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Human Matrix Attachment Regions Insulate Transgene Expression from Chromosomal Position Effects in *Drosophila melanogaster*

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Germ line transformation of *white*$^-$ *Drosophila* embryos with *P*-element vectors containing *white* expression cassettes results in flies with different eye color phenotypes due to position effects at the sites of transgene insertion. These position effects can be cured by specific DNA elements, such as the *Drosophila scs* and *scs'* elements, that have insulator activity in vivo. We have used this system to determine whether human matrix attachment regions (MARs) can function as insulator elements in vivo. Two different human MARs, from the apolipoprotein B and α1-antitrypsin loci, insulated *white* transgene expression from position effects in *Drosophila melanogaster*. Both elements reduced variability in transgene expression without enhancing levels of *white* gene expression. In contrast, expression of *white* transgenes containing human DNA segments without matrix-binding activity was highly variable in *Drosophila* transformants. These data indicate that human MARs can function as insulator elements in vivo.

Matrix attachment regions (MARs) are DNA elements that are identified and defined by their ability to bind to DNA- and histone-depleted nuclei, which are generally termed nuclear matrices (9, 33). MARs are typically AT-rich elements that contain consensus cleavage sites for topoisomerase II, and they may contain one or more loosely defined short sequence motifs, but, in general, their structures are not highly homologous. MARs are dispersed throughout eukaryotic genomes, having been found in centromeric DNA (47), within genes (9, 10, 20, 22, 40), and in intergenic regions (4, 11, 14, 20, 29, 33, 35). The matrix-binding activities of MARs have been conserved throughout eukaryotic evolution (9, 19). The functions of MARs in vivo are largely unknown, but one commonly held view is that MARs anchor individual chromatin loops to a proteinaceous matrix or scaffold in both interphase nuclei (14, 33, 47) and mitotic chromosomes (46).

An increasing body of evidence suggests that MARs may play a direct role in the regulation of gene expression. For example, the intronic MAR of the immunoglobulin κ gene is adjacent to a tissue-specific enhancer, and both elements are required for the proper regulation of the immunoglobulin κ gene during development (24, 30). Deletion of either the MAR or the enhancer resulted in constitutive hypermethylation of the gene in all cell types and in permanent repression of the locus (24). Moreover, replacement of the κ intronic MAR with MARs from other locations in the genome or from other species restored the normal pattern of both methylation and gene expression. These results indicate that MARs can be directly involved in the regulation of gene expression, and they also suggest that MAR function may be neither tissue nor species specific.

Another putative function of MAR elements, particularly those that flank individual genes or gene clusters, is to act as insulator elements. This is an attractive hypothesis because it equates the structural boundaries of a chromatin loop, the flanking MAR elements, with the functional boundaries of the domain, the putative chromosomal insulator elements. According to this hypothesis, MAR elements, or other elements at chromatin domain boundaries, may act as insulators, shielding genes within the domain from the regulatory elements of adjacent domains. However, this hypothesis has been difficult to test experimentally. Studies designed to test the ability of MARs to insulate transgenes from position effects have been reported in both plant (1, 6, 43, 51, 52) and animal systems (28, 31, 32, 34, 38, 45, 48, 49). While many of these studies have shown that transgenes flanked by MARs are more highly expressed than similar transgenes without MAR elements, conflicting views have been expressed as to whether MAR elements can render transgene expression position-independent. For example, different groups have reported that transgene expression from concatemeric arrays was silenced (1, 21), expressed in a copy number-dependent fashion (5, 15, 31, 36, 37, 45), or neither (38). These conflicting views are due, at least in part, to the inherent limitations of these transfection assays, because the numbers and arrangements of transgene sequences within the typically multimeric arrays are difficult to determine and could differ in a number of ways. First, the number and arrangement of transgenes within a single concatemeric array could affect transgene silencing versus activation. Second, some of the transfectant clones that have been studied contained multiple transgene insertions, with different transgene arrays integrated at different chromosomal sites. Patterns of gene expression among such genotypically complex transfectants might be difficult to discern. Finally, rearrangement of transgene sequences was a common event in some experiments, although transgene expression could still be detected (1). Therefore, meaningful genotype-phenotype correlations in such transfectant clones would be difficult to establish.

One reasonable, if inefficient, means to circumvent the limitations inherent in analyses of transfectants containing multiple transgene insertions would be to study only those transfectant clones that contain single, intact transgenes integrated in the recipient cell genome. We used this approach previously to study the functions of MAR elements from the human apoli-

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poprotein B-100 (apoB) locus (21). The human apoB gene is thought to be the sole resident of a 48-kb DNase I-sensitive domain that is flanked by MAR elements (29). When single-copy transfectants containing *lacZ* reporters with or without flanking apoB MARs were analyzed, a significant increase in transgene expression and a reduction in variability of expression among apoB MAR-containing clones were observed (21). This was consistent with the suggestion that the apoB MARs were insulating transgene expression from chromosomal position effects, although the possibility that the MARs resulted in integration-dependent enhancement could not be excluded by these data.

To more critically test the possibility that the apoB MARs were functional boundaries of the apoB domain, we studied their insulating activities in a position effect assay in *Drosophila melanogaster*, as first described by Kellum and Schedl (23). In this assay, germ line transformation of *white*$^-$ (*w*$^-$) *Drosophila* embryos with *P*-element vectors containing *white* transgenes results in transgenic flies with different eye color phenotypes, as each *white* transgene is expressed at levels dictated by regulatory elements at the site of insertion (17, 27). In contrast, *P* elements containing *white* transgenes that are flanked by insulating elements, such as the specialized chromatin structures (*scs* and *scs′*) from the *hsp70* locus of *Drosophila* (50), are expressed in a position-independent manner, and all of the transgenic lines display similar eye color phenotypes (23). A vertebrate regulatory element, hypersensitive site 4 (HS4) of the chicken β-globin locus control region (LCR), also functions as an insulator in this assay (8). In this study, we used the *Drosophila* assay to assess the insulating properties of human MAR elements. MARs from the human apoB and α1-antitrypsin (α1AT) loci displayed insulating activities much like those of *scs* itself. In contrast, human DNA segments without matrix-binding activity had no insulating activity in this assay. These results indicate that at least some human MAR elements can function as chromosomal insulators in vivo.

MATERIALS AND METHODS

Construction of transformation vectors. The *P*-element transformation vector RW$^+$, herein designated EmwS′, was kindly provided by Paul Schedl; the construction of this plasmid has been described (53). apoB3′MEmwS′ was generated by ligating a 786-bp *Xho*I fragment containing the apoB 3′ MAR (29) into the unique *Xho*I site upstream of *white* in EmwS′. The orientation of the apoB 3′ MAR fragment in the various subclones was determined by restriction analysis. The *P*-element vector with *scs* upstream of *white* (SEmwS′) was made by directional ligation of a gel-purified 1.7-kb *Kpn*I/*Sal*I fragment containing *scs* (23, 50) into the unique *Kpn*I/*Xho*I site upstream of *white* in EmwS′. SmwS′ was generated by digesting EmwS′ with *Kpn*I and *Spe*I, which removed the *white* upstream regulatory region, and inserting a gel-purified 1.7-kb *Kpn*I/*Spe*I fragment containing *scs* into the linearized plasmid at this site. This resulted in the replacement of the *white* upstream regulatory region with *scs*. All of the other constructs in which the *white* upstream regulatory region was deleted were derivatives of ΔXS, herein designated mwS′, which was provided by Paul Schedl. mwS′ was prepared from EmwS′ by removing an *Xho*I/*Spe*I fragment from the *white* upstream regulatory region. apoB3′MmwS′, ATRMmwS′, apoBmwS′, and apoB5′MmwS′ were generated by blunt-end ligation of the appropriate restriction fragments into the unique *Xba*I site upstream of *white* in mwS′. The inserts used in these constructions were a 786-bp *Xho*I fragment containing the apoB 3′ MAR (29) for apoB3′MmwS′; a 4.1-kb *Xho*I/*Sal*I fragment containing the ATR MAR (39a) for ATRMmwS′; an 800-bp *Eco*RI/*Nco*I fragment containing part of intron 5, exon 6, and part of intron 6 of the apoB gene (3) for apoBmwS′; and a 1.0-kb *Xba*I/*Xho*I fragment containing the apoB 5′ MAR (29) for apoB5′MmwS′. The orientations of the inserted DNA fragments were determined by restriction analysis. The constructs without the *scs′* element, apoB3′Mmw and apoB3′MEmw, were prepared by deleting an ~400-bp *Pvu*II/*Sal*I fragment from mwS′.

Transformation and line establishment. Samples (500 μg/ml) of each *P*-element transformation vector were coinjected with 150 μg of helper plasmid pπ25.7wcΔ2-3 per ml into *w*^{1118} embryos as described by Spradling and Rubin (44). Survivors were crossed to each other in groups of five (three females and two males), and transformants were identified by eye pigmentation. Mixed populations of transgenic flies were separated on the basis of eye color. Chromosome assignments of the various transgene insertions were made by crossing the transformants with *w*$^-$ balancer stocks containing dominant markers: *In(2LR)O,Cy* for the second chromosome, *In(3LR)TM3,Sb* for the third chromosome, and *In(1)FM6,B* for the X chromosome. All lines were maintained as balanced stocks. Transgene copy number was determined by Southern hybridization. Genomic DNA was isolated from 20 flies of each transformed line, digested with a restriction endonuclease that cuts once within the transgene (*Xba*I for *white* enhancer-containing constructs and for mwS′; *Spe*I for all other constructs), separated on 1% agarose gels by field inversion gel electrophoresis, and transferred to nylon membranes in 0.2 M NaOH–0.6 M NaCl denaturing solution. The filters were hybridized with various radiolabeled DNA probes: a 786-bp *Xho*I fragment containing the apoB 3′ MAR for apoB3′MEmwS′ and apoB3′MmwS′, a 4.1-kb *Xho*I/*Sal*I fragment containing the ATR MAR for ATRMmwS′, a 1.0-kb *Xba*I/*Xho*I fragment containing the apoB 5′ MAR for apoB5′MmwS′, and a 400-bp *Eco*RI/*Bam*HI fragment containing *scs′* for EmwS′, mwS′, SEmwS′, and SmwS′.

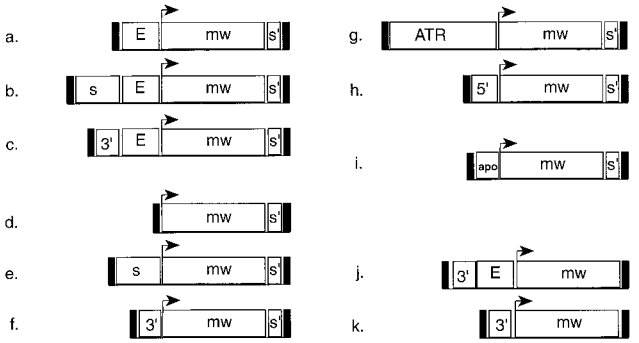

FIG. 1. *white P*-element transformation vectors. Symbols: E, the upstream regulatory region of *white* that contains the eye- and testes-specific enhancers; mw, *mini-white* cDNA expression cassette which includes the proximal promoter, with the *white* transcription start site depicted by the arrow; S′, *scs′*; S, *scs*; 3′, apoB 3′ MAR; 5′, apoB 5′ MAR; ATR, ATR MAR; apo, apoB transcribed sequence; black rectangles at the ends of each transposon, 5′ and 3′ *P* elements. Vectors: a, EmwS′; b, SEmwS′; c, apoB3′MEmwS′; d, mwS′; e, SmwS′; f, apoB3′mwS′; g, ATRMmwS′; h, apoB5′MmwS′; i, apoBmwS′; j, apoB3′MEmw; and k, apoB3′Mmw.

Eye pigment assay. Five mating pairs of flies for each line were placed at 25°C. After 5 days the adults were removed to prevent overcrowding of larvae, which can result in variations in head and body size among the progeny. Heterozygous virgin females were then collected and aged for 6 days at 25°C. To quantitate eye pigment, flies from each phenotypic class were collected, frozen, and decapitated by vortexing for 10 s. Heads from each eye color category (13 for pale yellow [phenotypic class I], 10 for yellow [II], 8 for orange [III], 5 for dark orange [IV], and 4 for red [V] and dark red [VI]) were pooled, and pigment was extracted by incubating the heads in 30% ethyl alcohol, pH 2, at room temperature for 4 days. Pigment absorption was determined at 450 nm. Each group of heads was assayed in triplicate, and absorption-per-head values were determined.

Photography. The eyes of 4-day-old heterozygous females were photographed using a Zeiss SR microscope fitted with a Nikon FX-35 WA camera. Illumination was supplied by a Nikon MK II fiber optic light source, and photographic images were prepared with Fujichrome tungsten 64T film. Transformed lines carrying *P*-element insertions in the X chromosome were backcrossed to *w*^{1118} stocks to remove the *In(1)FM6,B* balancer chromosome prior to photography. The eye color phenotypes were identical in both genetic backgrounds.

RESULTS

The human apoB 3′ MAR functions as an insulator element in *Drosophila*. The *P*-element transformation vectors used in this study are shown in Fig. 1. In the first set of experiments, three different *P*-element transposons were employed. EmwS′ contained the *white* gene promoter and enhancer (E), a *white* cDNA coding cassette (termed *mini-white* [mw]), and the *Drosophila scs′* (S′) element downstream of the *white* transcription unit (Fig. 1a). In addition, SEmwS′ (Fig. 1b) contained the *Drosophila scs* element (S) upstream of *white*, and apoB3′ MEmwS′ (Fig. 1c) contained the 3′ MAR from the human apoB locus (apoB3′M) inserted upstream of *white.*

Each *P*-element transposon was injected into *w*$^-$ *Drosophila* embryos, and transformed lines were established from flies

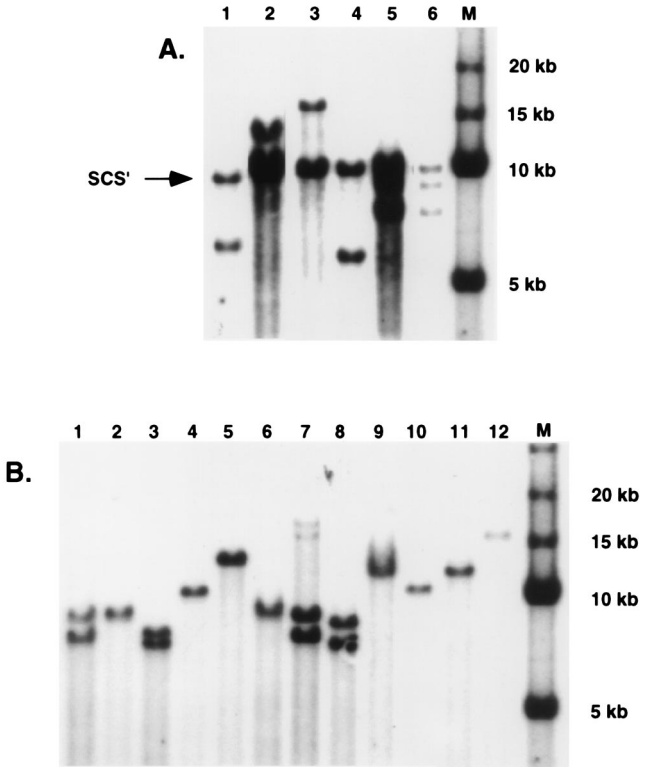

FIG. 2. Copy number determinations for the *P*-element transformants. (A) mwS′ transformants. Genomic DNA was isolated, digested with *Xba*I, separated on an agarose gel, and probed with a labeled, ~400-bp *Eco*RI/*Bam*HI DNA fragment containing *scs′*. The endogenous *scs′* DNA fragment of ~ 10 kb is indicated by the arrow. Each transformant contained, in addition, one (lanes 1 to 4) or two (lanes 5 and 6) additional *scs′* fragments, corresponding to single or double transgene insertions. The marker (M) lane contains DNA fragments of 5, 10, 15, and 20 kb. (B) apoB5′MmwS′ transformants. *Spe*I-digested genomic DNAs were probed with a labeled, ~1.0-kb *Xba*I/*Xho*I DNA fragment containing the apoB 5′ MAR. Single (lanes 2, 4, 5, 6, 9, 10, 11, and 12) and double (lanes 1, 3, 7, and 8) transgene insertions were obtained.

with any detectable eye pigment. Transgene copy number was determined in each transformed line by Southern hybridization (Fig. 2), and the eye color phenotypes of age-matched females heterozygous for the various *white* insertions were compared. Each transformant was assigned to one of six phenotypic classes: I (light yellow), II (yellow), III (light orange), IV (orange), V (red), and VI (dark red), as shown in Fig. 3, 4, and 5. Seven transformed lines were obtained using the control construct EmwS′, and all seven lines contained single transgene insertions (data not shown). As shown in Fig. 3a, three of these lines had light orange eyes (phenotypic class III), three had red eyes (V), and one had dark red eyes (VI). Thus, transgenes without an insulating element upstream of *white* were expressed at different levels in different transformed lines. This observation suggested that the *white* transgenes in these lines were sensitive to position effects at the sites of insertion. These results are in accord with those of Kellum and Schedl (23), who showed that transformants containing *white* transgenes without insulating elements had eye color phenotypes that ranged from orange to dark red. In contrast, all three single-copy lines that contained SEmwS′, in which the *white* transcription unit is flanked by *scs* and *scs′*, had dark red (VI) eyes (Fig. 3b). This suggested that expression of *white* transgenes containing *scs* and *scs′* was largely position independent, as shown previously by Kellum and Schedl (23).

To determine whether the MAR from the 3′ boundary of the human apoB domain could function as an insular element in *Drosophila*, transformants containing apoB3′MEmwS′ (Fig. 1c) were prepared. This *P*-element vector was derived from EmwS′ by inserting a 786-bp *Xho*I fragment containing the apoB 3′ MAR (29) upstream of the *white* transcription unit. Seventeen transformed lines were established, all of which contained single *P*-element insertions (data not shown). Among the 17 single-copy lines, 16 had red (V) or dark red (VI) eyes (Fig. 3c). One transformant had orange (IV) eyes (Fig. 3c). Thus, inserting the apoB 3′ MAR upstream of *white* in a vector that contained *scs′* downstream largely eliminated the phenotypic variation in *white* gene expression that was observed with a similar vector containing *scs′* alone. Furthermore, the predominant eye color phenotype of the apoB3′MEmwS′ transformants, dark red (VI), was the same as that observed in SEmwS′ transformants in which transgene expression was shielded from chromosomal position effects by the *Drosophila* insulators *scs* and *scs′*. These observations suggest that the apoB 3′ MAR can function as an insulator element in *Drosophila*. An alternate interpretation of these results would be that the apoB 3′ MAR was functioning as a strong enhancer in these experiments, stimulating but not insulating *white* gene expression. To address this possibility, experiments using enhancer-sensitive vectors were performed.

The human apoB 3′ MAR insulates but does not enhance *white* gene expression. To distinguish between the possibilities that the human apoB 3′ MAR was acting as an insulator versus an enhancer, we prepared *P*-element transformation vectors in which putative enhancement of *white* gene expression by the apoB MAR would be readily apparent. To do this, constructs similar to those employed in the experiments described above but lacking the *white* gene enhancers were prepared. The vectors mwS′, SmwS′, and apoB3′MmwS′ (Fig. 1d, e, and f) were similar to EmwS′, SEmwS′, and apoB3′MEmwS′ (Fig. 1a, b, and c), respectively, but they lacked an ~1.6-kb *Xho*I/*Spe*I DNA fragment upstream of *mini-white* that contains the *white* gene enhancers (53). *white* gene expression from such *P*-element vectors is reduced compared to that from enhancer-containing vectors, so that enhancement of *white* gene expression can be readily distinguished from insulation (23).

Nineteen mwS′ transformants were isolated, and the genotypes of the transformed lines were determined by Southern hybridization. DNA from each transformant was digested with *Xba*I, which cuts once within the *P*-element vector, resolved by electrophoresis, and hybridized with a *Drosophila scs′* probe. Figure 2a shows results for six of the mwS′ transformants. As shown in the figure, each line contained an ~10-kb fragment that corresponded to the endogenous *scs′* element at *hsp70*. In addition, each transformant contained one (Fig. 2a, lanes 1 to 4) or two (lanes 5 to 6) additional *scs′* fragments, indicating single or double transgene insertions. In total, 16 of the 19 mwS′ transformants contained single *P*-element insertions.

As expected, *white* gene expression in the mwS′ lines, as judged by eye pigmentation, was generally less than that of transformants expressing EmwS′, which includes the *white* enhancers (compare Fig. 3a and 4a). Nonetheless, the eye color phenotypes of the mwS′ transformants varied considerably, ranging from light yellow (I) to red (V) (Fig. 4a). The 16 mwS′ transformants were widely distributed in five phenotypic classes (I, 1; II, 5; III, 6; IV, 2; and V, 2). Thus, mwS′ transposons, like EmwS′ vectors, were sensitive to chromosomal position effects when integrated in the *Drosophila* genome. In contrast, all five lines derived from SmwS′-injected embryos fell into a single phenotypic class, light orange (III) (Fig. 4b),

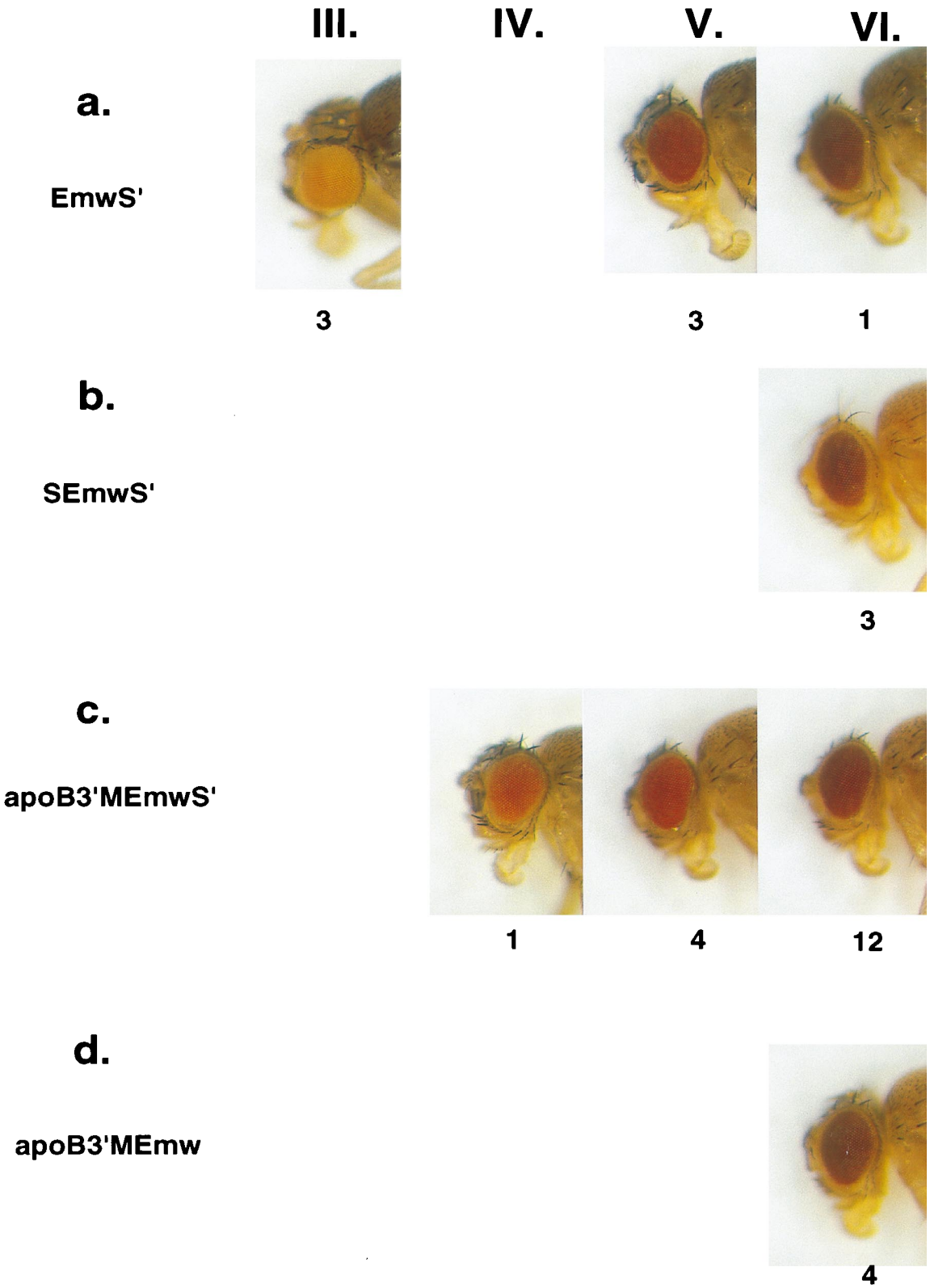

FIG. 3. Eye color phenotypes of *Drosophila* transformants expressing *mini-white* from *white* enhancer-containing vectors. Eyes of 4-day-old females heterozygous for each of the different, single-copy *P*-element insertions were classified as being light orange (phenotypic class III), orange (IV), red (V), or dark red (VI). A representative of each of the phenotypes obtained with the different *white* vectors is shown, and the number of independent transformed lines with that phenotype is indicated below each picture. (a) EmwS′ transformants had eye color phenotypes that varied widely. (b) SEmwS′ transformants had dark red (VI) eyes. (c) apoB3′MEmwS′ transformants had primarily red (V) or dark red (VI) eyes. (d) apoB3′MEmw transformants had red (VI) eyes.

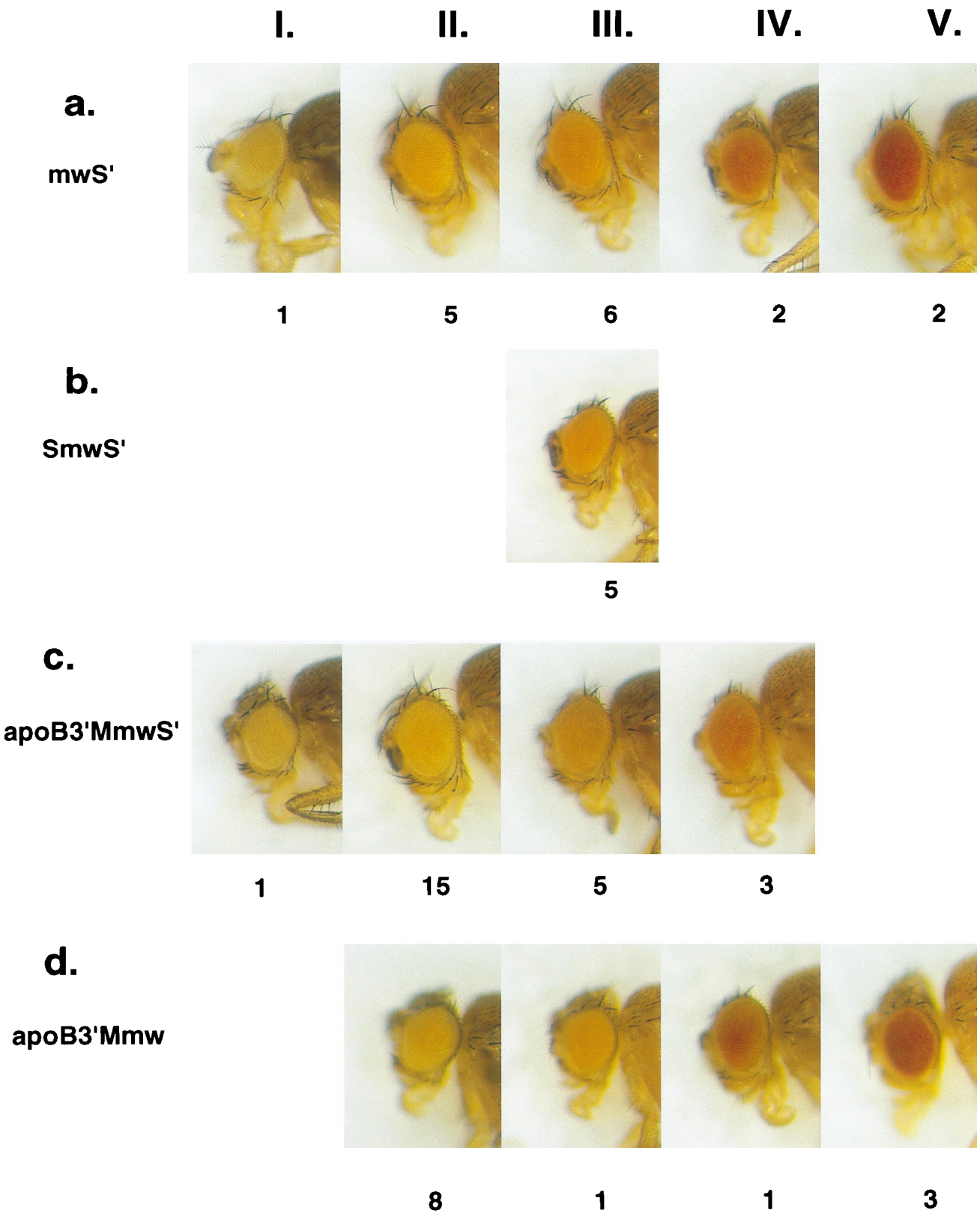

FIG. 4. Eye color phenotypes of *Drosophila* transformants expressing *mini-white* from *white* enhancerless vectors, series 1. Four-day-old females heterozygous for each of the different, single-copy *P*-element insertions were classified as having light yellow (phenotypic class I), yellow (II), light orange (III), orange (IV), or red (V) eyes. A representative of each of the phenotypes obtained with the different *white* vectors is shown, and the number of transformed lines with that phenotype is indicated below each picture. (a) mwS′ transformants were widely distributed in all five phenotypic classes. (b) SmwS′ transformants all had light orange (III) eyes. (c) apoB3′MmwS′ transformants had primarily yellow (II) or light orange (III) eyes. (d) Most apoB3′Mmw transformants had yellow (II) eyes, but flies with light orange (III), orange (IV) and red (V) eyes were also obtained.

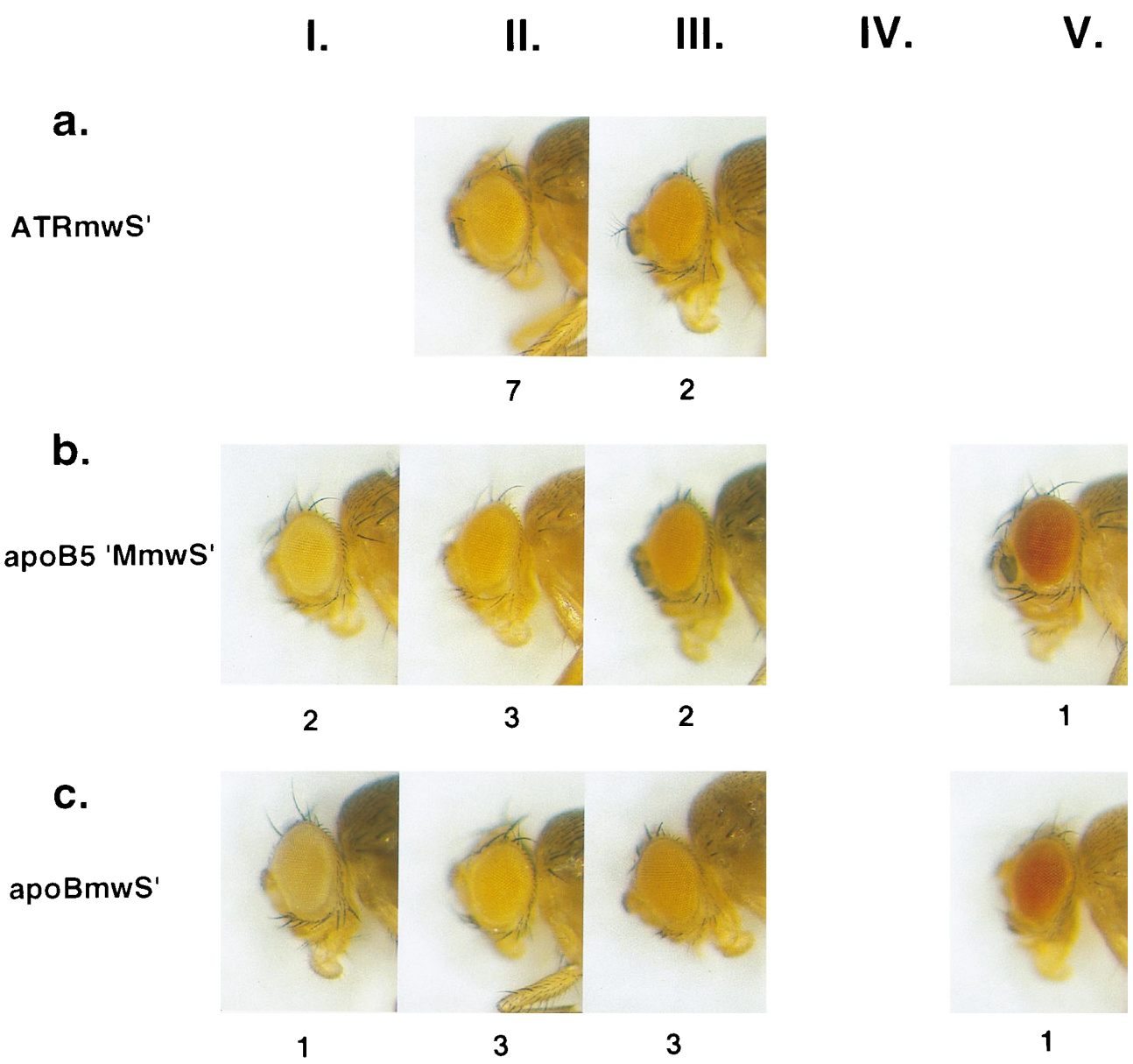

FIG. 5. Eye color phenotypes of *Drosophila* transformants expressing *mini-white* from *white* enhancerless vectors, series 2. Four-day-old females heterozygous for each of the different, single-copy *P*-element insertions were classified as having light yellow (phenotypic class I), yellow (II), light orange (III), orange (IV), or red (V) eyes. A representative of each of the phenotypes obtained with the different *white* vectors is shown, and the number of transformed lines with that phenotype is indicated below each picture. (a) ATRmwS′ transformants had yellow (II) or light orange (III) eyes. (b) apoB5′MmwS′ transformants and (c) apoBmwS′ transformants were widely distributed in the light yellow (I), yellow (II), light orange (III), and red (V) phenotypic classes.

indicating that *white* expression from this *P*-element vector, which contains *scs* and *scs′*, was position-independent.

Twenty-four of 27 lines transformed with apoB3′MmwS′ (Fig. 1f) contained single *P*-element insertions (data not shown). Twenty of these 24 lines had yellow (II) or light orange (III) eyes (Fig. 4c). These results were similar to those obtained with apoB3′MEmwS′, in that >80% of the transformed lines in each experiment fell into two similar phenotypic classes. Furthermore, only 3 of the 24 apoB3′MmwS′ transformants had orange (IV) eyes, and none of them had red (V) eyes, so the apoB 3′ MAR was clearly not acting as an enhancer in this system (Fig. 4a versus 4c). Thus, inserting the apoB 3′ MAR upstream of the *white* transcription unit of mwS′ enriched for yellow-light orange transformants and eliminated red transformants without enhancing *white* gene expression. These data indicate that the apoB 3′ MAR can function as an insulator element in *Drosophila*. However, the insulating activity of the apoB MAR appeared to be less than that of *scs* itself, because variation in *white* gene expression was reduced but not eliminated.

The human ATR MAR functions as an insulator element in *Drosophila*. To determine whether other human MARs can function as insulator elements in *Drosophila*, we utilized a MAR that is located approximately 2 kb downstream of the α1-antitrypsin-related (ATR) sequence on human chromosome 14q32.1. This MAR is one of five matrix-binding elements that we have identified in an ~120-kb segment of 14q32.1 that includes three related serine protease inhibitor

genes, α1AT, ATR, and corticosteroid-binding globulin (CBG) (39; unpublished data). A *P*-element vector in which the ATR MAR was inserted upstream of the *white* transcription unit (ATRMmwS′ [Fig. 1g]) was prepared and used to transform w^- *Drosophila* embryos. Nine of 10 transformed lines contained single-copy ATRMmwS′ insertions (data not shown). Seven of the nine single-copy lines had yellow (II) eyes, and two had light orange (III) eyes (Fig. 5a). This distribution of eye color phenotypes was similar to that observed in apoB3′MmwS′ transformants (Fig. 4c), suggesting that the human ATR MAR has insulating properties similar to those of the apoB 3′ MAR.

A putative apoB 5′ MAR does not function as an insulator element in *Drosophila*. MARs have been mapped both upstream and downstream of the human apoB gene, and these elements have been proposed to define the limits of the apoB chromatin domain (29). In view of our finding that the apoB 3′ MAR acted as an insulator element in *Drosophila*, it might have been expected that the apoB 5′ MAR would have similar properties. To test this possibility, an ~1-kb DNA fragment reported to contain the 5′ apoB MAR was inserted upstream of the *white* transcription unit in the *white* enhancerless *P*-element vector (Fig. 1h). Twelve apoB5′MmwS′ transformants were obtained; eight of these contained single transgene insertions and four contained double transgene insertions (Fig. 2b). The eye color phenotypes of the eight single-copy transformants varied considerably, ranging from light yellow (I) to red (V) (Fig. 5b). This range of phenotypes, without enrichment for any particular phenotypic class, was much like that seen in the mwS′ transformants (Fig. 4a), which do not contain an insulator element upstream of *white*. These results indicate that the putative apoB 5′ MAR does not function as an insulator element in *Drosophila*. This observation prompted us to reassess the matrix-binding activity of this human DNA fragment. Despite repeated attempts, we have been unable to detect matrix-binding activity of the apoB 5′ MAR DNA fragment in any of the standard assays (9, 33). Furthermore, DNA sequencing studies demonstrated that the "apoB 5′ MAR" fragment is not particularly AT-rich, nor does it contain the characteristic features of MAR elements (39a). Therefore, the status of this DNA element as a matrix-associated region is uncertain at present.

A DNA segment from within the apoB gene does not insulate in *Drosophila*. The results described above suggest that at least some human MARs can function as insulator elements in *Drosophila*. In contrast, a DNA fragment without matrix-binding activity failed to insulate *white* gene expression from position effects in *Drosophila*. To explore this difference further, we prepared a transformation vector in which an ~800-bp human DNA fragment from within the apoB gene was inserted upstream of the *white* transcription unit (apoBmwS′ [Fig. 1i]). This human DNA fragment is clearly devoid of matrix-binding activity. Eight of 11 transgenic lines containing apoBmwS′ had single-copy transgene insertions. The eye color phenotypes of these eight transformed lines varied widely, ranging from pale yellow (I) to red (V) (Fig. 5c). These results were similar to those obtained with the other position-sensitive *P* elements, mwS′ (Fig. 3a) and apoB5′MmwS′ (Fig. 5b). Therefore, this DNA fragment, which contains intron and exon sequences from the human apoB gene, does not function as an insulator element in *Drosophila*. Thus, human DNA does not have intrinsic insulating activity in this assay, nor is the insulating phenotype due to a distance effect between the *white* transcription units and *Drosophila* genomic elements upstream of the *P*-element insertions.

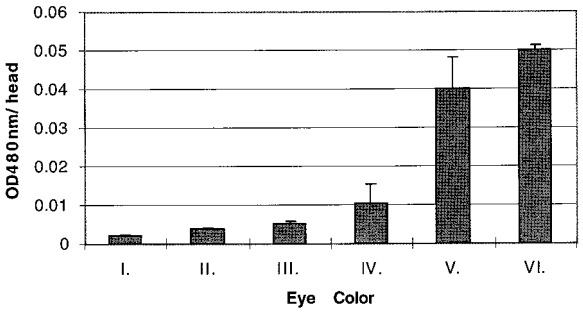

FIG. 6. Eye pigment expression in the different phenotypic classes. Eye pigment was extracted from pools of each phenotypic class and quantitated spectrophotometrically as described in Materials and Methods. The mean optical density at 480 nm per head is indicated. The phenotypic classes are light yellow (I), yellow (II), light orange (III), orange (IV), red (V), and dark red (VI).

***scs′* affects insulator function of the apoB 3′ MAR in *Drosophila*.** It has been reported that the *scs′* fragment used in these and other *mini-white* transformation vectors is not fully functional as an insulator element in *Drosophila* (16, 53). To determine whether *scs′* was required for insulator function of the apoB 3′ MAR to be apparent in our assays, transformation vectors containing the apoB 3′ MAR upstream of the *white* transcription unit, but without *scs′* downstream, were prepared. Two different transformation vectors were prepared, one containing the *white* gene enhancers (apoB3′MEmw [Fig. 1j]) and one without the *white* enhancers (apoB3′Mmw [Fig. 1k]). Four of seven apoB3′M Emw transformants had single *P*-element insertions (data not shown), and all four of these lines had dark red (VI) eyes (Fig. 3d). This was the same phenotypic class as most of the apoB3′MEmwS′ transformants, which contained *scs′* (Fig. 3c). However, the distribution of eye color phenotypes among the apoB3′Mmw transformants was more complex. Eight of 13 single-copy apoB3′Mmw transformants had yellow (II) eyes (Fig. 4d), like most of the *scs′*-containing apoB3′MmwS′ transformants (Fig. 4c). However, three of the apoB3′Mmw transformants had red (V) eyes, a phenotype that was not observed among 24 apoB3′MmwS′ transformants. These observations suggest that *white* transformation vectors containing the apoB 3′ MAR alone are more sensitive to chromosomal position effects than vectors containing both the apoB 3′ MAR and *scs′*.

Eye color phenotypes versus eye pigment expression—quantitative aspects. The eye color phenotypes of our transformants varied in a continuous fashion from light yellow to dark red (Fig. 3, 4, and 5). We divided these phenotypes into six classes, I through VI, which could be described as various shades of yellow, orange, and red. To determine how the different phenotypic classes corresponded to the amount of pigment in the *Drosophila* eyes, eye pigment was quantitated spectrophotometrically in the different phenotypic classes. Transformants from each phenotypic class were obtained, fly heads were pooled in triplicate, pigment was extracted, and absorbance at 480 nm was determined. The mean absorbance per head for each phenotypic class is shown in Fig. 6. The most common phenotypic classes among the enhancerless *white* transformants, light yellow (I), yellow (II), and light orange (III), differed from each other only about twofold, and the amount of pigment per head varied linearly in this range. Thus, small changes in eye pigment expression among the enhancerless transformants yielded readily discriminated eye color phenotypes. The orange (IV), red (V), and dark red (VI) phenotypes corresponded to approximately 2-, 9-, and 11-fold more

pigment than light orange (III), so that eye color readout was a less sensitive indicator of *white* expression in this range. These data demonstrate that eye pigment expression in our transformants varied over an approximately 20-fold range, and small variations within this range could be readily resolved into the different phenotypic classes. Thus, the eye color phenotypes of *white* transformants are very sensitive indicators of *white* gene expression, which makes this system particularly useful as a position effect assay. Among the *white* enhancerless transformants, the apoB 3′ MAR and the ATR MAR both enriched for yellow (II) to light orange (III) transformants (Fig. 4c and 5a). These phenotypes differed only about 1.2-fold in eye pigment expression. These observations provide further support for the conclusion that these two human MARs can function as insulator elements in *Drosophila.*

DISCUSSION

MARs are DNA segments that are defined by their abilities to bind to isolated nuclear matrices in vitro (9, 33). These binding properties are consistent with the view that at least some MAR elements might represent structural boundaries of individual chromatin domains, serving to tether the ends of individual chromatin loops to a proteinaceous nuclear matrix in vivo. One functional activity that is often ascribed to DNA elements that are located at or near chromatin domain boundaries is insulation, an activity by which a DNA sequence prevents interactions between neighboring regulatory elements. This suggests that specific DNA segments between neighboring chromatin domains might have both matrix-binding activity and insulator function. Although this model is consistent with currently available data, it has not been critically tested, and alternate interpretations of the data remain viable (26, 54).

The experiments reported here were designed to test whether human MAR elements can function as chromosomal insulators in vivo. To do this, we used the *mini-white* position effect assay of Kellum and Schedl (23) to study insulator function of human MAR elements in *Drosophila*. This assay was used previously to demonstrate the insulating properties of *scs* and *scs′*, which are well-characterized insulator elements from *Drosophila* (23). Using this approach, we have demonstrated that two different human MARs can insulate transgenes from position effects in *Drosophila*. w^- flies transformed with *P* elements in which either the apoB 3′ MAR or the ATR MAR was inserted upstream of a *white* transcription unit had substantially less variability in *white* transgene expression than control transformants without human MARs. Furthermore, variability of transgene expression was reduced without increasing the levels of *white* transgene expression. This contrasts with conclusions drawn from transfection experiments, in which MAR elements have been suggested to increase transgene expression without eliminating variation in expression levels (1, 38). However, the interpretation of transfection studies is complicated by the complex rearrangements of transgene sequences that generally occur in transfected cells. Our experiments clearly show that neither the apoB 3′ MAR nor the ATR MAR enhanced *white* gene expression in *Drosophila*, as most transformants that expressed *white* from enhancerless *P*-element vectors had yellow or orange eyes irrespective of the presence or absence of MAR elements. This conclusion is in accord with previous studies, in which the apoB 3′ MAR did not enhance expression of transiently transfected reporter genes in mammalian cells (21). Thus, the apoB 3′ MAR and the ATR MAR do not contain associated enhancers, unlike some *Drosophila* MARs (14), the chicken lysozyme 5′ MAR (45), and MARs from the human immunoglobulin κ and μ (10, 30) and beta interferon (25) genes.

In contrast to results obtained with the apoB 3′ MAR and the ATR MAR, insulator activity was not observed in human DNA segments that were devoid of matrix-binding activity. For example, an ~800-bp fragment of the apoB gene that included parts of introns 5 and 6 and all of exon 6 had no insulator function in *Drosophila*. Furthermore, an ~1,000-bp DNA fragment from the upstream region of apoB also failed to function as an insulator. This result was surprising in view of the fact that this DNA fragment had been reported to have matrix-binding activity; indeed, this element is thought to be the upstream boundary of a 48-kb chromatin domain that contains the apoB gene as its sole resident (29). However, we were unable to detect matrix-binding activity of this DNA fragment in any of the standard assays (9, 33). Moreover, the DNA sequence of this fragment has none of the features that commonly occur in MAR elements, except for a single core binding site for topoisomerase II (42). Thus, we conclude that the putative apoB 5′ MAR has neither matrix-binding nor insulator activities, and we view its identification as the upstream boundary of the apoB domain with skepticism. This suggests that the chromatin domain structure of the human apoB locus may be more complex and extensive than previously envisioned.

Both the apoB 3′ MAR and the ATR MAR insulated *white* gene expression from position effects in *Drosophila*. Transformants expressing control vectors without insulator elements upstream of *white* generally displayed a wide range of eye color phenotypes with no apparent enrichment for any particular phenotypic class. In contrast, most of the transformed lines containing either human MAR upstream of *white* fell into a single phenotypic class, and variation in *white* gene expression was greatly reduced. However, a few lines in each collection displayed slightly different eye color phenotypes. Thus, the human apoB 3′ MAR and the ATR MAR reduced, but did not completely eliminate, variability in transgene expression. This suggests that, in some lines, insulation was not complete. This could be due to any of several factors. First, it has been shown that two *Drosophila* insulators, *scs* and suppressor-of-hairy-wing [*su(HW)*], can block the effects of some, but not all, enhancers and repressors in enhancer-blocking assays (7, 41). These assays are thought to mimic some aspects of insulator function. Thus, some of our *P*-element insertions may have occurred in the vicinity of strong enhancers or repressors, resulting in incomplete insulation. The notion that insulator elements may vary in intrinsic activity is also consistent with the observation that two copies of HS4 of the chicken β-globin locus control region were required for insulator function in *Drosophila* (8). Second, recent reports suggest that *scs′* is a weaker insulator element than either *scs* or *su(HW)* (16, 53). The *scs′* fragment used in those experiments, and in those described here, is a derivative of a larger *scs′* fragment originally employed by Kellum and Schedl (23). This derivative seems to have less insulator activity than the larger *scs′* fragment, which may account for some of the variation in *white* expression in our transformants.

The demonstration that human MARs can function as insulator elements in *Drosophila* suggests that this activity is evolutionarily conserved. It has already been shown that the matrix-binding activities of MARs from different species are highly conserved (9, 19). However, it is not clear at present whether matrix attachment is required for insulator function. We presume that the two activities are distinct because other insulator elements, including *scs*, *scs′*, *su(HW)*, and HS4, are not known to have matrix-binding activity. Conversely, not all

MAR elements establish boundaries between chromatin domains, as some of them map within expressed genes (9, 10, 20, 22, 40), so it seems unlikely that all MARs will function as insulators. Thus, we anticipate that insulator function and matrix-binding activities will prove to be separable activities, although they may colocalize to discrete DNA fragments in some instances. These issues will be interesting to explore.

Finally, the genomic locations of the two human MAR elements that have insulator activity are consistent with the possibility that they might represent the boundaries of individual chromatin domains. The apoB 3′ MAR is just downstream of the apoB transcription unit (29), but the chromatin configuration of sequences further downstream has not yet been explored. The ATR MAR is located ~2 kb downstream of ATR, an antitrypsin-related sequence that may (18) or may not (2) be a pseudogene. The α1AT gene is located ~32 kb upstream of the ATR MAR, and the CBG gene is ~33 kb downstream (39). α1AT and CBG are highly expressed in hepatic cells but differentially expressed in macrophages and intestinal epithelium. The entire region is inactive in most other cell types. This provides an opportunity to determine whether the ATR MAR functions as an insulator between putative α1AT and CBG chromatin domains by constructing and analyzing specifically modified human chromosomes using recombination-proficient cell hybrids (12, 13).

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REFERENCES

1. **Allen, G. C., G. Hall, Jr., S. Michalowski, W. Newman, S. Spiker, A. K. Weissinger, and W. F. Thompson.** 1996. High-level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. Plant Cell **8:**899–913.
2. **Bao, J. J., L. Reed-Fourquet, R. N. Sifers, V. J. Kidd, and S. L. Woo.** 1988. Molecular structure and sequence homology of a gene related to α1-antitrypsin in the human genome. Genomics **2:**165–173.
3. **Blackhart, B. D., E. M. Ludwig, V. R. Pierotti, L. Caiati, M. A. Onasch, S. C. Wallis, L. Powell, R. Pease, T. J. Knott, M. L. Chu, et al.** 1986. Structure of the human apolipoprotein B gene. J. Biol. Chem. **261:**15364–15367.
4. **Bode, J., and K. Maass.** 1988. Chromatin domain surrounding the human interferon-β gene as defined by scaffold-attached regions. Biochemistry **27:**4706–4711.
5. **Bonifer, C., M. Vidal, F. Grosveld, and A. E. Sippel.** 1990. Tissue-specific and position-independent expression of the complete gene domain for chicken lysozyme in transgenic mice. EMBO J. **9:**2843–2848.
6. **Breyne, P., M. van Montagu, N. Depicker, and G. Gheysen.** 1992. Characterization of a plant scaffold attachment region in a DNA fragment that normalizes transgene expression in tobacco. Plant Cell **4:**463–471.
7. **Cai, H., and M. Levine.** 1995. Modulation of enhancer-promoter interactions by insulators in the *Drosophila* embryo. Nature **376:**533–536.
8. **Chung, J. H., M. Whiteley, and G. Felsenfeld.** 1993. A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. Cell **74:**505–514.
9. **Cockerill, P. N., and W. T. Garrard.** 1986. Chromosomal loop anchorage of the κ immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. Cell **44:**273–282.
10. **Cockerill, P. N., M. H. Yuen, and W. T. Garrard.** 1987. The enhancer of the immunoglobulin heavy chain locus is flanked by presumptive chromosomal loop anchorage elements. J. Biol. Chem. **262:**5394–5397.
11. **Conkling, M. A., C.-L. Cheng, Y. T. Yamamoto, and H. M. Goodman.** 1990. Isolation of transcriptionally regulated root-specific genes from tobacco. Plant Physiol. **93:**1203–1211.
12. **Dieken, E. S., E. M. Epner, S. Fiering, R. E. K. Fournier, and M. Groudine.** 1996. Efficient modification of human chromosomal alleles using recombination-proficient chicken/human microcell hybrids. Nat. Genet. **12:**174–182.
13. **Dieken, E. S., and R. E. K. Fournier.** 1996. Homologous modification of human chromosomal genes in chicken B-cell x human microcell hybrids. Methods **9:**56–63.
14. **Gasser, S. M., and U. K. Laemmli.** 1986. Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster*. Cell **46:**521–530.
15. **Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51:**975–985.
16. **Hagstrom, K., M. Muller, and P. Schedl.** 1996. *Fab-7* functions as a chromatin domain boundary to ensure proper segment specification by the *Drosophila bithorax* complex. Genes Dev. **10:**3202–3215.
17. **Hazelrigg, T., R. Levis, and G. M. Rubin.** 1984. Transformation of white locus DNA in *Drosophila*: dosage compensation, *zeste* interaction, and position effects. Cell **36:**469–481.
18. **Hofker, M. H., M. Nelen, E. C. Klasen, T. Nukiwa, D. Curiel, R. G. Crystal, and R. R. Frants.** 1988. Cloning and characterization of an α1-antitrypsin-like gene 12 kb downstream of the genuine α1-antitrypsin gene. Biochem. Biophys. Res. Commun. **155:**634–642.
19. **Izaurralde, E., J. Mirkovitch, and U. K. Laemmli.** 1988. Interaction of DNA with nuclear scaffolds *in vitro*. J. Mol. Biol. **200:**111–125.
20. **Jarman, A. P., and D. R. Higgs.** 1988. Nuclear scaffold attachment sites in the human globin gene complexes. EMBO J. **7:**3337–3344.
21. **Kalos, M., and R. E. K. Fournier.** 1995. Position-independent transgene expression mediated by boundary elements from the apolipoprotein B chromatin domain. Mol. Cell. Biol. **15:**198–207.
22. **Kas, E., and L. A. Chasin.** 1987. Anchorage of the Chinese hamster dihydrofolate reductase gene to the nuclear scaffold occurs in an intragenic region. J. Mol. Biol. **198:**677–692.
23. **Kellum, R., and P. Schedl.** 1991. A position-effect assay for boundaries of higher order chromosomal domains. Cell **64:**941–950.
24. **Kirillov, A., B. Kistler, R. Mostoslavsky, H. Cedar, T. Wirth, and Y. Bergman.** 1996. A role for nuclear NF-κB in B-cell-specific demethylation of the Igκ locus. Nat. Genet. **13:**435–441.
25. **Klehr, D., K. Maass, and J. Bode.** 1991. Scaffold-attached regions from the human interferon β domain can be used to enhance the stable expression of genes under the control of various promoters. Biochemistry **30:**1264–1270.
26. **Laemmli, U. K., E. Kas, L. Poljak, and Y. Adachi.** 1992. Scaffold-associated regions: *cis*-acting determinants of chromatin structural loops and functional domains. Curr. Opin. Genet. Dev. **2:**275–285.
27. **Levis, R., T. Hazelrigg, and G. M. Rubin.** 1985. Effects of genomic position on the expression of transduced copies of the *white* gene of *Drosophila*. Science **229:**558–561.
28. **Levy-Wilson, B.** 1995. Transcriptional control of the human apolipoprotein B gene in cell culture and in transgenic animals. Prog. Nucleic Acids Res. Mol. Biol. **50:**161–190.
29. **Levy-Wilson, B., and C. Fortier.** 1989. The limits of the DNase I-sensitive domain of the human apolipoprotein B gene coincide with the locations of chromosomal anchorage loops and define the 5′ and 3′ boundaries of the gene. J. Biol. Chem. **264:**21196–21204.
30. **Lichtenstein, M., G. Keini, H. Cedar, and Y. Bergman.** 1994. B cell-specific demethylation: a novel role for the intronic κ chain enhancer sequence. Cell **76:**913–923.
31. **McKnight, R. A., A. Shamay, L. Sankaran, R. J. Wall, and L. Hennighausen.** 1992. Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. Proc. Natl. Acad. Sci. USA **89:**6943–6947.
32. **McKnight, R. A., M. Spencer, R. J. Wall, and L. Hennighausen.** 1996. Severe position effects imposed on a 1 kb mouse whey acidic protein gene promoter are overcome by heterologous matrix attachment regions. Mol. Reprod. Dev. **44:**179–184.
33. **Mirkovitch, J., M. E. Mirault, and U. K. Laemmli.** 1984. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. Cell **39:**223–232.
34. **Palmiter, R. D., E. P. Sandgren, D. M. Koeller, and R. L. Brinster.** 1993. Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice. Mol. Cell. Biol. **13:**5266–5275.
35. **Phi-Van, L., and W. H. Stratling.** 1988. The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. EMBO J. **7:**655–664.
36. **Phi-Van, L., and W. H. Stratling.** 1996. Dissection of the ability of the chicken lysozyme gene 5′ matrix attachment region to stimulate transgene expression and to dampen position effects. Biochemistry **35:**10735–10742.
37. **Phi-Van, L., J. P. von Kries, W. Ostertag, and W. H. Stratling.** 1990. The chicken lysozyme 5′ matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. Mol. Cell. Biol. **10:**2302–2307.
38. **Poljak, L., C. Seum, T. Mattioni, and U. K. Laemmli.** 1994. SARs stimulate but do not confer position independent gene expression. Nucleic Acids Res. **22:**4386–4394.
39. **Rollini, P., and R. E. K. Fournier.** 1997. Molecular linkage of the human

α1-antitrypsin and corticosteroid-binding globulin genes on chromosome 14q32.1. Mamm. Genome **8:**913–916.

39a. **Rollini, P., and R. E. K. Fournier.** Unpublished results.

40. **Romig, H., J. Ruff, F. O. Fackelmayer, M. S. Patil, and A. Richter.** 1994. Characterisation of two intronic nuclear-matrix-attachment regions in the human DNA topoisomerase I gene. Eur. J. Biochem. **221:**411–419.
41. **Roseman, R. R., V. Pirrotta, and P. K. Geyer.** 1993. The *su(Hw)* protein insulates expression of the *Drosophila melanogaster white* gene from chromosomal position-effects. EMBO J. **12:**435–442.
42. **Sander, M., and T. S. Hsieh.** 1985. *Drosophila* topoisomerase II double-strand DNA cleavage: analysis of DNA sequence homology at the cleavage site. Nucleic Acids Res. **13:**1057–1072.
43. **Schoffl, F., G. Schroder, M. Kliem, and M. Rieping.** 1993. An SAR sequence containing 395 bp DNA fragment mediates enhanced, gene-dosage-correlated expression of a chimaeric heat shock gene in transgenic tobacco plants. Transgenic Res. **2:**93–100.
44. **Spradling, A. C., and G. M. Rubin.** 1982. Transposition of cloned *P*-elements into *Drosophila* germ line chromosomes. Science **218:**341–347.
45. **Stief, A., D. M. Winter, W. H. Stratling, and A. E. Sippel.** 1989. A nuclear DNA attachment element mediates elevated and position-independent gene activity. Nature **341:**343–345.
46. **Strick, R., and U. K. Laemmli.** 1995. SARs are *cis* DNA elements of chromosome dynamics: synthesis of a SAR repressor protein. Cell **83:**1137–1148.
47. **Strissel, P. L., R. Espinosa III, J. D. Rowley, and H. Swift.** 1996. Scaffold attachment regions in centromere-associated DNA. Chromosoma **105:**122–133.
48. **Talbot, D., P. Descombes, and U. Schibler.** 1994. The 5′ flanking region of the rat LAP (C/EBP β) gene can direct high-level, position-independent, copy number-dependent expression in multiple tissues in transgenic mice. Nucleic Acids Res. **22:**756–766.
49. **Thompson, E. M., E. Christians, M. G. Stinnakre, and J. P. Renard.** 1994. Scaffold attachment regions stimulate HSP70.1 expression in mouse preimplantation embryos but not in differentiated tissues. Mol. Cell. Biol. **14:**4694–4703.
50. **Udvardy, A., E. Maine, and P. Schedl.** 1985. The 87A7 chromomere. Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. J. Mol. Biol. **185:**341–358.
51. **van der Geest, A. H., A. H. M. Hall, S. Spiker, and T. C. Hall.** 1994. The β-phaseolin gene is flanked by matrix attachment regions. Plant J. **6:**413–423.
52. **van der Geest, A. H., and T. C. Hall.** 1997. The β-phaseolin 5′ matrix attachment region acts as an enhancer facilitator. Plant Mol. Biol. **33:**553–557.
53. **Vazquez, J., and P. Schedl.** 1994. Sequences required for enhancer blocking activity of *scs* are located within two nuclease-hypersensitive regions. EMBO J. **13:**5984–5993.
54. **Zhou, J., S. Barolo, P. Szymanski, and M. Levine.** 1996. The *Fab-7* element of the bithorax complex attenuates enhancer-promoter interactions in the *Drosophila* embryo. Genes Dev. **10:**3195–3201.