In vivo analysis of scaffold-associated regions in *Drosophila*: a synthetic high-affinity SAR binding protein suppresses position effect variegation

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Scaffold-associated regions (SARs) were studied in *Drosophila melanogaster* by expressing a synthetic, high-affinity SAR-binding protein called MATH (multi-AT-hook), which consists of reiterated AT-hook peptide motifs; each motif is known to recognize a wide variety of short AT-rich sequences. MATH proteins were expressed specifically in the larval eye imaginal discs by means of the tetracycline-regulated transactivation system and tested for their effect on position effect variegation (PEV). MATH20, a highly potent SAR ligand consisting of 20 AT-hooks, was found to suppress *white*^{mottled} variegation. This suppression required MATH20 expression at an early larval developmental stage. Our data suggest an involvement of the high AT-rich SARs in higher order chromatin structure and gene expression.

Keywords: chromatin structure/*Drosophila*/PEV/SAR

Introduction

Scaffold-associated regions (SARs), also called matrix attachment regions or MARs, are operationally defined as DNA sequences that specifically associate with the nuclear scaffold or matrix and possibly define the bases of chromatin loops (Mirkovitch *et al.*, 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986). SARs are very AT-rich regions of several hundred base pairs in length, that are possibly best described as being composed of numerous, irregularly spaced A tracts (short AT-rich sequences containing homopolymeric runs) (reviewed in Laemmli *et al.*, 1992). Proteins that specifically bind to SARs do not appear to interact with a precise base sequence but rather recognize certain structural features of non-B DNA, such as narrow minor grooves, DNA bends and a propensity to unwind (Bode *et al.*, 1992; Käs *et al.*, 1993).

SARs play roles in both chromosome condensation and gene expression. A recent report identified SARs as *cis*-elements of chromosome dynamics (Strick and Laemmli, 1995), while numerous publications demonstrated that SARs, in a flanking position, can strongly stimulate the expression of various heterologous reporter genes in

different biological systems (Laemmli *et al.*, 1992). The SAR-mediated stimulation of transgene expression is not observed in transient assays, but only after stable integration of the test constructs into the genome (Klehr *et al.*, 1991; Poljak *et al.*, 1994). Hence, these *cis*-acting elements may exert their effect via chromatin structure, since transiently transfected DNAs are known to be poorly organized into nucleosomes.

A recent model attempts to explain the general stimulatory effect of SARs on transcription by proposing that SARs facilitate the displacement of histone H1 (a process referred to as chromatin opening) through mutually exclusive interactions with proteins of similar DNA-binding specificity, such as the high mobility group protein HMG-I/Y (Käs *et al.*, 1993). HMG-I/Y contains three short DNA-binding domains, called AT-hooks, that bind the minor groove of A tracts similarly to the peptide antibiotic distamycin (Reeves and Nissen, 1990). Histone H1, HMG-I/Y and distamycin all bind selectively to the A tracts of SARs, and competition experiments between them have demonstrated that HMG-I/Y and distamycin are 'dominant': i.e. both can displace pre-bound histone H1 from an SAR template (Zhao *et al.*, 1993). The observation that distamycin, added to cells, markedly stimulated cleavage at SARs by topoisomerase II in internucleosomal linker DNA but not at hypersensitive sites, lends *in vivo* support to this model; increased accessibility for cleavage presumably arises from the displacement of proteins, possibly histone H1 (Käs *et al.*, 1993). The model is also consistent with elegant studies demonstrating that SARs are necessary to spread open chromatin from enhancers to gene promoters (Kirillov *et al.*, 1996; Jenuwein *et al.*, 1997).

To study the role of SARs in chromosome condensation, synthetic multi-AT-hook proteins (MATH), consisting of numerous reiterated AT-hook peptide motifs derived from HMG-I/Y (Strick and Laemmli, 1995), were synthesized. Since the AT-rich SARs have enough adjacent binding sites to accommodate all the multiple, covalently linked AT-hooks, these HMG-I/Y derivatives bind SARs (both as DNA and chromatin) with exquisite specificity (Strick and Laemmli, 1995). Their effects on chromosome assembly were tested in *Xenopus* egg extracts capable of converting added nuclei to mitotic chromosomes *in vitro*. Remarkably, adding low levels of MATH20, a protein containing 20 hooks, inhibited the normal events of chromosome condensation, suggesting that SARs are *cis*-elements of mitotic chromosome dynamics (Strick and Laemmli, 1995).

To address the importance of SARs in the fruit fly *Drosophila melanogaster*, we expressed MATH20 in the larval eye imaginal disc to test for an effect on position effect variegation (PEV). We found that regulated expression of MATH20 led to a suppression of PEV, suggesting

Fig. 1. Protein overexpression by means of the tetracycline-regulated transactivation system in *Drosophila*. (A) Schematic representation of the Tet system applied to *Drosophila* (see text for details). In (B–F), β -galactosidase staining of eye-antennae imaginal discs from early (B and E), mid (C) and late third instar larvae (D and F), showing the activation of a tetO–LacZ reporter construct by the *eyeless* enhancer-tTA strain. In (E) and (F), larvae were maintained on medium containing tet 0.2 μ g/ml, resulting in a complete inhibition of the tTA-induced LacZ expression.

an involvement of SARs or SAR-like AT-rich regions in long-range chromatin structure and gene regulation.

Results

Targeted expression of synthetic SAR-binding proteins in *Drosophila*

With the aim of improving our understanding of SAR function(s) *in vivo*, we have overexpressed MATH20, the synthetic SAR-binding protein, in *Drosophila* by means of the tetracycline (tet)-regulated transactivation system (Tet system) (Bello *et al.*, 1998). Our strategy consisted of targeting expression of these proteins specifically to the eye imaginal discs during larval development and scoring for effects on *white* variegation. The principle of the binary Tet system is depicted in Figure 1A. A fly strain expressing a tet-regulated transactivating protein [tTA, a fusion of the tet repressor DNA-binding domain and the VP16 transcriptional activation domain (Gossen and Bujard, 1992)] under the control of regulatory sequences [in our case, an eye-specific enhancer identified in the intronic sequences of the *eyeless* gene (Quiring *et al.*, 1994)] is crossed to a strain containing the coding sequence for the gene of interest downstream of the tet operator sequences. The expression of this gene is then obtained in the progeny, in the same pattern as tTA is expressed, and can be tightly controlled by tet (Bello *et al.*, 1998). As shown in Figure 1B–D, by monitoring tetO–LacZ reporter gene expression, the *ey*-tTA strain can be used to target protein expression in the third instar eye imaginal discs, primarily in the undifferentiated eye cells

(Figure 1B), and progressively only in the differentiated cells posterior to the morphogenetic furrow (Figure 1C and D). Additionally, β -galactosidase activity is also observed in the eye imaginal discs during the first and second instars (data not shown). Tet, when added to the food at a concentration as low as 0.2 μ g/ml, is able to completely silence the expression of the LacZ reporter gene, both in early (Figure 1E) and late third instar (Figure 1F).

MATH20 suppresses PEV

In *Drosophila*, PEV refers to the mosaic expression of a gene when chromosome rearrangements place it close to heterochromatin. This heterochromatin-mediated gene silencing is proposed to be a heritable, epigenetic event that involves no alteration in the DNA content. Silenced genes are believed to be packaged into a higher order chromatin structure, or alternatively might be localized to a special nuclear compartment that confers transcriptional repression (reviewed in Karpen, 1994; Elgin, 1996). A classical example of PEV is the *white*-mottled (w^{m4}) inversion, in which the *white* gene necessary for the red pigmentation of the eye is juxtaposed close to heterochromatin of the X chromosome.

The variegating phenotype of *white*-mottled is seen as numerous clones of red, wild-type cells in an otherwise *white* mutant background (Figure 2A). Since MATH proteins are very efficient in SAR binding, including satellite III found in the centromeric heterochromatin of the X chromosome (Strick and Laemmli, 1995, and below), we reasoned that overexpressing MATH proteins specifically in the eye imaginal discs of the developing larvae might modify *white* variegation, a process known to involve chromatin structure. Females of the *ey*-tTA strain in a w^{m4} background were crossed to males of the various independent tetO-MATH20 strains. After eclosion, males of the desired genotypes (which are heterozygous for both *ey*-tTA and tetO-MATH20 transgenes, and hemizygous for w^{m4}) were kept for 5 days at 25°C and photographed. As a control, we used a line containing an empty tetO vector, showing a typical ‘salt and pepper’-like mosaic expression of the *white* gene (Figure 2A). Expressing MATH20 resulted in a significant derepression of the *white* gene, and a general loss of the variegating phenotype. This suppression of PEV was clearly visible in various independent MATH20 lines (Figure 2B–F), and was shown to be highly reproducible. Quantitative analysis of the red eye pigment levels for seven independent MATH20-expressing strains is shown in Figure 3A, with PEV suppressor ratios ranging from 1.6 to 2.8 when compared with the control line (two independent control lines gave identical results). In contrast, MATH11, a less potent SAR DNA-binding protein (Strick and Laemmli, 1995), revealed no PEV-modifying effects in four independent strains (Figure 3A, dashed bars).

To test for the specificity of the MATH20-induced suppression of PEV, we made use of tet to silence the expression of the MATH20 transgene. Progeny of a cross between w^{m4} , *ey*-tTA females and tetO-MATH20-19 or empty tetO were grown on either normal or tet-containing food, under exactly the same conditions of temperature and population density as before. While tet treatment has no effect on the *white* mosaic expression in the control

Fig. 2. Eye-specific expression of MATH20 suppresses *white* variegation. (A–F) Females of the *ey-tTA* strain in a w^{m4h} background were crossed to males of the various *tetO-MATH20* strains. Progeny was grown on standard medium at 25°C. After adult eclosion, males of the desired genotype were kept for 5 days at 25°C before photography. Shown are photomicrographs of male heads of the following genotypes: (A) w^{m4h}/Y , control $tetO/+$, $ey-tTA/+$; (B, E and F) w^{m4h}/Y , $tetO-MATH20/+$, $ey-tTA/+$, with respectively strains 20-14, 20-2 and 20-19. (C and D) w^{m4h}/Y , $+/+$, $ey-tTA/tetO-MATH20$, with respectively strains 20-4 and 20-6. (G–J) Inhibition of MATH20-induced PEV suppressor effect by tet treatment. Females of the *ey-tTA* strain in a w^{m4h} background were crossed to males of the control *tetO* strain (G and H) or the *tetO-MATH20-19* strain (I and J). Progeny was grown at 25°C, either on standard medium (G and I) or medium containing tet 0.2 $\mu\text{g/ml}$ (H and J).

line (Figure 2G–H), it clearly inhibits MATH20-induced suppression of PEV (compare Figure 2I and J). Quantitative analysis is given for three MATH20 strains (Figure 3B). Results are shown as the ratio of OD_{480} MATH20 to OD_{480} control: while modification of *white* variegation is observed in the absence of tet (Figure 3B, open bars), tet treatment, by maintaining silent the MATH20 transgene expression, leads to eye pigment levels very similar to those of the control (Figure 3B, black bars).

We next made use of tet to examine whether suppression of PEV by MATH20 might require expression during an early developmental stage. For this purpose, 50 females of the w^{m4h} , *ey-tTA* strain were crossed to males of the control or *tetO-MATH20-12* strain. One hour egg collections were made, and kept on either normal or tet-containing food. Larvae were then transferred at regular intervals to normal food. In these conditions, the transgene is kept efficiently silent, but it is activated after shifting larvae off tet following a lag period of ~12 h (Bello *et al.*,

1998). As shown in Figure 3C, PEV suppression is still observed when larvae are exposed to tet up to 60 h after egg laying (AEL). If the gene is kept silenced longer (72–120 h), then no PEV suppression is observed. The *eyeless* enhancer activity and hence MATH20 expression can be detected throughout the larval stages and up through early pupal stages when differentiated eye cells develop from precursor cells. Since activation of the transgene, during the third instar period (72 h AEL), no longer reduced PEV, we conclude that MATH20 expression in the undifferentiated cells is required to achieve suppression of PEV.

MATH20 suppresses cleavage by topoisomerase II in satellite III heterochromatin of chromosome X

Is suppression of PEV by MATH20 mediated by interactions at SARs? The w^{m4} inversion juxtaposes the *white* gene to the heterochromatin of the X chromosome. Intriguingly, the predominant component of the

Fig. 3. Quantitation of MATH20-induced PEV suppression. **(A)** MATH20, but not MATH11, suppresses w^{m4} variegation. Females of the *ey-tTA* strain in a w^{m4h} background were crossed to males of the control tetO strain or the various tetO-MATH11 (dashed bars) and tetO-MATH20 strains (shaded bars). Quantitation of the red eye pigment levels was done on groups of 40 male heads 5 days after eclosion, and repeated 4–6 times. Standard deviations are shown as thin lines above the histograms. **(B)** Inhibition of MATH20-induced PEV suppression by tet. Females of the *ey-tTA* strain in a w^{m4h} background were crossed to males of the control tetO strain or three independent tetO-MATH20 strains. Progeny were grown at 25°C on either normal food (open bars) or food containing tet 0.2 µg/ml (black bars). Values are given as the ratio of the OD_{480 nm} of the tetO-MATH20 lines to the OD_{480 nm} of the empty tetO vector control line, and represent the average of three independent measurements. **(C)** Females of the *ey-tTA* strain in a w^{m4h} background were crossed to males of the control tetO strain or tetO-MATH20-12 strain. Progeny were kept on normal or tet-containing food (0.2 µg/ml). Larvae were transferred to normal food at the indicated times after egg laying. The red eye pigment levels were measured in groups of 30 male heads, and repeated three times. Values are given as the average ratio of the OD_{480 nm} of the tetO-MATH20-12 strain to the OD_{480 nm} of the control strain.

heterochromatin is satellite III, also called the 1.688 or the 359 bp repeat satellite (Hsieh and Brutlag, 1979). Two or three repeats (718–1017 bp) of this satellite behave as fully fledged SARs *in vitro*; they preferentially bind nuclear scaffolds, topoisomerase II and HMG-I/Y (Käs and Laemmli, 1992; Karpen, 1994). Thus, the inverted *white* gene appears juxtaposed to a giant, reiterated SAR of ~11 Mb.

Topoisomerase II is one of the growing number of proteins associated with heterochromatin (Rattner *et al.*, 1996). Although it is not known whether topoisomerase II is implicated in heterochromatin formation, it is one of the few proteins for which the site of interaction can be studied by stabilizing the so-called cleavage intermediate using cytotoxic drugs. Thus, this protein serves here as a convenient, heterochromatin-associated reporter protein which is expected to monitor the interaction of MATH20 in this chromatin. Moreover, topoisomerase II is known to be specifically enriched over satellite III heterochromatin, as revealed by microinjection of fluorescent topoisomerase II into *Drosophila* embryos (Denburg *et al.*, 1996). This preferential interaction was also borne out by previous studies that demonstrated a major topoisomerase II cleavage site once per satellite III repeat; this 359 bp repeat contains two positioned nucleosomes, and the major topoisomerase II cleavage site occurs in one of two nucleosomal linker regions as depicted in Figure 4B.

Does MATH20 interfere with topoisomerase II cleavage in satellite III? We addressed this question using *Xenopus* egg extracts. These extracts are known to carry out faithfully many cellular processes. Indeed, we noted that the endogenous topoisomerase II of such extracts generated a cleavage ladder in satellite III repeats of *Drosophila* Kc nuclei that is indistinguishable from the one observed in cells (Figure 4A, lanes 1 and 2). Interestingly, this cleavage ladder is suppressed specifically in a dose-dependent manner by MATH20 (lanes 2–5). In contrast, no inhibition is observed by added HMG-I/Y (lane 6) which binds much more dispersively to the genome. In conclusion, MATH20 can interfere specifically with topoisomerase II cleavage in satellite III, strongly suggesting that the MATH20 protein encoded by the transgene expressed in flies is very likely to bind specifically the heterochromatin of chromosome X.

Discussion

The variegated phenotype of *white* mottled flies is the result of a large inversion in the X chromosome that places the *white* gene adjacent to centomeric heterochromatin. PEV is due to a stochastic inactivation of the *white* gene in some but not other cells at an early stage of eye development, followed by clonal maintenance through later stages. The resulting pattern of *white* gene expression

Fig. 4. Specific suppression by MATH20 of topoisomerase II cleavage in satellite III of chromosome X. This figure demonstrates that MATH20 specifically inhibits topoisomerase II cleavage in satellite III, which is the predominant component of the heterochromatin of chromosome X. **(A)** Isolated Kc *Drosophila* nuclei were incubated in mitotic *Xenopus* egg extracts in the presence of different concentrations of MATH20 or HMG-I/Y. The topoisomerase II cleavage activity subsequently was monitored in satellite III by treating the extracts for 10 min with VM26. The DNA samples were displayed on a 1.2% agarose gel and the Southern blot hybridized with a satellite III repeat probe. VM26 and proteins were added as indicated at the top. The sample in lane 1 received no VM26 and no MATH20, and that in lane 2 received VM26 only. Samples in lanes 3–5 contained 80, 40 and 20 ng of MATH20, respectively. The sample in lane 6 contained 80 ng of HMG-I/Y. **(B)** The repeat structure of satellite III chromatin, which consists of two nucleosomes per 359 bp repeat unit. Topoisomerase II cleaves (arrow) once per unit, in every other nucleosomal linker region (Käs *et al.*, 1993).

is observed in the eye as patches of pigmentation. Morphologically, PEV is observed on polytene chromosomes as a spreading of heterochromatic structures into euchromatic genes (Elgin, 1996). Current evidence favors a model according to which silencing proteins of the centromeric heterochromatin spread into the juxtaposed euchromatic region by a cooperative assembly process. This spreading may be a consequence of the inversion by removing putative boundary elements that otherwise delimit heterochromatin (Locke *et al.*, 1988).

We have shown here that specific expression of the

artificial high-affinity SAR-binding protein MATH20 in the developing eye imaginal discs results in suppression of *white* variegation. Using the tetracycline gene regulation system, we demonstrated that suppression of PEV requires the expression of MATH20 in the undifferentiated eye cells and is observed if this transgene is activated up to 60 h after egg laying. In contrast, no suppression is observed upon activation of MATH20 during the late third instar period. Interestingly, suppression of PEV by MATH20 was also observed for the *Brown^D* variegating rearrangement (data not shown). We found no effect on *white* variegation by expression of MATH11; this protein with only 11 AT-hooks has a 7-fold lower binding affinity ($K_D = 18.2$ pM) for SARs than MATH20 (2.6 pM). Consequently, proportionally higher amounts of protein were required to affect chromosome condensation in *Xenopus* extracts (Strick and Laemmli, 1995). Hence, it might be necessary to express MATH11 in the eye imaginal disc with a stronger promoter to achieve an effect on *white* variegation. The differential effect of MATH11 versus MATH20 underscores the notion that suppression of PEV is a specific phenomenon; it appears to be related to the binding strength of the effector. Adding MATH to a mitotic *Xenopus* extract led to the formation of abortive condensation products (Strick and Laemmli, 1995), and microinjection of MATH20 (but not HMG-I/Y) blocked HeLa cells in late G₂-phase following passage through S-phase (R.Strick, R.Peperkok and U.K.Laemmli, in preparation). In agreement with this, we have observed that higher levels of embryonic and larval expression of either MATH20 or MATH11 led to lethality. Thus, suppression of PEV required tissue-specific expression of MATH20 at a low level that does not interfere with cell division.

The inverted *white* gene is juxtaposed to a giant 11 Mb reiterated SAR in the form of satellite III repeats. MATH20 binds there with great specificity (Strick and Laemmli, 1995) and is able to interfere with the activity of topoisomerase II (Figure 4). This observation establishes proof of the principle that MATH20 can interact with or displace a pre-bound protein associated with heterochromatin. Although we cannot rule out other possible models, it is tempting to explain the effect of SAR on gene expression, chromatin opening and PEV by extending a model put forward by Laemmli and colleagues (Laemmli *et al.*, 1992). In this model, certain proteins (called compacting proteins here) interact with SARs or certain AT-rich satellites cooperatively; this can lead to either chromatin folding, chromosome condensation (looping) or formation of heterochromatin. Conversely, other proteins such as HMG-I/Y and their monster derivatives, MATH, bind non-cooperatively to SARs and can displace the compacting proteins by disrupting their cooperative interactions, hence resulting in chromatin unpacking. These alternative chromatin states are governed by the binding strength and the relative local level of the chromatin packaging proteins versus those that undo it. Of importance for these considerations is the extent of SAR repetition at a given region. Since an assembly of cooperatively interacting proteins becomes energetically more favorable with increasing repeats, certain packaging proteins are expected to polymerize preferentially onto satellite III chromatin over individual SARs. In contrast, the non-cooperative MATH

proteins would bind dispersively to single and reiterated SARs. Thus in a cell, while a single SAR in euchromatin may promote chromatin opening and stimulation of gene expression (Jenuwein *et al.*, 1997), a reiterated SAR could result in silencing.

It is easy to explain the effect of MATH20 on PEV by simple considerations based on the above model. The high affinity of MATH20 for SARs could allow it to bind to the satellite III region or other AT-rich regions, thus disrupting the cooperative interaction of the compacting proteins. This in turn would energetically disfavor the spreading of the polymerizing proteins into the flanking euchromatic region. As the probability of inactivating an adjacent euchromatic gene diminishes, one expects to observe suppression of PEV. It is impossible at present to obtain direct evidence for the model, and for a direct *in vivo* proof for MATH20 binding to satellite III. PEV appears to be a complex, poorly understood process, and the nature of the *cis*-DNA elements involved in heterochromatin formation has yet to be elucidated. The results reported here could also provide a clue about the nature of these *cis*-acting elements involved in PEV; the data suggest that this chromatin state might be mediated in part by proteins that interact with AT-rich repeats, such as those of satellite III.

Materials and methods

Fly strains

The yw^{67c23} strain was used as recipient for injections. P-element-mediated germ line transformation was done using standard procedures (Spradling and Rubin, 1982a,b). For each construct, multiple independent lines were established, and the chromosomal location of the inserted transgene was determined by standard genetic analysis using balancer chromosomes. The *white*^{mottled 4} inversion, *In(1)w^{m4h}* strain, was used to score the effects of MATH proteins on *white* variegation. PEV-modifying effects were quantified by red eye pigment measurement (Ashburner, 1989), on groups of 40 male heads of the desired genotype, kept for 5 days at 25°C after eclosion. Flies were grown at 25°C on standard medium, unless specified otherwise in the text. Tetracycline (Sigma) was used at 0.2 µg/ml as described (Bello *et al.*, 1998). Fly strains expressing tet-VP16 transactivator are described elsewhere (Bello *et al.*, 1998).

Plasmid constructs

Further details of the cloning procedures are available upon request. MATH11 and MATH20 cDNAs (Strick and Laemmli, 1995) were inserted into pWTP (marked with *miniwhite*) (Bello *et al.*, 1998) and/or pYTP (marked with *yellow*). pYTP was constructed by inserting a tet operator-P promoter-SV40 polyadenylation signal cassette from pWTP into pY vector, made by inserting the *yellow* gene from the YES vector (Patton *et al.*, 1992) into Carnegie 4 (Rubin and Spradling, 1983).

β-Galactosidase activity

β-Gal stainings were done as described (Ashburner, 1989), on glutaraldehyde-fixed eye-antennal imaginal discs from early and late third instar larvae.

Topoisomerase II cleavage assay

Kc nuclei were isolated (Mirkovitch *et al.*, 1984) and added to *Xenopus* egg extracts (Strick and Laemmli, 1995) to a final concentration of 10 000 nuclei/µl of extract, incubated at 21°C for 1 h and subsequently treated with 50 µM VM26 for 10 min. The reactions were stopped and isolated DNA samples were electrophoresed in a 1.2% agarose gel, electroblotted to nylon membranes and hybridized to satellite III repeat probes as described (Käs *et al.*, 1993).

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