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Red flag on the white reporter: a versatile insulator abuts the *white* gene in *Drosophila* and is omnipresent in *mini-white* constructs

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ABSTRACT

Much of the research on insulators in *Drosophila* has been done with transgenic constructs using the *white* gene (*mini-white*) as reporter. Hereby we report that the sequence between the *white* and CG32795 genes in *Drosophila melanogaster* contains an insulator of a novel kind. Its functional core is within a 368 bp segment almost contiguous to the *white* 3′UTR, hence we name it as Wari (*white*-abutting resident insulator). Though Wari contains no binding sites for known insulator proteins and does not require Su(Hw) or Mod(mdg4) for its activity, it can equally well interact with another copy of Wari and with unrelated Su(Hw)-dependent insulators, gypsy or 1A2. In its natural downstream position, Wari reinforces enhancer blocking by any of the three insulators placed between the enhancer and the promoter; again, Wari–Wari, Wari–gypsy or 1A2–Wari pairing results in mutual neutralization (insulator bypass) when they precede the promoter. The distressing issue is that this element hides in all *mini-white* constructs employed worldwide to study various insulators and other regulatory elements as well as long-range genomic interactions, and its versatile effects could have seriously influenced the results and conclusions of many works.

INTRODUCTION

The two definitive properties of insulator elements are (i) the ability to block stimulation of a downstream gene promoter by an upstream enhancer (supposed to restrict 'cross-talk' in complex genetic loci) and (ii) the ability to put up a barrier between active and suppressive chromatin (1–7). We more or less understand now what molecular mechanisms may be involved in the chromatin barrier function (1,3,4,7). In contrast, no one really knows how a single insulator can block the enhancer–promoter communication. Perhaps for this reason the widespread models simply shun this question and regard insulators just as 'clothes pegs' that tether the chromatin fibre to the nuclear matrix/scaffold/envelope, or as 'snap halves' that bind with each other to close a chromatin loop; in either case, this is supposed to result in partitioning of the genome into 'independent transcription units'. Only the enhancer-blocking function is considered in this work.

Most of the progress in this challenging field of research has been achieved in the transgenic approach, examining the effects of insulator(s) in a construct comprising enhancer(s) and reporter gene(s) that is inserted into the genome. With the standing problem of genomic position effects, it is still more important to manipulate a well-defined 'autonomous unit' with predictable/controllable interactions at least within the construct. Again, the general methodological requirement that data obtained in different laboratories must be comparable and reproducible should have been enforced by the availability of standard tools such as expression vectors with convenient reporters.

One such instance is the *white* gene, required for eye pigmentation in *Drosophila* and regulated by its eye-specific enhancer (8). The changes in gene expression are phenotypically obvious (brick red eyes in wild type, paling through shades of red and yellow with decreasing stimulation by the enhancer, down to white eyes when

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the gene is inactive) and easily assessed. The *mini-white* gene, which is an abridged *white* with most of the first intron deleted (9), is one of the most popular reporters in transgenic studies, which include testing the insulator properties of various sequences (10–18).

Here we expose a serious pitfall in the use of these 'standard' constructs: the *mini-white* insert proves to contain a 3′-adjacent insulator of a novel kind, which can pair not only with its twin but also—not less efficiently—with unrelated insulators, reinforcing or nullifying their enhancer-blocking activity depending on the position relative to the gene promoter.

MATERIALS AND METHODS

Drosophila strains, transgenes, germ line transformation and genetic crosses

All flies were maintained at 25°C on the standard yeast medium. The mutant alleles and chromosomes used in this study and the balancer chromosomes are described elsewhere (19). Transgenes were obtained with standard cloning techniques (Supplementary Data).

The construct, together with a *P* element containing defective inverted repeats (P25.7wc) that was used as a transposase source (20), was injected into *y ac* w^{1118} preblastoderm embryos as described (21,22). The resulting flies were crossed with *y ac* w^{1118} flies, and the transgenic progeny were identified by their eye and/or cuticle colour. The chromosome localization of various transgene inserts was determined by crossing the transformants with the *y ac* w^{1118} balancer stock carrying dominant markers: *In*(*2RL*),*CyO* for chromosome 2 and *In*(*3LR*)*TM3*,*Sb* for chromosome 3. The transformed fly lines were tested for transposon integrity and copy number by Southern blot hybridization. Only single-copy transformants were taken into study.

The lines with DNA fragment excisions were obtained by crossing the flies bearing the transposons with the Flp (w^{1118}; *S2CyO*, *hsFLP*, *ISA*/*Sco*; +) or Cre (y^1, w^1; *Cyo*, *P*[*w*+,*cre*]/*Sco*; +) recombinase-expressing lines or with the I-*Sce*I endonuclease-expressing line (*v P*{*v*+; *hsp70*-I-*Sce*I}) (23–25). The Cre recombinase induces 100% excisions in the next generation. High levels of Flp recombinase (almost 90% efficiency) and I-*Sce*I endonuclease (90% efficiency) were produced by heat shock treatment (2 h daily) during the first 3 days after hatching. All excisions were confirmed by PCR analysis; for details, see 'Supplementary Data'.

The $su(Hw)^v/su(Hw)^2$ and $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ mutations were combined with transgenes as previously described (26).

The phenotypic scoring assay

To estimate the levels of *yellow* and *white* expression, we visually determined the degree of pigmentation in the abdominal cuticle and wing blades (*yellow*) and in the eyes (*white*) of 3- to 5-day-old males developing at 25°C, with reference to standard colour scales. In the five-grade scale of *yellow* (Supplementary Figure S1 for the abdominal stripes), grade 5 corresponds to wild type and grade 1 to total loss of *yellow* expression. Identical data were obtained for the wing and body pigmentation in all experiments. In the nine-grade scale of *white* (Supplementary Figure S2), brick red (R) eyes correspond to wild type and white (W) to total loss of *white* expression. Intermediate levels of eye pigmentation are brownish red (BrR), brown (Br), dark orange (dOr), orange (Or), dark yellow (dY), yellow (Y) and pale yellow (pY) in the order of decreasing gene expression.

Two experts separately inspected 30–50 flies from each of two independent crosses for every transgenic line. For all data considered, there was full agreement between crosses and between experts. Each line thus assessed contributed a unit to the corresponding bin of the scoring table. Hence, each numerical entry in the distributions shown in the figures under the scales is the number of fly lines with the specified pigmentation grade (corresponding to the gene expression level decreasing from left to right).

Additionally, the central tendency in the distribution was estimated as the arithmetic mean (for this purpose, the R–W grades of *white* were temporarily converted into numerical grades 9–1). The values thus obtained proved stable against truncation (tested up to 10%), the shift never exceeding 0.1 of the mean. These statistical estimates are shown in Figures 2 and 3 as the positions of shaded 'cursors' on the distribution frames relative to the scale above. In an alternative assessment, the medians of these distributions always were either one or both grades enclosing the means; therefore, they are not shown.

Assessment of changes in gene expression

The effects of insulator elements and their combinations and rearrangements on gene expression are deduced by comparing the phenotypic distributions of fly lines carrying the basic constructs and their derivatives produced by *in vivo* excision of a particular element (such elements were flanked with appropriate sites as shown in the construct schemes and parenthesized in construct names).

The data are presented as 'tabular figures' where the position of the frame enclosing the entire sample on the horizontal colour scale gives a rough idea of the expression range. Note that practically always the range is broader for *white* than for *yellow*, for the obvious reason that it is spread on a more detailed scale (nine standard grades versus five).

The study is made robust against the genomic position effects (including the possible influence of other nearby insulators) by the double assessment protocol whereby all essential conclusions are drawn from comparisons not between single transgenic lines but between groups of independent lines with single copies of the construct inserted at random in different places of the genome. Considering that interplay of insulators can both reinforce and neutralize their enhancer-blocking activity (14,15), the occasional effects of such extraneous elements are likely to be stochastically 'levelled off'

besides being simply 'diluted' in group comparison. The processed data are presented as follows:

(i) In the N/T column on the right of the framed sets of scoring data, the only or the last figure (T) is the total number of fly lines examined for the given construct, and the numerator N is the number of lines from this sample that acquire a new phenotype upon manipulation indicated by the change in the construct name on the left. A ratio $N/T \geq 0.5$ is taken to be a reliable indication of the influence of the tested element on gene expression.
(ii) The distribution frames also carry 'shaded cursors' marking the central tendency determined as in the preceding subsection, so one can see the shifts in the averaged expression caused by removal of each element. Note that the cursors indicate the 'mean colour' on the scale above and should not be directly associated with the numerals they may be superimposed on.

The two kinds of assessment are to some extent complementary: e.g. if excision of an insulator is expected to increase gene expression but some opposite responses also occur within a group of fly lines (broadening of the distribution), the changes of interest will be overestimated by N/T but underestimated by the arithmetic mean, so one can always make a sober judgement.

In as much as insulator elements are thought to influence not the gene transcription as such but the stimulatory action of enhancers, it should be admitted that a more sophisticated analysis might include (apart from the conventional wild type and null extremes) a third reference point on the gene expression scale: a 'ground level' that can be established from the phenotypes of transgenic lines with the corresponding enhancer-less constructs. Thus in our experience the overwhelming majority of such flies exhibit wing and body pigmentation about grade 2 and eye pigmentation not exceeding orange; the same is observed for the enhancer-excised controls in this study [a single dOr 'outlier' (Figure 3B) out of 49 lines for *white* and none out of 37 for *yellow*]. *A propos*, these data confirm again that incidental activation of either transgene by resident genomic enhancers is a very rare event. Estimation of the central tendency from the aggregate data gives exactly grade 2 for *yellow* and exactly the 3rd grade (Y) for *white*.

Notwithstanding, for the purposes of the present study, we decided against any cut-offs or editing, and compared the whole samples; clearly, this most conservative assessment attenuates the relative changes in the enhancer action, i.e. the quantitative effects of insulators and their interactions, but on the other hand, augments the reliability of the qualitative conclusions drawn from such consideration.

Plasmid constructs, transient transfection and luciferase expression assay

See Supplementary Data

RESULTS AND DISCUSSION

An insulator resides immediately downstream of the *white* gene

In our previous experiments concerning the role of insulators (enhancer blockers) in gene expression control, from time to time we encountered some strange or equivocal data; retrospective analysis suggested that the *mini-white* used as a reporter gene might have itself carried insulator-like activity associated with its 3′ end. Indeed, the *mini-white* module in the pCaSpeR series routinely contains almost a 1000 bp of genomic DNA after the coding part (9).

To check this surmise, we isolated the corresponding stretch of pCaSpeR2 DNA totalling 825 bp to include the end of the *white* 3′UTR and the beginning of the *P* element that follows the reporter gene in the plasmid (Figure 1A), flanked it with recombinase sites for *in vivo* excision, and inserted it in a 'standard' *white* expression construct (pCaSpeR3) between the eye enhancer and the *mini-white* (scheme in Figure 1B-1).

Eye pigmentation in such transgenic flies (ranging from orange to brown, 1st row of expression data in Figure 1B-1) was markedly weaker than usual (10–12), which meant that the action of the eye enhancer on the *white* promoter was partly blocked upon interposing the 825 bp duplicate; indeed, excision of this sequence largely restored the gene expression (red to brown eyes) (Figure 1B-1, 2nd row).

The same phenomenon was clearly observed with an analogous two-gene construct where *white* was preceded by *yellow* [another popular reporter gene, responsible for dark cuticular pigmentation (27,28)], the corresponding enhancers were grouped upstream (wing and body enhancers for *yellow* surrounding the eye enhancer for *white*, collectively designated W·E·B), and the 825 bp duplicate was placed in between (Figure 1B-2): the promoters of both genes enclosed by two copies of the supposed insulator were only weakly stimulated by their enhancers, proving that the enhancer-blocking effect of the tested sequence was not unique for the 'aboriginal' gene.

Next, we tested the position dependence of the enhancer-blocking activity. The 825 bp duplicate on the other side of the enhancers (Figure 1B-3) had no influence on the expression of either gene, be it with (cf. 1st and 2nd rows) or without enhancers (cf. 3rd and 4th rows). The duplicate inserted into the *yellow* intron (Figure 1B-4) allowed full stimulation of the (upstream) *yellow* promoter, as reported for other insulators and genes (10–12,29,30), but prevented stimulation of the (downstream) *white* promoter (1st row). Expectedly, removal of this insert (2nd row) did not change the expression of *yellow* but restored normal expression of *white*.

Thus, the 825 bp sequence from the 3′ end of *white* exhibits all the definitive features of an enhancer blocker: it can hinder the stimulatory action of enhancers on the promoters of different genes in a strictly position-dependent manner (as opposed to silencing) without irreversibly inactivating either element. It may be

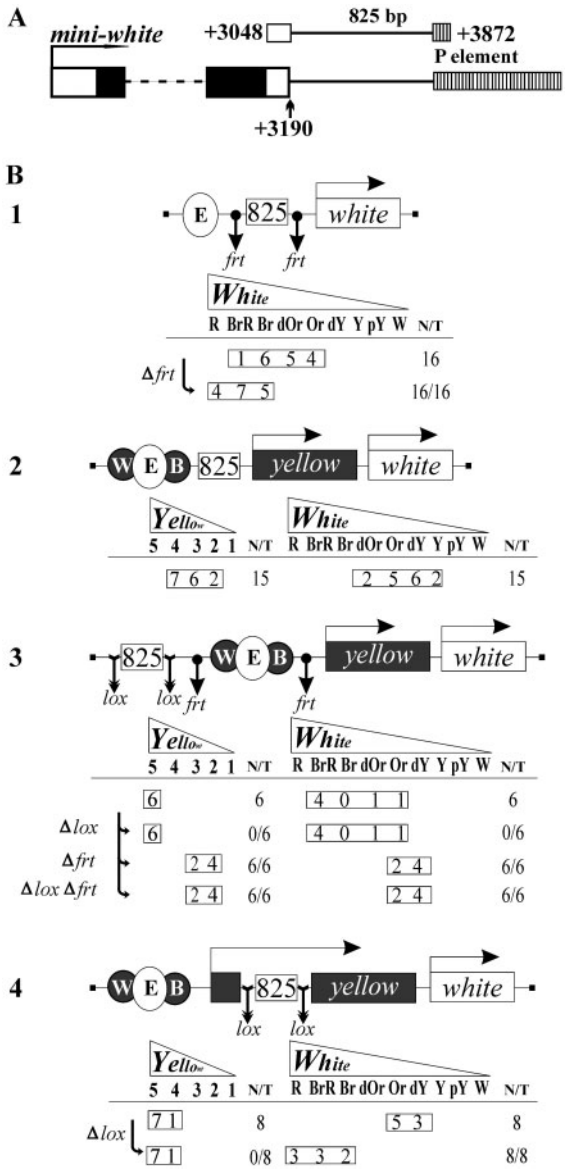

Figure 1. Identification of an insulator at the 3′ end of the *mini-white* gene. (**A**) Demarcation of the pCaSpeR2 825 bp segment taken into study; positions given relative to the *mini-white* transcription start site. (**B**) Transgenic constructs used in the enhancer-blocking assay. The *yellow* and *white* genes are shown as rectangles with arrows indicating the direction of transcription. The Wing, Eye and Body enhancers are encircled and shaded as their target gene. The tested 825 bp sequence is boxed. Downward arrows indicate sites for Flp or Cre recombinases. Below the schemes are the expression data for each parental construct shown in the scheme and those derived from it by *in vivo* excision of the elements flanked by the specified sites. The horizontal colour scales are headed by tapered gene names, the reference images are shown in Supplementary Figures S1 and S2. For *yellow*, grade 5 pigmentation is that in wild type, grade 2 corresponds to complete blocking of wing and body enhancers and grade 1 is characteristic of completely lost expression. For *white*, the scale is from red (R) in wild type through brownish red (BrR), brown (Br), dark orange (dOr), orange (Or, maximal colour without eye enhancer), dark yellow (dY), yellow (Y) and pale yellow (pY) to white (W) in the absence of any expression. Each entry in the frame gives the number of transgenic lines with the corresponding pigmentation grade, while the frame itself shows the range; *T* is the total number of lines examined for each particular construct; for derivative constructs, *N* is the number of lines where the phenotype (i.e. expression level) changed as compared with the parental construct.

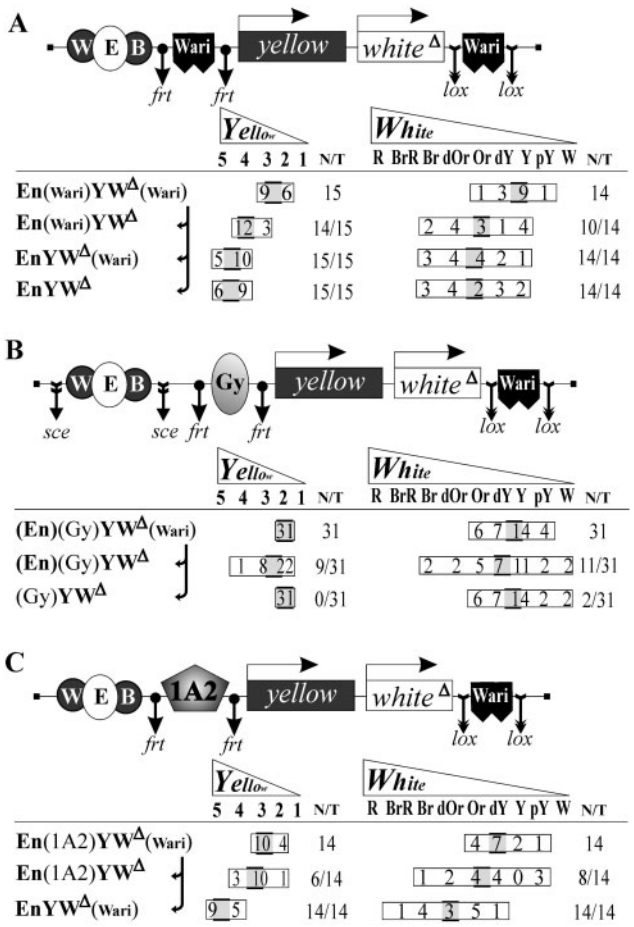

Figure 2. Reinforcing influence of downstream Wari on the apparent activity of Wari (**A**), Gy (**B**) and 1A2 (**C**) insulators in standard enhancer blocking assays. Presentation and designations as in Figure 1B, except that all parental and excision-derived constructs are spelled out at the left of the expression data, with excisable elements parenthesized; *sce* in scheme B denotes the sites for I-*Sce*I endonuclease; *white*$^{\Delta}$ (W$^{\Delta}$) is the 'purified' *mini-white* from which the downstream insulator-containing sequence was removed; Wari is the novel *white*-abutting resident insulator, Gy is the Su(Hw)-dependent insulator from the *gypsy* retrotransposon, 1A2 is the endogenous Su(Hw)-dependent insulator found after the *yellow* gene. The shaded cursor at each distribution frame marks the 'mean colour' on the scale above; thus, cursor positions and shifts in different rows are directly comparable, but the cursors themselves are not associated with the numerals they may cover.

provisionally called *white*-abutting resident insulator (Wari, as an acronym).

Wari is not related to any known *Drosophila* insulator

Sequence analysis of the Wari-containing segment revealed no similarity with already known elements reported to have enhancer-blocking activity. Since insulators are generally held to exert their functions through specific associated proteins, we first of all looked for consensus binding sites, but TRANSFAC(R) Professional r10.2 found none for Su(Hw), dCTCF, GAGA or Zw5 (data not shown). This immediately set Wari apart from the most studied groups such as gypsy and other Su(Hw)-dependent insulators (31,32); Fab-6, Fab-8 and Mcp elements (33); or scs (34). As the binding consensuses

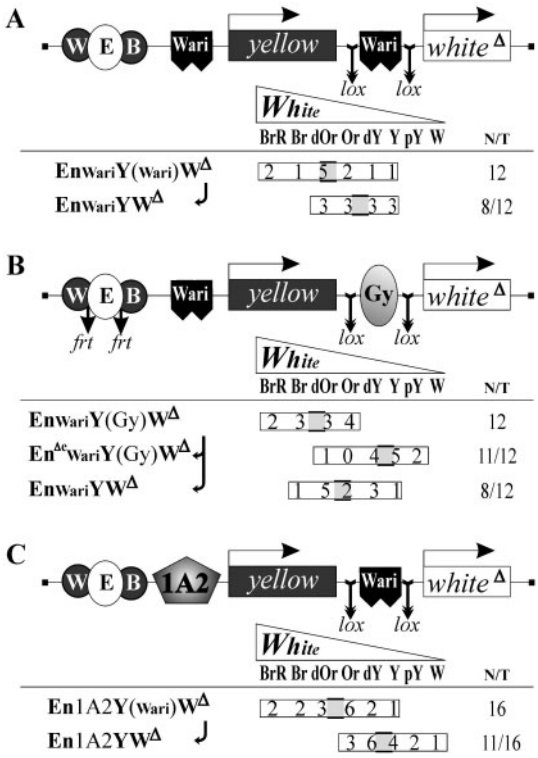

Figure 3. Mutual neutralization of two insulators around *yellow* in the expression of *white*. (**A**) By two Wari insulators. (**B**) By Wari and Gy insulators. (**C**) By Wari and 1A2 insulators. Presentation as in Figure 2; $En^{\Delta e}$ in panel B denotes excision of the eye enhancer. The data for *yellow* expression (without any appreciable changes) are given in Supplementary Figure S3.

are still a matter of debate, we nonetheless tried to check whether any of the major insulator-related proteins could be somehow involved in the Wari function.

In particular, the 825 bp DNA fragment was tested in electrophoretic mobility shift assays using *in vitro*-synthesized Su(Hw) or CTCF proteins. As positive controls, we used the 1A2 insulator, which has two sites for Su(Hw) (35,36), and Fab-8 with two sites for CTCF (37). However, no binding of these proteins to the Wari-containing sequence could be observed (data not shown).

In another approach, nine transgenic fly lines with two Waris around *yellow* and *white* were tested in the $su(Hw)^{v}/su(Hw)^{2}$ (38) or $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ (39) background, i.e. in the absence of functional Su(Hw) or Mod(mdg4) proteins; these mutations did not influence the enhancer-blocking ability of the novel insulator (data not shown, being similar to those in Figure 1B-2 or Figure 2A).

It is yet to be discovered what Wari-binding protein(s) might mediate its function; thus far we can only say that Wari is clearly distinct from any type of insulator element known to date.

The resident insulator aggravates the effect of the same or unrelated enhancer blocker on the enclosed gene(s) in standard assays

It should be noted that in the first subsection (Figure 1) the enhancer-blocking properties of Wari were tested with constructs that originally contained this sequence within the *mini-white* module. Thus, the effect of the insulator placed between enhancers and genes could have been modulated by interaction between the two copies. There is ample, though often inconclusive, evidence for such functional interactions, concerning twin pairs as well as different insulator elements (14,15,17,40–43).

In the constructs tested further, the 825 bp Wari-containing stretch was removed from the 3′ end of conventional *mini-white* (this 'purified' module is denoted as W^{Δ}) and, where specified, reinserted in the same (and/or other) position, *frt*- or *lox*-flanked to be excisable *in vivo* (which is denoted in parentheses) [W^{Δ}(Wari)]. The procedure of assessing the changes in gene expression is detailed and substantiated in section 'Methods'.

As demonstrated in Figure 2A, insertion of two Waris around *yellow* and *white* (i.e. reconstruction of the arrangement shown in Figure 1B-2) resulted in markedly attenuated expression of both genes (1st row: no flies with wing and body pigmentation exceeding grade 3 or eyes darker than orange). Removal of Wari from its 'natural' position largely restored the gene activities (2nd row); i.e. a single Wari between the enhancers and the promoters was only a modest blocker. Excision of the interposed duplicate admitted the same extent of gene stimulation as excision of both Waris (Figure 2A, 3rd and 4th rows), which means that the resident downstream insulator by itself does not perceptibly affect reporter gene expression. This is the most likely reason why it has remained hidden heretofore. These data once again confirm the positional dependence of Wari action, but more importantly, they strongly suggest functional interaction (pairing) between the two copies [as reported for other insulators (14,15,17,40–44)]; most plausibly, such pairing gives rise to a loop sequestering the two genes, which may indeed make their promoters less accessible to the enhancer 'signals'.

The next obvious step was to test whether this hidden element could also modulate the effects of other, unrelated insulators. We made two analogous constructs with the gypsy insulator (Gy) or another Su(Hw)-dependent 1A2 insulator between the enhancers and the reporter genes, as required in a standard assay, and excisable Wari reinserted after *white*.

Figure 2 shows that the action of enhancers on *yellow* and *white* in such transgenic flies was completely blocked with Gy (panel B, compare 1st row with the 3rd where the enhancers had been excised from the construct) and largely blocked with 1A2 (panel C); this was quite in line with the literature data (11,35,36,42,44). However, the apparent blockage by 1A2 was appreciably weakened upon removal of the downstream Wari, and even with Gy quite a few Wari-excision lines showed increased pigmentation (cf. 1st and 2nd rows in each panel). Closer inspection of the expression data reveals that the less strong is the first (interposed) insulator (Gy > 1A2 > Wari), the greater is the 'reinforcing contribution' of Wari in its natural position, without discernible difference between the two enclosed genes. At the same time, one can see that removal of the downstream Wari may result in even lower *white*

expression in occasional fly lines (e.g. two more pY lines in the 2nd row of panel C and even two W lines in panel C). Note that these changes occur in the very low expression range that is not associated with enhancer action, and hence can hardly be due to altered enhancer blocking. Most probably, in some genomic positions such 'open' constructs become more susceptible to the influence of neighbouring repressive chromatin. These observations suggest that Wari may also have the second, barrier function (see 'Introduction') and thus may be a full-fledged insulator element; work along this line is under way.

Since the constructs where Gy or 1A2 is combined with Wari (Figure 2B and C) behave very similarly to those with two interacting Waris (Figures 1B-2 and 2A), and there are no reasons to suppose that the same effects in analogous constructs are caused by basically different events, these results are the first evidence for pairing between insulators totally unrelated in nucleotide sequence and apparently having no common proteins. Of course the molecular mechanism(s) involved are yet to be elucidated, but this is so even for twin pairs of long-studied insulators.

'Mutually neutralizing' interaction of Wari with another Wari, Gy or 1A2 between the eye enhancer and the *white* promoter in *white* expression

Insulator pairing is still more vividly demonstrated with *white* expression in the other series of constructs where one of the insulators followed the enhancers and another was inserted between the two reporter genes (Figure 3).

With two identical or different insulators between the eye enhancer and the *white* promoter, the gene was expressed to the same or even higher level than in constructs without any interposed insulator (cf. 1st rows in all Figure 3 panels with, e.g. 3rd rows in Figure 2A and C). The 2nd row in Figure 3B proves that this high expression was due to promoter stimulation by the eye enhancer within the construct. Conversely, excision of the insulator right in front of the promoter (bottom rows in each Figure 3 panel) resulted in marked attenuation of *white* expression (consistent with the data for the corresponding arrangements in Figure 2). Note that *yellow* showed no response to these manipulations, remaining weakly or moderately insulated in accordance with the strength of the insulator preceding its promoter (Supplementary Figure S3).

This seemingly paradoxical behaviour of *white* is, however, another manifestation of the 'insulator bypass', 'mutual neutralization' or 'cancellation' phenomenon (14,15,17,40–43). It was first observed with tandemly placed identical insulators (14,40); later we demonstrated (42,45) that insulators such as Gy could interact with each other at considerable distances, over enhancers or promoters and coding sequences. Indeed, the only reasonable explanation of the data in Figure 3 is that here the insulators (even unrelated ones) pair around *yellow* to form a loop but no longer present any obstacle for stimulation of *white*; moreover, the distance between its enhancer and promoter becomes much shorter. That is, in one and the same construct, the same pair of elements acts as blockers for *yellow* but as facilitators for *white*.

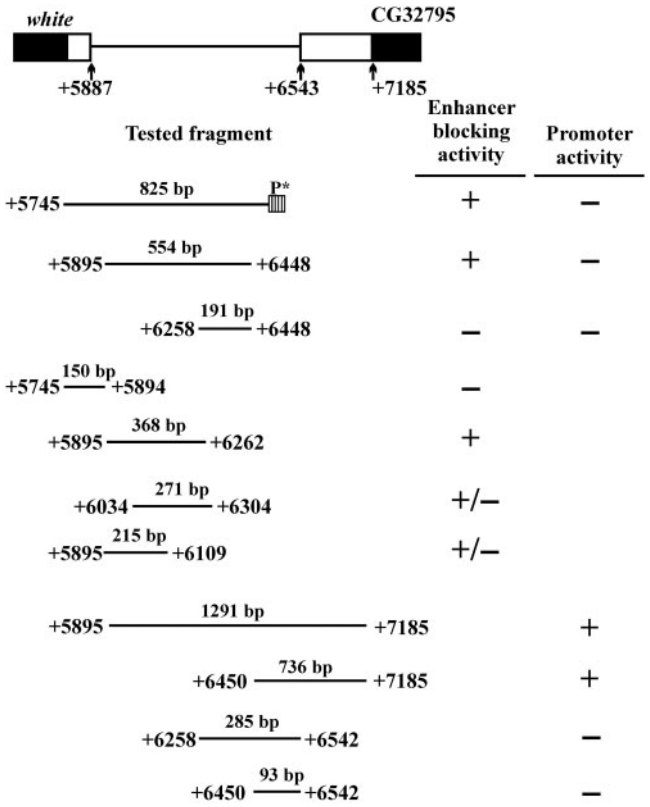

Figure 4. Functional anatomy of the sequence between *white* and CG3295. All positions are given relative to the transcription start site of the unabridged *white* gene. Specified fragments were tested for enhancer-blocking activity as in Figure 1B2 and for promoter activity in the luciferase reporter assay. The '+/–' marks indicate weak activity (mean expression shifts <1 grade) for *yellow* and only trace activity for *white*.

Mapping of the Wari core

Finally, we undertook an attempt to locate more precisely the novel insulator in the genomic sequence between the coding part of *white* and the next gene CG32795. Specifically, we wanted to check overlapping with the CG32795 promoter, overlapping with *white* 3′ UTR, and to isolate the Wari functional core. To this end, we obtained fragments of different lengths and positions (specified in Figure 4) and tested them for (i) enhancer-blocking activity in transgenic constructs analogous to the 'standard' shown in Figure 1B-2 and (ii) promoter activity in the luciferase reporter assay (*Drosophila* S2 cells, see 'Supplementary Data').

The full-sized 825 bp sequence ending shortly before the reported CG32795 regulatory region (see scheme in Figure 4) proved devoid of promoter activity (line 1), which indeed mapped to the most downstream part of the fragments tested (line 3 from bottom).

Removal of the 5′-terminal 150 bp did not affect the enhancer-blocking activity (line 2), and the fragment

thus removed had none of its own (line 4), meaning that the insulator proper does not overlap with the *white* gene sequence. Moreover, the 3′-terminal quarter proved also inessential (meaning that the insulator is not even contiguous to the promoter region of the next gene), while the central 368 bp segment (45% of the initial length) retained full enhancer-blocking capacity (line 5). Another dissection in the middle part, however, reduced the activity to weak for *yellow* and insignificant for *white* (as in the two overlapping fragments of 271 and 215 bp). The 368 bp sequence encompassing the Wari core is given in Supplementary Figure S4.

Inferences and implications

Thus, we have found an insulator residing immediately downstream of the *white* gene in the *Drosophila* genome. This is a novel and somewhat surprising kind of enhancer blocker, as its functional requirements apparently do not include Su(Hw), CTCF, Zw5 or GAGA factor, though such zinc finger proteins heretofore appeared almost universal in insulator functions. Nonetheless, it interacts not only with another copy of itself in model transgenic constructs but equally well with the totally unrelated Su(Hw)-dependent insulators, markedly modulating their apparent enhancer-blocking activity; furthermore, this interaction implies physical pairing, as suggested by the bypass/neutralization phenomenon (Figure 3). All these findings make the obscure question of protein-mediated insulator function still more puzzling and still more challenging.

Anyway, it is clear that no responsible conclusions about the properties of any insulator element(s) can be drawn without considering the possible interactions with other insulators. Hence the general impact of our finding, stemming from the fact that this versatile insulator was inadvertently included in all transgenic constructs with *white* as reporter for testing the enhancer-blocking activity of various insulators (10–18,34,35,41–43), the anti-insulator ability of promoter-targeting sequences (46–49), the boundary activity of insulators and matrix attachment regions (50–53), or simply as selection marker (29–32,36,37,40,44,54,55). The cryptic downstream Wari could have aggravated the effects of single insulators, just as shown here for Gy and 1A2 (quantitative distortion of data); conversely, it could have disrupted their tandem pairing or simply masked their mutual neutralization in 'insulator bypass' assays (qualitative distortion).

In our previous work (45), pairing between Gy copies located in the same sites on homologous chromosomes facilitated the enhancer action *in trans* throughout the *Drosophila* genome. However, in some genomic positions the phenomenon was also observed in the absence of Gy; this 'residual' *trans*-activation could actually be due to pairing between Waris. Much the same applies to works concerning various kinds of long-distance genomic interactions presumably mediated by insulator elements (45,56–58).

Overall, the results and interpretations in a number of works (including ours) perhaps require re-evaluation, and care should be exercised in future studies regarding not only insulators proper but also enhancer–promoter communication and genomic control in general.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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