene expression available in other metazoans, such as cell-free transcription or transient expression in injected oocytes or transfected tissue culture cells. The transduced gene is present intrachromosomally in proper copy number in all cells and those cells are in their normal environment in the developing animal.

The expression of the *white* gene is required for the normal pigmentation in the adult fly of the compound eyes, the ocelli (simple eyes), the Malpighian tubules and the testis sheath of the male. These structures contain varying amounts of two groups of pigments, the red pteridines and the brown ommochromes. In the absence of *white* gene product all of these structures are colorless. The role of the *white* gene in pigmentation is uncertain, but *white* is not thought to code for any of the enzymes directly involved in the biosynthetic pathway for either group of pigments (for reviews, see Dickinson and Sullivan, 1975; Judd, 1976; Phillips and Forrest, 1980; Summers *et al.*, 1982). It has been proposed that the *white* product is a membrane protein necessary for the transport of intermediates of both types of pigment into the cell (Summers *et al.*, 1982).

The expression of *white* is temporally as well as spatially regulated. Transcripts of the *white* gene appear in the precursors of the Malpighian tubules during embryogenesis (Fjose *et al.*, 1984) and pigment accumulates in the differentiating Malpighian tubules of the embryo prior to hatching (Poulson, 1950). Several days after their first appearance in the Malpighian tubules, *white* transcripts accumulate in the eye-antennal imaginal discs of third instar larvae, from which the adult eyes will develop (Fjose *et al.*, 1984). Deposition of pigment in the eyes and male testis sheath begins during the mid-pupal period. The expression of *white* in these tissues is cell-autonomous, i.e., only cells expressing *white* are pigmented (Demerec and Slizynska, 1937; Schultz, 1957; Van Breugel, 1973; Ready *et al.*, 1976).

The expression of the *white* gene is subject to dosage compensation, as are most other genes located on the X chromosome. Males, which have one copy of the *white* gene, have the same amount of pigment in their eyes as females, which have two copies. Dosage compensation is thought to modulate the transcription rate of genes subject to this regulation (reviewed by Stewart and Merriam, 1980; Baker and Belote, 1983).

The phenotypic expression of *white* is also influenced in *trans* by the *zeste* (*z*) locus. The function of the $zeste^+$ product is unknown, but certain mutations of *zeste* drastically reduce pigmentation in the eye. This reduction in pigment has been reported to be accompanied by a reduction in the absence of the *white* transcripts specifically in the head (Bingham and Zachar, 1985). *zeste* is a particuarlly interesting *trans* regulator, because its interaction with *white* and at least two other genes appears to depend on the chromosomal synapsis of genes with which it interacts (Gans, 1953; Kaufman *et al.*, 1973; Jack and Judd, 1979; Gelbart and Wu, 1982).

The major mature transcript of *white* is a 2.6-kb polyadenylated RNA (O'Hare *et al.*, 1983; Pirrotta *et al.*, 1983). This transcript predominates at all stages of development, although smaller, less abundant transcripts can also be detected at some stages (Pirotta and Bröckl, 1984; Fjose *et al.*, 1984). When adult heads are separated from bodies, the 2.6-kb RNA is the major *white* transcript in the RNA of both fractions (Bingham and Zachar, 1985; Zuker and Rubin, unpublished results). The transcripts of *white* are rare at all stages; their maximal abundance has been estimated at 0.003% of the polyadenylated RNA of late third in-

star larvae (Fjose *et al.*, 1984).

Several putative *cis*-acting regulatory mutations of *white* map genetically at one end of the *white* locus (Green, 1959; Judd, 1976). These are associated with deletions or with transposable element insertions (Zachar and Bingham, 1982; Davison *et al.*, 1985) that are located near to and upstream from the sequences that code for the 5′ end of the major transcript (O'Hare *et al.*, 1983, 1984; Pirotta and Bröckl, 1984). The *white* gene DNA segments which have been previously transduced have contained this putative 5′ regulatory region defined by mutants. The proper regulation of these transduced gene segments demonstrated that the *cis*-acting control sequences for *white* must be located within an 11.7-kb region which includes the RNA coding sequences, 2.9 kb of 3′-flanking and 3.0 kb of 5′-flanking DNA (Hazelrigg *et al.*, 1984; Gehring *et al.*, 1984).

The analysis of the transduced segments reported here delimits the DNA sequences required in *cis* to a 9.9-kb region including 1.9 kb 5′ and 2.1 kb 3′ to the mature RNA coding sequences. Our results suggest that there are multiple *cis*-acting regulatory elements in the 5′-flanking region. Sequences in the interval between 1.1 and 1.9 kb upstream from the 5′ end of the transcript augment pigmentation in the eye and are required for detectable pigmentation of the testes and for interaction with *zeste-1*. In contrast, sequences further than 0,42 kb upstream from the 5′ end of the transcript are not required for pigmentation of the ocelli or Malpighian tubules or for dosage compensation.

Results

Recovery of transformants varying in the amount of flanking white DNA

The proposed structure of the major mature *white* transcript is diagrammed in Figure 1. We will refer to the interval between the 5′ and 3′ ends of this mature transcript as the transcribed region, even though other sequences may be transcribed as part of the primary *white* transcript. Figure 1 also shows the limits of the genomic *white* DNA segments which were inserted into P-element vectors (Figure 3) and transduced into the germ lines of *Drosophila* embryos. The phenotypes of transformants with the P[(*w,ry)A*] transposon, containing 3.0 kb of 5′-flanking and 2.9 kb of 3′-flanking DNA, have been reported (Hazelrigg *et al.*, 1984). Segments D, E, and F constitute a deletion series of the 5′-flanking sequences; they carry 1.9, 1.1 and 0.42 kb of upstream flanking sequences, but share the same 3′ end, 2.1 kb downstream from the transcribed region. Segment G lacks the putative 5′ exon entirely. Segment H tests the requirement for the 3′-flanking DNA; it contains only 157 bp of DNA downstream of the position of the 3′ end of the mature transcript.

The P-element transposons also carry the *rosy (ry)* gene inserted adjacent to the 5′ end of the *white* DNA (Figure 3). We initially injected plasmids containing these transposons into w^+ ry^- embryos and screened for ry^+ transformants. In this way we could recover transposon insertions of *white* DNA without requiring *white* gene activity. The presence or absence of the same *rosy* DNA segment 3′ to the 11.7-kb *white* segment A appears to have no influence on *white* gene expression (Hazelrigg *et al.*, 1984; Gehring *et al.*, 1984). The inserted transposons in the ry^+ transformants were then transferred, by conventional genetic crosses, into a w^- genetic background in order to observe the *white* phenotype conferred by them. In some cases (see Materials and methods), once a given *white* DNA segment was found to be expressed phenotypically, further injections were done directly into w^- embryos.

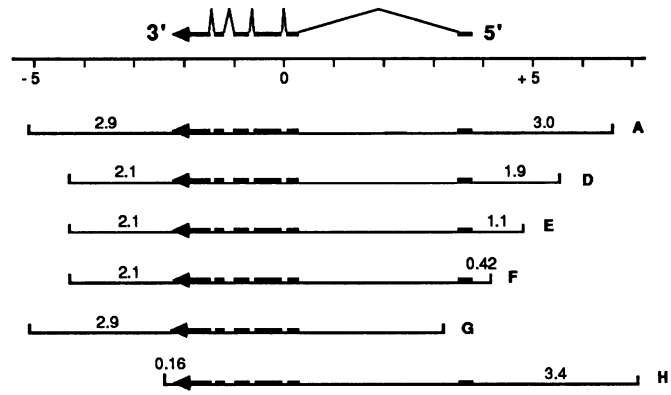


Fig. 1. *white* DNA segments contained within the P[*w,ry*] transposons. The interrupted arrow at the top represents the exon-intron structure of the major *white* transcript proposed by O'Hare *et al.* (1984). The 5′ end has been repositioned slightly to reflect a correction in the S1 nuclease determination of the size of the 5′ exon (Steller and Pirrotta, 1985). In the coordinate scale below the arrow the zero point is the point of insertion of the copia element in the w^a allele and each unit in the scale is 1 kb. The coordinates of the proposed endpoints of the mature transcript are +3.69 and −2.23. The *white* DNA segments in transposons A and D−H are indicated by the brackets below the scale. The distance in kb between the endpoints of the segments and the transcribed region are indicated. The 3′ end point of segments A and G is the end point of the insert of λm2.1 (coordinate −5.1) (Levis *et al.*, 1982). The 3′ end point in segments D, E and F is a *Kpn*I site (−4.3). The 3′ end point of segment H is a *Bss*HII site (−2.38). The 5′ end points are at the following sites, with the coordinates in parentheses: A - *Eco*RI(+6.62), D - *Hinc*II(+5.55), E - *Bgl*II(+4.79), F - *Sca*I(+4.11), G - *Hind*III(+3.18), and H - *Hpa*I (+7.13).

For each different *white* segment we recovered several independent transformants, having different chromosomal sites of insertion of the transposon, so that we could evaluate how much the expression of the transduced *white* gene varied as a function of its genomic position. Transformant lines containing a single copy of an injected transposon are listed in Table I. The transformants are named with a capital letter designating the *white* segment inserted and those with the same segment are numbered consecutively. In a few cases insertions at more than one site were isolated from the progeny of a single injected individual; for example, transformant lines H2-1 and H2-2 are two lines, each containing a different single H insert, that were isolated from the progeny of a single injected individual.

The genomic DNA of each transformant line was analyzed by DNA blot hybridizations to establish that each carried a single copy of the transposon and that the *white* DNA within the transposon was intact (see Materials and methods for details). The *white* DNA of two transformant lines was found to be rearranged; these transformants have not been included in Table I and will not be considered further. We also hybridized a *white* DNA probe *in situ* to the polytene salivary gland chromosomes of each transformant line as a further confirmation that each contained a transposon inserted at a single site. The cytogenetic locations of these insertion sites are listed in Table I.

Measuring white gene expression

We have used pigmentation as a measure of *white* gene expression. It is impractical to measure small differences in *white* transcript levels in particular tissues, due to the low abundance of the transcript and difficulties in obtaining large quantities of pure tissues at the appropriate times during development. A qualitative evaluation of pigmentation can be made by visual inspection of the tissues of a single individual. The pigments can

Table I. Transformants: location, eye color and pigment level

Transformant	Abbreviated name	Cytogenetic location[a]	Eye color[b] (one copy female)	Pigment level[c] (one copy male) (% wild-type)
P[(*w*,*ry*)D]1	D1	25C (2L)	Red, slightly brownish	50
P[(*w*,*ry*)D]2	D2	62A (3L)	Red, slightly brownish	76
P[(*w*,*ry*)D]3	D3	90E (3R)	Wild-type red	
P[(*w*,*ry*)D]4	D4	24AB (2L)	Wild-type red	
P[(*w*,*ry*)E]1	E1	19F (X)	Orange-brown	45
P[(*w*,*ry*)E]2	E2	12B (X)	Orange	33
P[(*w*,*ry*)E]3[g]	E3	13EF (X)	Orange-brown	
P[(*w*,*ry*)E]4	E4	ND (2)[e]	Orange	19
P[(*w*,*ry*)E]5	E5	2A (X)	Orange-brown	
P[(*w*,*ry*)E]6	E6	57B (2R)	Dark yellow	<10[d]
P[(*w*,*ry*)E]7	E7	94D (3R)	Yellow	
P[(*w*,*ry*)E]8	E8	47D (2R)	Red-brown	20
P[(*w*,*ry*)F]1	F1	50A (2R)[f]	Orange	
P[(*w*,*ry*)F]2	F2	86C (3R)	Red-brown	
P[(*w*,*ry*)F]3	F3	64B (3L)	Dark orange	
P[(*w*,*ry*)F]4-1	F4-1	57B (2R)	Yellow	<10[d]
P[(*w*,*ry*)F]4-2	F4-2	21D (2L)	Orange-brown	
P[(*w*,*ry*)F]4-3	F4-3	97B (3R)	Orange	14
P[(*w*,*ry*)G]1	G1	21A (2L)	White	
P[(*w*,*ry*)G]2	G2	37A (2L)	White	
P[(*w*,*ry*)G]3	G3	96AB (3R)	White	
P[(*w*,*ry*)G]4	G4	13EF (X)	White	
P[(*w*,*ry*)H]1	H1	78CD (3L)	Brown	38
P[(*w*,*ry*)H]2-1	H2-1	85F (3R)	Red, slightly brownish	42
P[(*w*,*ry*)H]2-2	H2-2	75C (3L)	Brown	
P[(*w*,*ry*)H]3	H3	85D (3R)	Wild-type	59
P[(*w*,*ry*)H]4	H4	6AB (X)	Red, slightly brownish	

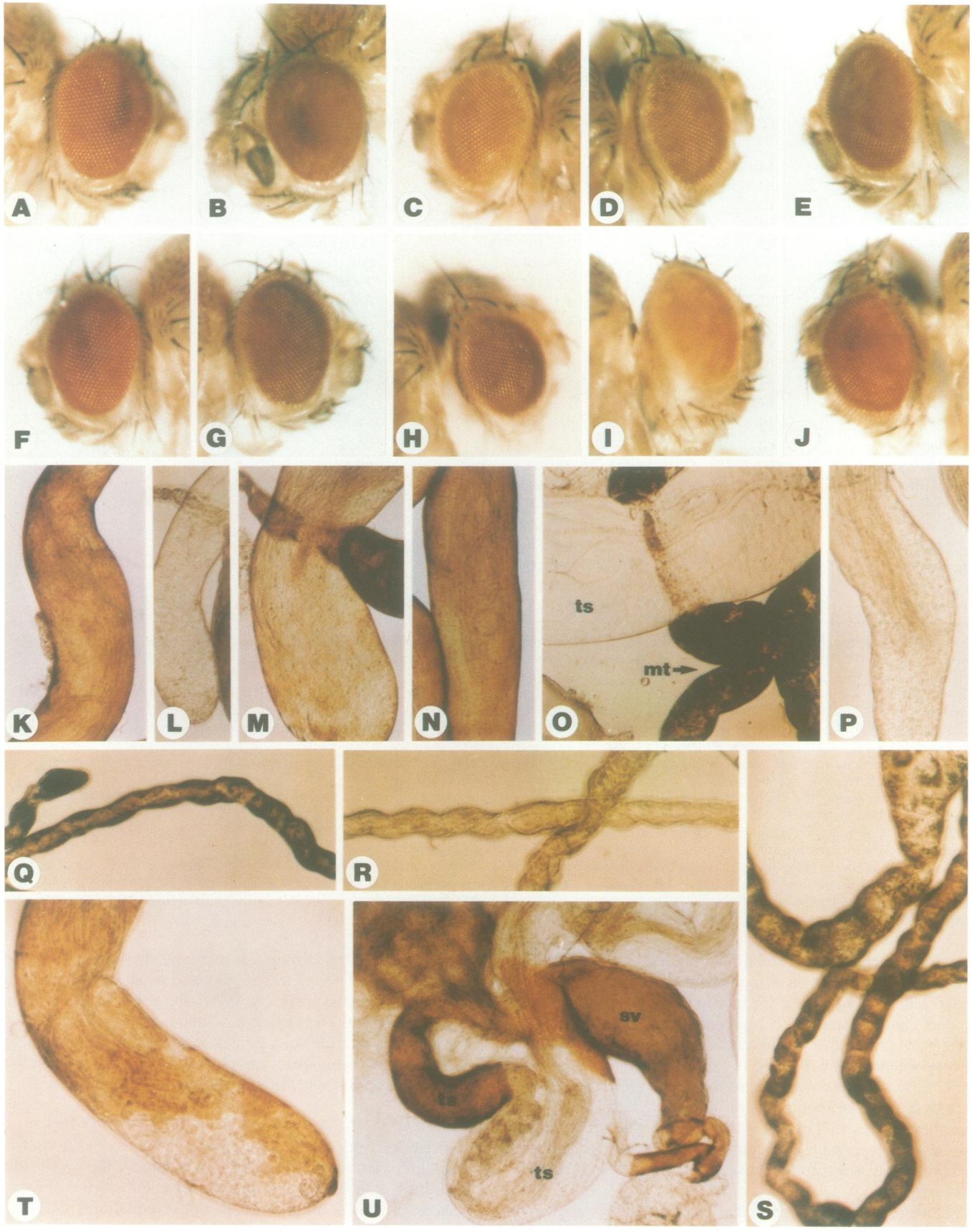
[a]The number and letter give the polytene chromosome location of the insertion in the map of Bridges (1935). The chromosome arm is given in parentheses.
[b]Eye color of a w^{1118}/w^{1118} female with one copy of the transduced gene, except for transformant E3 in which the females were *sc* $w^{11E4}cv/sc$ $w^{11E4}cv$ *P*[(*w*,*ry*)E]3. Flies were grown at 25°C and aged 5 days.
[c]Pigment was extracted from the heads of w^{1118} males with one copy of the transduced *white* gene (except for the E1 line in which *sc* $w^{11E4}cv$ *P*[(*w*,*ry*)E]1 males were used) and wild-type (Canton S) males. The optical densities from three replicates were averaged. For the H2-1 transformant only two replicates were done. The optical density of w^{1118} (=0.017) or *sc* $w^{11E4}cv$ (=0.028) control males was subtracted from each. The average optical density for the wild-type males following this subtraction was 0.903.
[d]Following subtraction of the null allele control, the optical density of the pigment of these transformants was <0.100. While we are confident that these are $<10\%$ of the wild-type, we feel the values are too small to be accurate beyond that conclusion.
[e]The chromosome bearing this insertion was part of a complex rearrangement. The transposon was not located at any of the breakpoints. Due to this rearrangement we could not figure out the map position of the site of hybridization.
[f]There is an inversion in the chromosome bearing this transposon. None of the breakpoints of the inversion coincided with the insertion site. Inversions of the 2nd chromosome have been reported to be present in many isolates of the ry^{506} stock (Bourouis and Richards, 1985).
[g]Transformation in the E3 line was accompanied by the induction of a recessive mutation at the *scalloped (sd)* locus. This mutation in the E3 flies failed to complement the sd^1 mutant, which has a similar wing phenotype. The transposon is inserted at the cytogenetic position of the *sd* locus, strongly suggesting that the *sd* mutation resulted from the insertion. Daniels *et al.* (1985) have described the mutation sd^{ry+}, induced by the insertion of a *P(ry)* transposon in or near the *sd* locus.

be extracted from the eyes of a few flies and measured spectrophotometrically to obtain a more quantitative measure of eye pigmentation. A significant decrease in the level of the *white* gene product can be expected to be accompanied by an observable decrease in pigmentation. This decrease in pigmentation may not be proportional to the decrease in expression, especially for flies with nearly wild-type *white* expression. The *white* gene product is probably not the limiting factor in eye pigmentation in wild-type flies. In flies with mutant *white* alleles that reduce gene function, eye pigmentation is roughly proportional to gene dosage (Muller, 1950) suggesting that the *white* product in these cases is limiting.

The pigmentation of a particular tissue probably requires the *white* gene product during a particular period during development. If a normally pigmented tissue is unpigmented in a transformant, we cannot distinguish between the inability of the transduced *white* gene to be expressed at all in the tissue and its inability to be expressed in that tissue at the proper time during development. Therefore our definition of expression is operational; when we refer to a transduced segment being expressed in a tissue, we mean that it can provide *white* gene product there during the period when it is required for pigmentation.

Pigmentation of the eyes and its dosage compensation

A brief description of the eye color of females of each transformant line with a single dose of the transduced *white* gene is given in Table I. It should be noted that the eye color which we observed was the product of the pigmentation of several different cell types in the eye. In partially pigmented *white* mutants the pigment in different cell types is not always reduced to the same extent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(Nolte, 1950, 1959, 1961; Gersh, 1952). We have not yet analyzed whether transduced *white* genes are differentially expressed in the various pigmented cells of the eye.

Comparing transformants with the D, E and F *white* DNA segments shows the relationship between the presence of portions of the 5′-flanking sequences and the eye pigmentation. Flies of two of the four transformant lines with segment D, with 1.9 kb of upstream flanking DNA, had an eye color indistinguishable from the red color of the wild-type, while the remaining two had a slightly browner eye color. A photograph of one of each type is shown in Figure 2B and F and of a wild-type fly in Figure 2A. Spectrophotometric measurements of the pigments extracted from heads of two lines transduced with the D segment showed them to be within 2-fold of wild-type (Table I). This is roughly equivalent to the amount of pigment in the eyes of transformants with segment A that has 3.0 kb of 5′-flanking DNA (Hazelrigg *et al.*, 1984). The eyes of flies transduced with the E and F segments, carrying 1.1 and 0.4 kb of 5′-flanking DNA, respectively, were all noticeably less pigmented than wild-type. The colors varied from yellow to a dull red. Pigment extractions from representative transformants showed that these corresponded to from $<10\%$ to $\sim 50\%$ of wild-type (Table I). None had the 'eye spot' characteristic of wild-type eyes and those transduced with segments A or D. Figure 2C and G shows the eye colors of two different E transformants and Figure 2D and I shows the eye colors of two F transformants. The eye pigmentation of the eight independent E transformants was, on average, closer to wild-type than the six F transformants. However, there was a considerable overlap between the ranges of pigmentation of these two groups. Given the range of pigmentation within each group transduced with the same segment and the limited number of insertion sites of each that we have studied, we cannot be certain whether the eye pigmentations conferred by the E and F segments are significantly different from each other. As expected, segment G, which lacks all 5′-flanking sequences and the entire putative 5′ exon, failed to provide any detectable pigmentation to the eyes of any of the four lines transduced with it. From our comparison of transformants with the A, D, E and F segments, we conclude that sequences that modulate the eye pigmentation are present in the interval from 1.1 to 1.9 kb upstream of the transcribed region of *white*. Nevertheless, 0.4 kb of 5′-flanking DNA is sufficient for partial pigmentation of the eye.

Only 157 bp of 3′-flanking DNA is contained within segment H. Different transformant lines with segment H had eye colors varying from brown to essentially wild-type (Figure 2E and J). The pigment extracted from the heads of three H transformant lines ranged from 38% to 59% of the wild-type (Table I). We will defer interpretation of this result to the Discussion.

If a transduced *white* gene retains the *cis*-acting sequences required for dosage compensation, one copy in a male should confer more pigment than one copy in the female. We have reported that for three transformant lines with segment A, a male with a single copy had $1.54-1.93$ times as much eye pigment as a female with a single copy (Hazelrigg *et al.*, 1984). We compared the eye pigmentation of males of each of the new transformant lines with females of the same line by visual inspection, as a qualitative assay for the dosage compensation of the P[*white*] gene. Both the males and females carried a single dose of the transduced *white* gene. Pigment differences caused by dosage compensation are most easily seen in flies having mutant *white* alleles rendering them partially pigmented (Muller, 1950). Thus, for each of the eight E transformant lines and six F transformant lines it was obvious that the eyes of males of a given line were more pigmented than the eyes of females of the same line. The eye colors of a female and male of the E6 line are shown in Figure 2C and H. The male/female ratio was quantified by pigment extractions for the E8 and F4-3 lines and found to be 2.6 and 2.3, respectively. The transduced *white* gene is located on an autosome in four of the eight E lines and all six F lines. We can therefore conclude that dosage compensation of the *white* gene eye pigmentation does not require flanking sequences located >0.4 kb upstream of the transcribed region.

We have also compared the eye pigmentation of males and females with a single dose of each H segment. The H transformants have a more nearly wild-type eye color than the E and F transformants, making these comparisons more difficult. However, we believe the male to have more eye pigment in all five cases, four of which have autosomal sites of insertion. Together with the results stated above, this indicates that sequences required in *cis* for the dosage compensation of eye pigmentation must either lie within the transcribed region, within 0.16 kb downstream from it or within 0.42 kb upstream from it.

We noticed that among the lightly pigmented E and F transformants, males or females homozygous for an autosome bearing a P[*white*] transposon had more darkly pigmented eyes than would be predicted from the eye pigmentation of a fly with a single copy of the same chromosome. This impression was borne out by quantitative pigment comparisons in two cases tested. For the E2 and F4-3 transformants, females with two doses of the P[*white*] gene had 4.8 and 5.1 times as much eye pigment as females with one dose.

Pigmentation of tissues other than the eye

Expression of the *white* gene is necesary for pigmentation not only in the eye, but also in the adult ocelli, the larval and adult Malpighian tubules and the testis sheath of the adult male. Segment G does not contain the 5′ exon of *white*, and does not confer pigmentation to any of these tissues. Transformants carrying any one of the other segments were pigmented in the eyes, ocelli and larval and adult Malpighian tubules. The levels of pigmentation in these tissues varied somewhat among transformants. Figure 2 shows the Malpighian tubules of a wild-type fly (panel Q), a w^- mutant (panel R), and an E (panel O) and F (panel S) transformant. The Malpighian tubules of a wild-type fly are pale yellow and those of a null mutant are colorless under normal illumination; the photographs of the Malpighian tubules and testes in Figure 2 were taken using phase illumination to enhance the contrast of the unpigmented tissues. We conclude that the *cis*-acting sequences required to provide sufficient *white* expression to give detectable pigmentation to the eyes, ocelli and

Fig. 2. Colors of the eyes, Malpighian tubules and testes of transformants. **Panels A – J** show the eyes of transformants of the following genotypes: (**A**) w^+(CantonS)/w^{1118} female; (**B**) w^{1118}/w^{1118};P[(*w*,*ry*)D]1/+ female; (**C**) w^{1118}/w^{1118};P[(*w*,*ry*)E]6/+ female; (**D**) w^{1118}/w^{1118};P[(*w*,*ry*)F]1/+ female; (**E**) w^{1118}/w^{1118};P[(*w*,*ry*)H]1 female; (**F**) w^{1118}/w^{1118};P[(*w*,*ry*)D]3/+ female; (**G**) w^{1118}/P[(*w*,*ry*)E]5 w^{1118} female; (**H**) w^{1118}/Y;P[(*w*,*ry*)E]6/+ male; (**I**) w^{1118}/w^{1118};P[(*w*,*ry*)F]4-3/+ female; (**J**) w^{1118}/w^{1118};P[(*w*,*ry*)H]3/+ female. **Panels K – U** show portions of the following organs of males: (**K**) testis of w^+ (CantonS)/Y; (**L**) testis of w^{1118}/Y; (**M**) testis with seminal vesicle overlying and to the right of it of w^{1118}/Y; P[(*w*,*ry*)H]2-2/*TM3*,*Sb*; (**N**) testis of w^{1118}/Y; P[(*w*,*ry*)D]3/P[(*w*,*ry*)D]3; (**O**) testis (ts) and Malpighian tubule (mt) of a w^{1118}/Y;P[(*w*,*ry*)E]8/*CyO*; (**P**) testis of w^{1118}/Y;P[(*w*,*ry*)F]2/P[(*w*,*ry*)F]2; (**Q**) Malpighian tubule of w^+ (CantonS)/Y; (**R**) Malpighian tubule of w^{1118}/Y; (**S**) Malpighian tubule of w^{1118}/Y;P[(*w*,*ry*)F]2/P[(*w*,*ry*)F]2; (**T**) testis of w^{1118}/Y;P[(*w*,*ry*)A^R]4-3/P[(*w*,*ry*)A^R]4-3; ((**U**) testis (ts) and seminal vesicle (sv) of w^{1118}/Y;P[(*w*,*ry*)A]4-4/P[(*w*,*ry*)A]4-4.

Table II. Interaction of transformants with z^1

Transformants	Eye color		
	z^+w^-;*P[w,ry]*/*P[w,ry]*	z^1w^-;*P[w,ry]*/*P[w,ry]*	Interaction[a]
Wild-type	Wild-type red	Yellow	+++
D1	Wild-type red	Orange-brown	++
D2	Wild-type red	Red-brown	+
D3	Wild-type red	Red-brown	+
D4	Wild-type red	Yellow with red spots	+++
E1	Dull red	Dull red	−
E2	Dull red	Dull red	−
E3	Dull red	Dull red	−
E5	Nearly wild-type red	Nearly wild-type red	−
E6	Red-brown	Red-brown	−
E7	Orange-brown	Orange-brown	−
F2	Red-brown	Red-brown	−
F4-1	Yellow-orange + spots	Yellow-orange + spots	−
F4-2	Red-brown	Red-brown	−
F4-3	Red-brown	Red-brown	−
H1	Red-brown	Brown	+
H2-1	Red, slightly brownish	Yellow	+++
H2-2	Red-brown	Brown	+
H3	Wild-type red	Orange with dark spots	++
H4	Wild-type red	Yellow	+++

[a]Comparing the eye color conferred in a z^+ and a z^1 background, the degree of interaction has been graded from − (no interaction) to +++ (interaction equivalent to that of a wild-type *white* locus).

Malpighian tubules are located between a point 0.16 kb downstream and a point 0.42 kb upstream from the transcribed region.

The testes of flies transduced with different segments of *white* showed striking differences in pigmentation which were correlated with the length of 5′-flanking DNA. Figure 2 shows the testis of a wild-type male (panel K) and a w^- mutant (panel L). The testis of the wild-type male is bright yellow and that of the null mutant is colorless under normal illumination. The testes of the D and H transformants were all pigmented, varying in color from pale to bright yellow. Figure 2N shows an example of the testis of a D transformant. The testis of the H2-2 transformant in Figure 2M can be seen to be more lightly colored than that of the wild-type or D transformant (ignore the seminal vesicle which lies over and to the right of the testis in Figure 2M; the seminal vesicle appears dark under this illumination due to the refractility of the sperm bundles it contains). In contrast to the pigmented testes of the D and H transformants, the colorless testes of E and F transformants (panels O and P) were indistinguishable from those of the w^- mutant. We conclude that a transduced *white* gene with at least 1.9 kb of 5′-flanking DNA is able to provide sufficient *white* function in the testis sheath to produce detectable pigmentation, but that 1.1 kb of 5′-flanking DNA is not sufficient for testis sheath pigmentation.

We believe that the absence of the 5′-flanking sequences from 1.1 to 1.9 kb upstream from the transcribed region has a qualitatively greater effect on pigmentation of the testis sheath than of the other tissues. An alternative hypothesis is that there is a proportional decrease in the pigmentation of all tissues but that this decrease is more evident in the testis sheath. We reject this alternative for the following reason: the testes of wild-type males and of D and H transformant males were more darkly pigmented than the Malpighian tubules (the Malpighian tubules appear darker in Figure 2 only as a consequence of the phase illumination used). The fact that pigment was visible in the Malpighian tubules of the E and F transformants, but not in their testes, indicates that either there was no pigment in their testes sheaths, or it was present at a very reduced level relative to the levels in other tissues. We cannot rule out the possibility that there is a threshold level of *white* product required for detectable testes pigmentation, while pigmentation of other tissues is more nearly proportional to the level of the *white* product. This seems unlikely, however, since there are several *white* mutants, such as *white-apricot-2, white-apricot-3* and *white-carrot*, which have eyes that are less pigmented than our E or F transformants, but still have detectably pigmented testes (D.Thiery-Mieg, unpublished results).

The isolation of two *white* transformants, A^R4-3 and A4-4, which have a mosaic eye pigmentation, has been described (Hazelrigg *et al.*, 1984). The larval and adult Malpighian tubules, ocelli and testes pigmention in these transformants is described here. Pigment is present in all of these tissues in both cases. We did not observe mosaic expression in the ocelli or Malpighian tubules (larval or adult). The testes did show mosaic expression of *white* in both the A4-4 and A^R4-3 transformants. In the case of A4-4, which in the eye produces a background color of yellow with small red spots scattered across the eye, the testes tended to be colorless in the distal tips, and have large yellow patches on the seminal vesicles and proximal testes sheaths. An example is shown in Figure 2U. A^R4-3 transformants have eyes in which the anterior part of the eye is more darkly pigmented than the posterior part. The testes of A^R4-3 males have large yellow and colorless patches as shown in Figure 2T.

Interaction with zeste

To investigate the interaction of the transduced *white* genes with z^1, each homozygous-viable P[*w,ry*] transposon was made homozygous in combination with a z^1w^- and a z^+w^- X chromosome. The eye colors of each transformant in a z^1 and z^+ background are compared in Table II. Bear in mind that for previously described *white* transformants, with as much as 5.7 kb 5′- and 2.9 kb 3′-flanking DNA, the degree of interaction of the P[*white*] gene with z^1 varies considerably among independent transformants, presumably as a consequence of the different genomic positions of the transposons (Hazelrigg *et al.*, 1984).

The D, E and F transformants, which differ in the amount of

5′-flanking sequences, differed in their interaction with z^1. The transduced D segment, with 1.9 kb of upstream flanking DNA, interacted with z^1 in all four independent transformants. In contrast, in all six E transformants and all four E transformants which could be made homozygous, the z^1 and z^+ flies had an identical eye color. Therefore it appears that interaction of a transduced *white* gene with z^1 requires sequences in the interval from 1.1 to 1.9 kb upstream of the transcribed region to be present.

The interaction with z^1 does not require extensive 3′-flanking DNA, since all five of the H transformants interacted with z^1 to some degree and two (H2-1 and H4) interacted as strongly as a wild-type *white* gene. The H transduced segments carry only 0.16 kb of 3′-flanking DNA.

Each transduced *white* gene was also tested for its interaction with the z^{op6} allele. The interactions of this *zeste* allele with various alleles of *white* have not been studied as thoroughly as those of z^1, but z^{op6} is able to interact with a single w^+ gene (Lifschytz and Green, 1984), whereas z^1 interacts only with pairs of w^+ genes (Gans, 1953; Jack and Judd, 1979; Gelbart and Wu, 1982). Therefore, transduced *white* genes that cannot be made homozygous for testing the z^1 can be tested with z^{op6}. Transformants carrying segment A (3.0 kb 5′-flanking DNA) and segment B or C (5.7 kb 5′-flanking DNA) (Hazelrigg *et al.*, 1984) were first tested. For all seven of the transduced A, B and C segments inserted at autosomal sites there was a perfect correlation between those that interacted with z^1 and with z^{op6} (data not shown). Similarly, the D and H transduced genes interacted with z^{op6} in all nine transformant lines and the relative degree of interaction at different genomic positions corresponded to that with z^1. The seven E transformants all failed to interact detectably with z^{op6}. Three of the five F transformants (F4-1, F4-2 and F4-3) showed a slight lightening in their eye color when combined with z^{op6}; however, this may reflect the influence of other unidentified modifiers differing between the z^+ and z^{op6} chromosomes used.

Discussion

A gene product, or several closely related gene products, must often be produced in different cell types and at different times during the development of a metazoan. This is sometimes accomplished by the duplication of the gene (e.g., actin, tubulin, globin and keratin). In such cases, each gene is presumably linked to different regulatory elements so that transcription of each gene can be activated independently in a specific cell type or developmental stage. However, for other genes, such as *white*, a single gene must be regulated in a complex manner. Is there a single, indivisible, *cis*-acting regulatory element which controls the gene in these diverse cellular/developmental circumstances? The conclusion of most general interest of our analysis is that the *white* gene is linked to two or more separable *cis*-acting regulatory elements. Separable regulatory elements have been demonstrated for the expression of *Drosophila* yolk protein genes in the ovary and fat body (Garabedian *et al.*, 1985).

Upstream sequence requirements for expression of the white gene

Our deletion analysis of the *white* gene has localized at least one of its regulatory elements to the 5′-flanking region. Table III summarizes the phenotypes conferred by segments differing in the length of 5′-flanking DNA they contain. We interpret these results to indicate that sequences in the interval between 1.1 and 1.9 kb upstream from the transcribed region are crucial for the interaction with z^1 and for expression in the testis sheath. This region also augments *white* expression in the eyes. Our experiments do not rule out the possibility of other *cis*-acting regulatory elements necessary for these functions located elsewhere within the DNA segments analyzed here. The cell-type specificity of immunoglobulin gene expression has been reported to be controlled by three or more separate *cis*-acting sequence elements (Grosschedl and Baltimore, 1985). Sequences >0.42 kb upstream from the transcribed region of *white* are not required in *cis* to give dosage compensation to the pigmentation of the eye or to provide detectable pigmentation to the Malpighian tubules and ocelli.

Table III. Requirements for the 5′-flanking region

Segment	A	D	E	F
5′ flanking DNA (kb)	3.0	1.9	1.1	0.42
Dosage compensation (eyes)	+	+	+	+
Pigmentation				
Eyes	Wild-type	Wild-type	Partial	Partial
Ocelli	+	+	+	+
Malpighian tubules	+	+	+	+
Testes sheath	+	+	−	−
z^1 interaction	Variable	Variable	−	−

For pigmentation of the ocelli, Malpighian tubules and testes a + sign indicates that detectable pigment was present in these structuress in all tranformants. The degree of pigmentation of these structures varied somewhat among transformants and was difficult to compare with wild-type.

The 5′-flanking sequences which we have studied by *in vitro* deletion analysis overlap with the sites of lesions of previously known mutants. Four mutants, *white-spotted* (w^{sp}) *1, 2, 3* and *4*, have deletions of or insertions into the region between 0.9 kb and 1.3 kb upstream from the transcribed region (Zachar and Bingham, 1982; O'Hare *et al.*, 1983, 1984; Pirrotta and Bröckl, 1984; Davison *et al.*, 1985). The w^{sp4} allele has a deletion of the sequences between 1.1 and 2.2 kb upstream from the proposed start site of transcription; the downstream end point of this deletion is only 3 bp from the 5′ end of our segment E. All four of these *white-spotted* mutants have a similar yellow-brown speckled eye color. None of the four interact with z^1. The reduced eye pigmentation and lack of z^1 interaction of the w^{sp} mutants and segment E transformants are in agreement. However, all four w^{sp} mutants have variegated eye pigmentation, while among our 14 segment E and F transformants only one (F4-1) had a comparable variegated eye. Moreover the alteration in dosage compensation of the w^{sp} mutants does not appear to be a property of the E and F transformants. The four w^{sp} mutants are all more darkly pigmented in the eyes of the male (with one dose) than the female (with two doses), as if the gene in the male is overcompensated. In none of our transformants were the eyes of the male with one copy of P[*white*] more darkly pigmented than those of the female with two copies. This issue is complicated by the observation that flies with two doses of an E or F P[*white*] segment appear to have more than twice as much pigment as flies with one dose.

We believe that the sequences required for detectable testis pigmentation that are absent in segments E and F are upstream regulatory sequences. An alternative is that these segments lack the promoter or coding sequences for a hypothetical testis-specific transcript. There is some evidence for a *white* transcript detectable only in males (Pirrotta and Bröckl, 1984; Fjose *et al.*, 1984; Bingham and Zachar, 1985) but no evidence that the 5′ end of this transcript differs from that of the major 2.6-kb transcript. The available transcript mapping data cannot exclude the possibility of a very small (<50 bp) exon upstream of the putative start

of transcription. The best evidence against a testis-specific transcript having an essential exon in the proposed regulatory region comes from the germ line transformation experiments of Pirrotta and his colleagues. Transformants in which the sequences between 0.8 and 1.9 kb 5′ to the proposed start of transcription are inverted have pigmented testes (Pirrotta *et al.*, 1985).

The interaction of *white* in *trans* with z^1 is unusual in that it depends on the number and chromosomal position of *white* genes (Gans, 1953). In most cases two *white* genes must be present in close chromosomal proximity, either in tandem on the same chromosome or on chromosome homologues (Jack and Judd, 1979; Gelbart and Wu, 1982). Previous genetic mapping of partial *white* duplications or deletions has indicated that the centromere-proximal (5′) portion of *white* is critical for the interaction; a single *white* gene can interact with z^1 if it is linked in *cis* to a duplication of the proximal portion of *white*; conversely, two *white* genes fail to interact if the proximal portion of one of them is missing (Judd, 1961; Green, 1963). In agreement with these studies, we found that a segment with only 157 bp of 3′-flanking DNA interacted with z^1 when homozygous, whereas segments with only 1.1 kb 5′-flanking DNA did not interact. Consistent with our results, Zuker *et al.* (in preparation) have found that a transduced segment containing a complete *white* gene (coordinates −4.3 to +6.62), linked to a second copy of the segment from 1.1 to 2.5 kb upstream from the start site of transcription, interacted much more strongly, on average, than the same segment without the partial duplication.

The sequences between 1.1 and 1.9 kb upstream from the transcribed region of *white* are necessary, but are not sufficient, for the P[*white*] gene at all genomic positions to interact as strongly with z^1 as does a wild-type *white* gene. Even *white* segments with 5.7 kb of 5′-flanking DNA show a variable degree of interaction as a function of their genomic positions (Hazelrigg *et al.*, 1984). The *zeste-white* interaction is more position sensitive than are other examples of gene regulation that have been studied by P-element transformation in *Drosophila*. It is not known whether the position sensitivity of the interaction should be viewed as a matter of sequences surrounding the site of insertion facilitating the interaction in cases of strong interaction or of interfering in the cases of weak interaction. Nevertheless, because of this position sensitivity, conclusions concerning the requirements of *cis*-acting sequences flanking *white* for its interaction with *zeste* must be made cautiously. In 13 of 14 cases a transduced gene containing the 1.1 − 1.9 kb upstream region interacted with z^1, while in none of six cases did the gene without this region interact detectably. Furthermore, in none of four cases did a transduced segment with 0.42 kb of 5′ flanking DNA interact detectably. Therefore, we can state that the presence of 1.9 kb or more of 5′-flanking DNA dramatically increases the likelihood that the P[*white*] segment will show an interaction with z^1.

We have observed that, even in a z^+ genetic background, the eye color conferred by segment E or F (1.1 and 0.42 kb upstream flanking DNA, respectively) varied over a wider range, as a function of the genomic location where it was inserted, than segment A or D (3.0 and 1.9 kb upstream flanking DNA, respectively). A reduction of flanking sequences has been reported to be accompanied by more pronounced position effects on the expression of the *Drosophila rosy* (Spradling and Rubin, 1983) and *sgs-3* genes (Bourouis and Richards, 1985). We have argued above that segments A and D carry control elements missing from segments E and F. One explanation for the greater position sensitivity of the smaller segments is that the function of the missing control elements can be partially supplied by nearby sequences at some genomic positions.

Downstream sequence requirements for expression of the white gene

The 3′ end of the mature major *white* transcript has been precisely mapped (O'Hare *et al.*, 1983, 1984; Pirrotta and Bröckl, 1983). It cannot be excluded that some rare transcripts, from some particular tissue or time during development, have other 3′ ends. Transduced *white* DNA segments with 2.9 or 2.1 kb of 3′-flanking *white* DNA conferred a nearly wild-type *white* phenotype at almost all genomic locations, so long as at least 1.9 kb of 5′-flanking DNA was included. However, when the 3′-flanking *white* DNA was reduced to 0.16 kb (segment H), some of the transformant lines had a brown eye color and others had a wild-type eye color. Dosage compensation and the interaction with z^1 did not appear to be affected by this truncation of downstream flanking DNA.

One possible reason for the reduced eye pigment in some H transformants is that the removal of the normal 3′-flanking sequences of *white* may have interfered with a post-transcriptional cleavage step in the maturation of the *white* mRNA. In the few cases in which transcriptional termination *per se* of eukaryotic genes has been carefully examined, transcription has been found to continue hundreds of nucleotides past the end point of the mature transcript (reviewed by Birnstiel *et al.*, 1985). The removal of the normal 3′-flanking sequences of a gene has been shown to reduce the efficiency of 3′ end maturation *in vivo* (Birchmeier *et al.*, 1984; McDevitt *et al.*, 1984). It is intriguing that several *white* mutants mapping to the distal (3′) end of the locus also have a brownish eye color (e.g., *white-colored, white-coffee* and *white-satsuma*). Unfortunately, in none of these mutants has a lesion in the DNA been identified (Zachar and Bingham, 1982) so that it is not known whether these mutations are within the mature RNA coding region.

An alternative is that the brown eye color of some of the H transformants may not be indicative of a requirement for specific *cis*-acting 3′-flanking sequences, but may be due to a more passive 'buffering' effect of these sequences on the transduced *white* gene. As noted above, buffering effects have been previously hypothesized for genes within P-element transposons, yet are poorly understood. To discriminate between these alternatives, a more sophisticated deletion strategy would be to remove various small internal portions of the normal flanking sequences without significantly reducing the overall length of the flanking DNA.

Pirrotta *et al.* (1985) have carried out an independent analysis of the requirements for the *cis*-acting flanking sequences of the *white* gene. The 5′ end of the *white* segment carried by their Bg-w transformants is identical to that carried out by our E transformants. The major results of our study are in complete agreement with those of Pirrotta *et al.* for flanking sequences examined in both studies.

Materials and methods

P-element transposon plasmids

A map of the structures of the P[*w,ry*] plasmids is shown in Figure 3 and a more detailed map of the *white* region in Figure 4. The vector for all of these plasmids except G is a modified fragment of Carnegie 30. Carnegie 30 was constructed by inserting the 7.2-kb *ry* *Hind*III fragment into the *Hind*III site of the polylinker of Carnegie 3 (Rubin and Spradling, 1983). Carnegie 30 was cut within the polylinker with *Sal*I, the 5′ overhang was filled in using the large fragment of DNA polymerase, and then the polylinker was cut with *Kpn*I. This created a linear molecule having a P element-*Hind*III-*Pst*-blunt (the filled-in *Sal*I site) end and a *Kpn*I-*Eco*RI-P element end. This vector fragment would therefore accept a *white* DNA segment having a *Kpn*I end and a blunt end. It will be referred

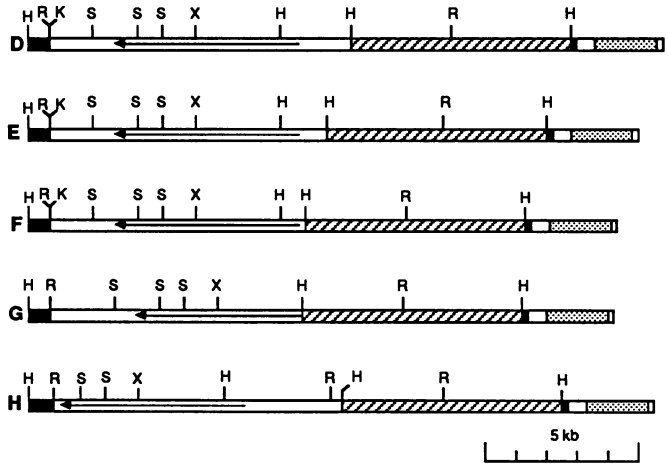

Fig. 3. Structure of the P[*w,ry*] transposon plasmids. The circular plasmids are represented in a linear form with the 5′ end of the P element at the extreme left. Solid bar - P element; open bar - *white* gene; striped bar - *rosy* gene; stippled bar - bacterial vector. Restriction enzyme cleavage sites: H - *Hin*dIII; K - *Kpn*I; R - *Eco*RI; S - *Sal*I; X - *Xba*I. The arrow within the *white* gene bar shows the direction of transcription and the proposed limits of the major mature transcript (O'Hare *et al.*, 1984), but does not show the position of the introns.

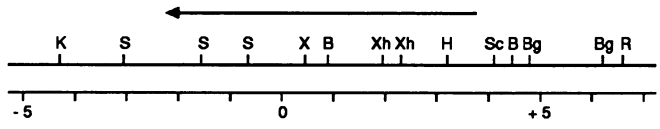

Fig. 4. Restriction map of the *white* gene region. See legend to Figure 1 for explanation of the coordiante system. Restriction enzyme cleavage sites: B - *Bam*HI; Bg - *Bgl*II; H - *Hin*dIII; K - *Kpn*I; R - *Eco*RI; S - *Sal*I; Sc - *Sca*I; X - *Xba*I; Xh - *Xho*I. The positions of all sites except the *Kpn*I site have been determined by DNA sequencing (O'Hare *et al.*, 1984). The arrow shows the direction of transcription and proposed limits of the major mature transcript.

to below as the C30KB fragment.

The plasmid pC1 was the source of the *white* DNA fragment in pP[(*w,ry*)D]. pC1 was constructed by cloning the 11.7-kb *Eco*RI fragment (coordinates −5.1 to +6.6) of λm2.1 (Levis *et al.*, 1982) in the *Eco*RI site of the P element vector pCIW4 (Rubin and Spadling, 1983). pC1 has the same structure as pP[(*w*)B] (see Figure 7 of Hazelrigg *et al.*, 1984) except that the latter has an additional 2.7 kb of the 5′-flanking DNA of *white* (coordinates +6.6 to +9.3). For the construction of pP[(*w,ry*)D], pC1 was cut with *Hin*dIII and the 5.8-kb *Hin*dIII fragment (+3.18 to +8.97) was gel purified. This was digested with *Hin*cII and the 1.6-kb *Hin*dIII (+3.18)-*Hin*cII (+4.79) fragment was gel purified. pC1 was cut separately with *Kpn*I and *Hin*dIII and the 7.3-kb *Kpn*I (−4.1)-*Hin*dIII (+3.18) fragment was gel purified. The C30KB vector fragment, the *Kpn*-*Hin*dIII fragment and the *Hin*dIII-*Hin*cII fragment were joined in a tri-molecular ligation and used to transform *Escherichia coli* HB101. The final plasmid, pP[(*w,ry*)D], contained the *Kpn*I (−4.1)-*Hin*cII (+3.18) segment of *white*.

For the construction of pP[(*w,ry*)E], λm2.1 was digested with *Bgl*II, the 5′ overhang was filled in using the large fragment of DNA polymerase, the DNA was then cut with *Kpn*I and ligated with the C30KB vector fragment. Colony hybridization was used to identify colonies having the plasmid with the *Kpn*I (−4.1)-*Bgl*II (+4.79) fragment of *white*. P[(*w,ry*)F] was constructed by ligating together the *Kpn*I (−4.1)-*Sca*I (+4.11) fragment of *white* from pC1 (see origin above) and the C30KB vector fragment.

P[(*w,ry*)G] was derived from pC1 by partially digesting it with *Hin*dIII, gel purifying the 12.9-kb fragment, and ligating this together with the 7.2-kb *Hin*dIII *ry* fragment. This had the overall effect of deleting the DNA of pC1 between the *Hin*dIII site at nucleotide 880 in the P element vector (O'Hare and Rubin, 1983) and the *Hin*dIII site at coordinate +3.18 in *white* and substituting the *ry* *Hin*dIII fragment.

The construction of P[(*w,ry*)H] began by cloning the *Kpn*I (−4.1)-*Hpa*I (+7.13) fragment of *white* into the C30KB vector fragment to create Carnegie 30[*w*(Kpn-Hpa),*ry*)]. The 3′-flanking DNA between the *Kpn*I (−4.1) and *Bss*HII (−2.38) sites was then removed by substituting the *Bss*HII (−2.38)-*Xba*I (+0.44) fragment of pC1 for the *Kpn*I (−4.1)-*Xba*I (+0.44) fragment of Carnegie 30[*w*(Kpn-Hpa),*ry*)]. The *Bss*HII-*Xba*I fragment of pC1 was prepared by first gel purifying the *Kpn*I (−4.1)-*Xho*I (+1.96) fragment, filling in the 5′ overhangs, then cutting with *Xba*I. Carnegie 30[*w*(Kpn-Hpa),*ry*)] was cut with *Kpn*I, the ends were blunted with T4 DNA polymerase, and the DNA was cut with *Xba*I. The *Bss*HII (blunted)-*Xba*I fragment of pC1 was ligated to the *Kpn*I (blunted)-*Xba*I vector portion of Carnegie 30[*w*(Kpn-Hpa),*ry*)] to form P[(*w,ry*)H]. The DNA sequence of the 3′ end of the *white* DNA segment of pP[(*w,ry*)H] was checked by subcloning the *Eco*RI (polylinker)-*Sal*I *(white*, −1.52) fragment into the M13 vector mp93 and sequencing the polylinker-*white* DNA junction as described by O'Hare *et al.* (1984). Nucleotide −2387 was found to be the 3′-most nucleotide of the *white* segment.

The Carnegie 30 vector was constructed in two forms, differing in the orientation of the *rosy* *Hin*dIII fragment. Transposon plasmids D, E, F and G were also constructed in two forms with alternative orientations of *rosy*. For all of the D and F transformants produced, *white* and *rosy* are oriented so that they are divergently transcribed. For the E and H transformants they are oriented so that they are transcribed in the same direction. In transformants G1 and G2, the two genes are transcribed in the same direction, in G3 and G4 they are transcribed in opposite directions (imagining that the *white* DNA in this transposon could be transcribed).

Drosophila transformation

The preparation of plasmid DNA and microinjection into embryos have been described by Spradling and Rubin (1982). The E1, E2, E3 transformants and all four G transformants were recovered from injections in which the co-injected helper plasmid was pπ25.1. All other transformants were recovered from injections using the pπ25.7wc plasmid helper (Karess and Rubin, 1984). All of the E, G and H transformants and transformants D3, D4, F1 and F2 were recovered as ry^+ transformants of the ry^{506} host strain (Daniels *et al.*, 1985). The D1, D2, F3 and F4 transformants were recovered from injections of w^{1118} hosts (Hazelrigg *et al.*, 1984).

The G1 transformants from a single G0 of either host were mated together to produce a parent line. For segregation analysis and balancing, 5 − 10 G2 transformant males were mated to w^{1118};*CyO/Sco* females having a dominantly marked second chromosome balancer (see Lindsley and Grell, 1968 for a description of balancer chromosomes and other genetic markers). Five to twenty *CyO* male progeny having pigmented eyes were mated individually to w^{1118} females. For transformants in which eye pigmentation segregated from *Cy* in all cases, a second chromosome isochromosome line was established, homozygous for the transposon-bearing chromosome or balanced with *CyO*. The whole procedure was carried out in parallel with w^{1118}; *TM3,Sb/CxD* females having a dominantly marked third chromosome balancer. Insertions on the w^+X chromosome of the ry^{506} host were transferred by recombination to the *sc* w^{11E4}*cv* X chromosome, and in most cases to the w^{1118} chromosome.

Tests of the interaction with zeste alleles

All of the crosses described below were carried out at 25°C. For tests of interaction with z^1, each transduced *white* gene listed in Table II was made homozygous in combination with z^1w^{11E4}. For autosomal P[*w*] inserts, males of the w^{1118};P[*w*] stock were mated to females homozygous for the $z^1w^{11E4}f^5$ X chromosome and heterozygous for an appropriate balancer chromosome (*CyO* for the 2nd and *TM3,Sb* for the 3rd chromosome). A single male progeny with the balancer was back-crossed to virgin females of the same stock as before. Males and females heterozygous for the balancer and P[*w*] chromosome were mated to each other and progeny homozygous for the P[*w*] chromosome were selected. For X chromosome inserts, P[*w*] was combined with z^1w^{11E4} by recombination between the parent w^+ P[*w*] stock and the $z^1w^{11E4}f^5$ X chromosome. A homozygous stock was established from a single male recombinant using the FM7c balancer (Merriam, 1968).

For X chromosome P[*w*] inserts that did not interact with z^1 (E1, E2, E3 and E5), we verified that the $w_{11}{}^{E4}$P[*w*] recombinant did in fact carry the z^1 allele rather than the z^+ allele. This was done by testing for the interaction between the putative zw^{11E4}P[*w*] recombinant chromosome and a heterozygous combination of dpp^{19}/dpp^4 (Gelbart and Wu, 1982). Males with the putative z^1w^{11E4}P[*w*] recombinant chromosome were mated to *FMA3/Y;CyO/Sco* dpp^4 females. The z^1w^{11E4}P[*w*];*Sco* dpp^4/+ male offspring were mated to z^1/z^1;*CyO*/dpp^{19} females. The wings of the z^1w^{11E4}P[*w*]/z^1;*Sco* dpp^4/dpp^{19} females were examined in comparison with a positive and negative control. z^+/z^1;*Sco* dpp^4/dpp^{19} females have wings of almost normal size whereas z^1/z^1;*Sco* dpp^4/dpp^{19} females have very small, poorly formed wings. The *dpp* tester chromosomes were obtained from C.-T.Wu.

For comparison of the eye colors of a transduced *white* gene in a z^1 and z^+ background, cultures of the z^+w^{1118};P[*w*]/P[*w*] and the z^1w^{11E4};P[*w*]/P[*w*] stock were grown in parallel. Flies from the two cultures were collected within 1 day of eclosion, aged at 25°C for 5 days, and their eye colors were compared side by side under the dissecting microscope. To test the interaction with z^{op6}, z^+w^{1118}/Y;P[*w*]/P[*w*] males were mated separately to *sc* z^{op6} w^1 *sn* and to z^+w^1 females. The male offspring were collected, aged and compared as above. For

X-linked P[*w*] genes, $z^1 w^{11E4}$P[*w*]/Y males were mated to the *sc* z^{op6} w^1 *sn* and z^+ w^1 females and the eye colors of the heterozygous female progeny were compared. The *sc* z^{op6} w^1 *sn* stock was constructed by E.Lifschytz and sent to us by W.Gelbart. We confirmed by DNA blot analysis that the *white* allele carried by this stock is w^1.

Pigmentation of the eyes and other tissues

The descriptions of eye colors given in the text refer to flies grown at 25°C, collected within 1 day of eclosion, and aged 5 days at 25°C. The eye colors were examined and photographed with a Nikon SMZ-10 binocular dissecting microscope equipped with a Nikon FX-35A camera and Nikon UFX exposure control using Kodachrome Type 40A film. They were illuminated with an MKII fiberoptics ring light source (Ehrenreich Optical Industries, Garden City, NY).

For quantitative measurements of eye pigments, five males of the autosomal w^{1118}/Y;P[*w*] stock were mated to five virgin w^{1118} females. For the E1 X-linked transformant, *sc* w^{11E4} *cv*/Y males were mated to *sc* w^{11E4}*cv*P[(*w,ry*)E]1/*sc* w^{11E4}*cv*P[(*w,ry*)E]1 females. For the E2 X-linked transformant, *w*1118/Y males were mated to w^{1118}P[(*w,ry*)E]2/w^{1118}P[(*w,ry*)E]2 females. The flies were grown, harvested, aged, decapitated, and the pigments extracted from their heads as described by Hazelrigg *et al.* (1984). The optical density spectrum from wavelength 350 – 550 nm was determined using a Carey model 219 spectrophotometer. The optical density at 450 nm was used for comparisons between transformants.

To assay for pigmentation in other tissues, five pairs of each transformed line were grown in 6-ounce plastic bottles at 25°C and the parents were discarded after 3 days. The Malpighian tubules of third instar larvae were dissected in Ringer's solution, and observed and photographed through a Zeiss Neoflure 10X objective with a total magnification of 125×, phase 2 condenser setting, using a Zeiss Universal Photomicroscope. The Malpighian tubules and testes of adult males were dissected from 7 – 10 day old males and observed in the same manner. Ocelli were examined with a dissecting micrsocope in 7 – 10 day old males. The following transformants were observed as homozygotes, or hemizygotes (if X-linked): D1, D2, D3, E1, E2, E3, E5, E7, F2, F4-1, F4-2, F4-3, H1, H3 and H4. The following transformants were observed heterozygous with the *CyO,Cy cn bw* chromosome 2 balancer: E4, E6, E8 and F1. The following transformants were observed as heterozygotes with the *TM3,*p^p bx^{34e} e^s *Sb* chromosome 3 balancer: F3, H2-1 and H2-2. Transformants with autosomal insertions of segment G were observed in w^{1118}/Y;P[(*w,ry*)G]/+ males.

DNA blot analysis

DNA was extracted from adult flies as described by Hazelrigg *et al.* (1984). For the G transformants DNA was extracted from w^+P[(*w,ry*)G] stocks. To verify that the *white* DNA segment in the G transformants was intact, the genomic DNA was digested with *Hin*dIII+*Eco*RI, electrophoresed on a 0.5% agarose gel, blotted to nitrocellulose and hybridized with a ^{32}P-labeled DNA probe prepared from a plasmid, pmXR3′, containing the cloned *white* DNA fragment coordinates −5.1 to +0.44 (the *Eco*RI-*Xba*I fragment of λm2.1). This probe hybridized in each sample to the 8.3-kb *Eco*RI(−5.1)-*Hin*dIII(+3.18) band from the transduced segment and the 9.2-kb *Eco*RI(−6.0)-*Hin*dIII(+3.18) band from the w^+ gene.

For all other transformants DNA was extracted from $z^1 w^{11E4} f^5$P[*w,ry*] lines. The w^{11E4} deletion removes all of the sequences of the host *white* gene complementary to any of the probes used (Zachar and Bingham, 1982). The integrity of the *white* DNA segment was checked by means of two separate blots assaying the 5′ and 3′ portions of the segment. To assay the 5′ portion, the DNA was digested with *Xba*I+*Hin*dIII and probed with the plasmid pmXR5′ containing the *white* segment coordinates +0.44 to +6.62 (the *Xba*I-*Eco*RI fragment of λm2.1). This probe hybridized in each sample to the 2.7-kb *Xba*I(+0.44)-*Hin*dIII(+3.18) fragment and to a fragment extending from the *Hin*dIII(+3.18) site to the *Hin*dIII site in the polylinker at the junction of the *white* and *rosy* segments (see Figure 3). To assay the 3′ portion of the *white* segments, the genomic DNAs were digested with *Xba*I+*Eco*RI and probed with the plasmid pmXR3′. This hybridized to a fragment extending from the *Xba*I (+0.44) site to the *Eco*RI site of the polylinker joining the *white* segment to the P element vector.

A DNA blot was also used to determine the number of junction fragments between the left end of the transposon (as drawn in Figure 3) and flanking DNA as a measure of the number of copies of the transposon. Genomic DNA of the H transformants was digested with *Xba*I+*Bam*HI and probed with a plasmid subclone containing *white* DNA coordinates −1.52 to −0.66. This probe hybridized to the junction fragment from the *Xba*I site at +0.44 to the next *Bam*HI or *Xba*I site in flanking DNA. All other transformant DNAs were digested with *Sal*I+*Xba*I and probed with a plasmid subclone containing the *white* DNA segment between coordinates −5.9 and −3.03. This hybridized to the junction fragment from the *Sal*I site at −3.03 to the next *Sal*I or *Xba*I site in the flanking DNA. In the case of the G transformants this also hybridized to the 20-kb fragment of the host *white* gene.

In situ hybridization to polytene chromosomes

Males of each homozygous or balanced lethal transformant line were mated to w^{1118} females. Polytene salivary gland chromsome squashes prepared from the larvae were hybridized with [^{3}H]cRNA (Hazelrigg *et al.*, 1984) or a biotinylated DNA probe (Zuker *et al.*, 1985) prepared from the DNA of λm2.1 (Levis *et al.*, 1982) which carries a genomic *white* DNA insert.

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