Modification of position-effect variegation by competition for binding to *Drosophila* satellites

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white-mottled (w^{m4}) position-effect variegation (PEV) arises by translocation of the *white* gene near the pericentric AT-rich 1.688 g/cm³ satellite III (SATIII) repeats of the X chromosome of *Drosophila*. The natural and artificial A•T-hook proteins D1 and MATH20 modify w^{m4} PEV in opposite ways. D1 binds SATIII repeats and enhances PEV, presumably via a recruitment of protein partners, whereas MATH20 suppresses it. We show that D1 and MATH20 compete for binding to identical sites of SATIII repeats *in vitro* and that conditional MATH20 expression results in a displacement of D1 from pericentric heterochromatin *in vivo*. In the presence of intermediate levels of MATH20, we show that this displacement becomes selective for SATIII repeats. These results strongly suggest that the suppression of w^{m4} PEV by MATH20 is due to a displacement of D1 from its preferred binding sites and provide additional support for a direct role of D1 in the assembly of AT-rich heterochromatin.

INTRODUCTION

Overexpression of the *Drosophila* D1 chromosomal protein, which contains 10 A•T-hook motifs, enhances position-effect variegation (PEV) of the *white-mottled* (w^{m4}) allele of the *white* gene. Conversely, heterozygous D1 mutants suppress w^{m4} PEV (Aulner *et al.*, 2002). The D1 C-terminal domain thought to be involved in protein–protein interactions is required for modification of PEV: expression of a deletion mutant, D1ΔE, has effects opposite to those of the expression of a D1 transgene. D1 localizes to the 1.688 g/cm³ satellite III (SATIII) repeats of the X chromosome that the *white* gene is placed near in the w^{m4} chromosomal inversion and to the 1.672 g/cm³ satellite I (SATI) AATAT repeats found on chromosomes 3 and 4 and on the Y chromosome.

Thus, the DNA-binding characteristics of D1 can account for its localization in heterochromatin and, presumably, for its effects on PEV, and we proposed that one of the functions of D1 might be the recruitment to AT-rich satellites of protein partners involved in heterochromatin-mediated silencing (Aulner *et al.*, 2002).

MATH20 is an artificial protein containing 20 A•T-hook motifs derived from the mammalian HMGA protein (Strick and Laemmli, 1995). These repeated motifs allow MATH20 to bind DNA A•T tracts with extraordinary affinity, a property that has been exploited to study the role of AT-rich sequences in chromosome dynamics (reviewed in Hart and Laemmli, 1998). Its expression in *Drosophila melanogaster* leads to a strong suppression of w^{m4} PEV (Girard *et al.*, 1998), an effect opposite to that of D1. These observations raise the possibility that suppression of w^{m4} PEV by MATH20 might be due to a direct perturbation of the association of D1 with SATIII and/or SATI heterochromatin. We demonstrate here that the artificial MATH20 protein can indeed displace D1 from its binding sites *in vivo*.

RESULTS

MATH20 and D1 compete for binding to identical SATIII sites *in vitro*

To test the possibility that MATH20 might compete with D1 for SATIII binding sites *in vivo*, we first measured their binding to a cloned 359-bp SATIII fragment (Hsieh and Brutlag, 1979). Addition of increasing amounts of each protein under stringent binding conditions resulted in the apparition of retarded complexes of different mobilities (Figure 1). The ladder of D1

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Fig. 1. Binding of D1 and MATH20 to SATIII repeats *in vitro*. Samples were incubated as described in Methods with purified D1 (lanes 2–8) or MATH20 (lanes 10–16) as indicated above the gel. Alternatively, complexes preformed with D1 were challenged with MATH20 (lanes 17–19), and complexes preformed with MATH20 were challenged with D1 (lanes 20–22).

complexes most likely reflects stepwise binding as a function of increasing protein concentrations (lanes 2–8). Addition of MATH20 led to the apparition of a larger complex in a dose-dependent fashion (lanes 10–16). It is most likely that the bulkier MATH20 protein with its 20 A•T-hook motifs and its high affinity for AT-rich DNA can span multiple binding sites on the SATIII fragment (Strick and Laemmli, 1995).

Competition experiments were carried out as follows. Preformed D1/SATIII or MATH20/SATIII complexes were challenged by the addition of purified recombinant MATH20 or D1 protein, respectively. As these complexes migrate to different positions, it is possible to distinguish binding reactions that occur upon challenge according to changes in the electrophoretic mobility of the complexes. D1/SATIII complexes were perturbed in their mobility by the addition of MATH20 (compare lanes 17–19 with lane 7). This indicates that MATH20 can displace D1. It is likely that, at intermediate amounts of added MATH20 (lanes 17 and 18), both D1 and MATH20 are bound to the complex (compare with lanes 14 and 15). However, the mobility of the complex formed at the highest amount of added MATH20 was similar to that formed in the presence of MATH20 alone (compare lanes 19 and 16), in line with the possibility of a complete displacement of D1 by MATH20. In contrast, the mobility of MATH20/SATIII complexes formed under conditions corresponding to those in lane 15 was not altered after challenge with D1 (lanes 20–22, compare with lane 15), suggesting that MATH20 could not be displaced by D1. Note that samples in lanes 18 and 21, which correspond to a reversed order of addition of identical amounts of MATH20 and D1, differed significantly in their mobilities. Taken together, these results indicate that MATH20 competes with D1 for binding to SATIII repeats *in vitro*.

Do MATH20 and D1 bind identical or overlapping sites? We addressed this question by performing DNase-I-footprinting

experiments on SATIII DNA. As shown in Figure 2, well-defined footprints could be observed in both cases, corresponding to identical sites that were mapped to the numerous A•T tracts present in the SATIII sequence. These results reinforce the model that MATH20 may displace D1, as both proteins bind to identical sites in SATIII repeats and MATH20 has an extremely high affinity for AT-rich DNA.

MATH20 competes for binding of D1 to SATIII repeats *in vivo*

A competition between MATH20 and D1 would account for the suppression of *w^{m4}* PEV by MATH20. The availability of polyclonal antibodies raised against D1 (Aulner *et al.*, 2002) and against the mammalian HMGA protein from which the MATH20 artificial protein is derived (Beaujean *et al.*, 2000) allowed us to test for a direct displacement of D1 by MATH20 *in vivo*. As shown for purified D1 and MATH20 proteins and for *Drosophila* Kc nuclei (Figure 3A), the D1 antibody reacted exclusively with D1 and did not cross-react with MATH20 (Figure 3B). Similarly, the HMGA antibody only recognized MATH20 and, significantly, did not cross-react with any protein present in Kc nuclei (Figure 3C).

We used these antibodies to study the localization of D1 in MATH20-expressing cells of eye imaginal disks from third-instar larvae. In this case, expression of a MATH20 transgene driven by a tetO promoter is directed to the region of eye disks posterior to the morphogenetic furrow by a tetR-VP16 transactivator under the control of the *eyeless* enhancer (Bello *et al.*, 1998; Girard *et al.*, 1998). In line with its specificity, the HMGA serum detected MATH20 in the posterior region of eye disks only (Figure 3D, top panel), but not in the salivary glands (middle panel) or in the wing disk (bottom panel). For comparison, D1 staining in the

Fig. 2. D1 and MATH20 bind identical sites of SATIII repeats. DNase-I-footprinting analyses were performed on complexes formed as described in the legend to Figure 1 using 0.75, 1.5 or 3 ng D1 (lanes 2–4) or 10, 5 and 2 ng MATH20 (lanes 5–7). Lanes marked NP contained no added protein. The gels to the left and to the right correspond to the 3' end-labeled lower and upper strands, respectively, of a *HinfI*-digested SATIII 359-bp monomer (Hsieh and Brutlag, 1979). Shaded boxes represent protected regions, and the scissors indicate the position of cleavage by topoisomerase II *in vivo* (Käs and Laemmli, 1992).

salivary gland of MATH20-expressing larvae is shown in the inset of the middle panel.

We next ascertained the association of MATH20 with SATIII and/or SATI repeats and assessed the localization of D1 as a function of MATH20 expression. Figure 4A and B show the results of control immunofluorescence experiments for MATH20 and D1 in larvae carrying the MATH20 transgene without its transactivator (see Methods). In this case, no MATH20 could be detected in the posterior region of eye disks, whereas D1 immunolocalized to SATI and SATIII repeats detected by staining with

Lex9F, a fluorescein-labeled A•T-hook-like oligopyrrole that interacts specifically with the minor groove of dA•dT-rich sequences (Janssen *et al.*, 2000a; Aulner *et al.*, 2002).

In MATH20-expressing larvae, the HMGA antibody detected the protein in the posterior part of eye disks (Figure 4C) but not in the anterior part (Figure 4E). To assess whether MATH20 was associated with DNA, we used an antibody directed against a synthetic A•T-hook peptide that does not interact with DNA-bound A•T-hook proteins (Beaujean *et al.*, 2000). This antibody recognized MATH20 in western blots but not in eye disks, suggesting that the protein is indeed DNA bound (data not shown). The MATH20 signal was found to co-localize strictly with SATI and SATIII repeats revealed by Lex9F staining, showing that the artificial protein binds sites in heterochromatin that are identical to those recognized by D1. In support of this conclusion, the D1 signal was found to be strongly reduced or absent from the posterior region of eye disks from MATH20-expressing larvae (Figure 4D) but was not affected in the anterior part of the disk (Figure 4F). Although we cannot rule out the possibility that the loss of the D1 signal reflects a loss of accessibility of the protein to the antibody due to the presence of MATH20, we consider this unlikely in view of the *in vitro* competition and binding results shown in Figures 1 and 2.

MATH20-expression levels are lower in the region immediately posterior to the morphogenetic furrow and which we call the *ante-posterior* region of eye disks (see inset in the upper panel of Figure 3D). A partial displacement of D1 from discrete sites might occur in this region, a possibility difficult to test in interphase nuclei. For this reason, we treated growing larvae with colchicine to assign D1 and MATH20 signals to SATI and SATIII repeats on individual chromosomes (Aulner *et al.*, 2002). As shown in Figure 5, we could detect D1 and MATH20 on chromosome spreads from the anterior and posterior regions, respectively, of eye disks from larvae expressing the artificial protein (Figure 5A and B). In comparison with the Lex9F signal, the MATH20 signal was found to be indistinguishable from that seen for D1, confirming that the localization pattern of MATH20 is identical to that of D1 *in vivo*. Again, D1 could not be detected in the posterior region of eye disks from MATH20-expressing larvae (Figure 5E), whereas MATH20 was absent from the anterior region (Figure 5F). When metaphase spreads from the *ante-posterior* region were examined, we could reproducibly detect a selective loss of D1 from SATIII repeats in the pericentric region of the X chromosome (see arrowheads in Figure 5C, and compare with the localization pattern of the *ante-posterior* D1 control, Figure 5D, or to the anterior region of MATH20-expressing larvae, Figure 5A).

Taken together, our results show that MATH20 competes with the *Drosophila* D1 protein for identical binding sites in SATIII DNA *in vitro* and displaces D1 from SATI and SATIII repeats *in vivo*. Furthermore, D1 can be selectively displaced from the SATIII repeats located in the pericentric heterochromatin of the X chromosome and which are thought to be involved in *white-mottled* variegation.

DISCUSSION

The *white-mottled* inversion places the *white* gene near the pericentric heterochromatin of the X chromosome of *D. melanogaster*, a region containing tandem arrays of 359-bp SATIII

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Fig. 3. Selectivity of the antibodies against D1 and MATH20. (A–C) 500 ng each of purified D1 (lane 1) and MATH20 (lane 2) or 0.5 A₂₆₀ units of Kc nuclei (lane 3) were subjected to SDS–PAGE and stained with Coomassie Brilliant Blue (A) or western blotted using antibodies against D1 (B) or HMGA (C). (D) An eye-antenna disk (top), a salivary gland (middle) and a wing disk (bottom) from MATH20-expressing larvae immunostained with the HMGA antibody. The inset in the top panel shows the enlarged *ante*-posterior region of the eye disk where MATH20 expression levels are reduced. The inset in the middle panel shows a salivary gland from MATH20-expressing larvae immunostained for D1. Scale bar = 250 μ m.

repeats over ~11 million base pairs (Lohe *et al.*, 1993). While not formally proven, it seems plausible that SATIII plays a role in heterochromatin-mediated silencing via its interaction with the multi-A•T-hook D1 protein. D1 localizes to SATIII repeats *in vivo* and is a modifier of *w^{m4}* variegation, as demonstrated by the effects of the EP473 P-element insertion in the promoter region of the D1 gene (Aulner *et al.*, 2002). Significantly, expression of D1 Δ E, a deletion of the acidic C-terminal domain of D1 that resembles a shorter MATH20 protein, acts as a dominant-negative mutation of D1 and strongly suppresses *w^{m4}* PEV, an effect similar to that of MATH20 (Janssen *et al.*, 2000b). The correlation that we establish between these opposite genetic effects and the displacement of D1 by MATH20 from SATIII sequences *in vitro* and *in vivo* strongly supports a model whereby the suppression of PEV by MATH20 is due to the displacement of D1. D1 is also massively recruited to SATI heterochromatin and might act there as well (Aulner *et al.*, 2002). It is possible, however, that this satellite might be less sensitive to perturbations in D1 levels, as D1 binds SATI better than it binds SATIII (Levinger, 1985).

Many other modifiers of *white-mottled* PEV, such as HP1, Su(var)3-9 and HDAC1, have been identified (Eissenberg and Elgin, 2000; Czermin *et al.*, 2001; Schotta *et al.*, 2002). Interestingly, HP1 and Su(var)3-9 also appear to be involved in gene-specific repression (Hwang *et al.*, 2001), and recent models suggest that reading of the histone code could be essential to the maintenance/propagation of a silenced state (Bannister *et al.*,

2001; Lachner *et al.*, 2001). How such a state is initiated remains unclear; for instance, HP1 binds DNA and nucleosomes but without clear sequence preference (Zhao *et al.*, 2000). It seems likely that sequence-specific DNA-binding proteins could be involved in recruiting proteins such as HDAC1 and Su(var)3-9 to selected genomic sites. Deacetylation/methylation events would then provide recognition sites for HP1, leading to assembly of silenced chromatin (Bannister *et al.*, 2001; Czermin *et al.*, 2001; Lachner *et al.*, 2001; Schotta *et al.*, 2002). Our results suggest that D1 possesses the properties required to effect such a targeting function.

Recently, Su(var)3-7, a partner of HP1, has been shown to bind DNA and it exhibits a preference for AT-rich satellite sequences such as SATI repeats and a 353-bp satellite DNA sharing sequence similarities with SATIII (Cléard and Spierer, 2001). Our own results suggest that D1 and Su(var)3-7 act in a common pathway: the enhancement of *w^{m4}* PEV by expression of an inducible Su(var)3-7 transgene (Cléard *et al.*, 1997) can be abolished by expression of D1 Δ E (data not shown). In this case, it would appear that the A•T hooks of D1 provide more affinity for binding to AT-rich sequences than the modular zinc fingers of Su(var)3-7. However, the latter have been proposed to bind to a wider range of DNA sequences, whereas the effects of D1 might be more specifically exerted in SATIII heterochromatin. It will be of interest to examine binding of Su(var)3-7 and D1 to SATI and SATIII repeats *in vitro* and *in vivo*, as the localization of Su(var)3-7 has only been examined in interphase nuclei and in

Fig. 4. Displacement of D1 by MATH20 *in vivo*. Eye imaginal disks were processed as described in Methods for MATH20 and D1 immunofluorescence (IF). Photographs are identified on the top and bottom and show merged DAPI and IF signals. Insets show the red IF signal and the green SATI and SATIII Lex9F signal. Control samples in (A) and (B) correspond to the posterior region of eye disks from larvae carrying the MATH20 transgene without its transactivator, and samples in (C–F) correspond to posterior and anterior regions of eye disks from MATH20-expressing larvae. Scale bar = 5 μm.

Fig. 5. Selective displacement of D1 from X-chromosome SATIII repeats. Eye disks from colchicine-fed larvae were processed as described in the legend to Figure 4. Samples are identified above and below each panel. X and Y chromosomes are marked as appropriate, and arrowheads in (C) (*ante-posterior* cells of a male larva) point to the pericentric region of the X chromosome where the absence of D1 signal is to be noted. Scale bar = 5 μm.

polytene chromosomes, which do not allow a precise assignment to individual chromosomes and specific satellite repeats (Clard *et al.*, 1997).

We propose that D1 might initiate heterochromatin formation by recruiting other heterochromatin proteins to SATI and SATIII DNA. The recruitment function of D1 would most likely be provided by its C-terminal domain, which has also been shown to be required for its direct interaction with the BEAF32 protein involved in boundary activity (Cuvier *et al.*, 2002). Our model is in line with the localization of D1 *in vivo*, its DNA-binding

properties and its effects on PEV and is further supported by direct evidence for a displacement of D1 by the MATH20 artificial suppressor. Moreover, the synthetic P9 oligopyrrole that mimics the properties of the A•T-hook motif (Janssen *et al.*, 2000a) also suppresses *w^{m4}* PEV (Janssen *et al.*, 2000b). As in the case of MATH20, P9 displaces D1 from its binding sites *in vivo* (C. Monod, O. Cuvier, N. Aulner and E. Ks, manuscript in preparation). This system now allows us to examine directly the localization of other heterochromatin-associated proteins as a function of a specific and controlled displacement of D1 *in vivo*.

METHODS

DNA binding and footprinting assays. Purified bacterially expressed recombinant D1 and MATH20 proteins were incubated with 1 ng of *Hinfl*-digested end-labeled SATIII DNA in the presence of 230 ng of salmon sperm DNA (average size 2 kb) in a 20 µl volume of 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol for 30 min at 28°C. Gel shifts were performed as described previously (Zhao et al., 1993). For competition experiments, complexes preformed for 15 min were challenged with increasing concentrations of MATH20 or D1, incubated for an additional 15 min and processed as above. Footprinting experiments were performed on similarly prepared samples, except that glycerol was omitted and MgCl₂ was added to a final concentration of 5 mM. Samples were digested for 1 min at 28°C with 5–25 ng of DNase I (20 000 U/mg), purified and electrophoresed on sequencing gels.

Western blotting, immunostaining and immunolocalization. Experiments with the D1 and HMGA antibodies were performed exactly as described previously (Beaujean et al., 2000; Aulner et al., 2002). For immunofluorescence, we used a short acid fixation protocol that results in optimal detection while preserving nuclear and chromosome morphology (Aulner et al., 2002). Samples were stained with Lex9F (Janssen et al., 2000a), mounted in DAPI and visualized by epifluorescence microscopy. For the experiment shown in Figure 5, actively feeding medium-sized third-instar larvae were starved for 3 h before being transferred to plates containing 100 µg/ml colchicine and yeast paste supplemented with the same concentration of the drug. The following day, larvae dissected in 0.5% sodium citrate yielded eye imaginal disks containing metaphases that were processed as above. Fly strains used in this study are as follows: strain 31 carries the tetO-MATH20 responder transgene and strain 53 expresses the *eyeless*-tetR-VP16 transactivator (Bello et al., 1998; Girard et al., 1998). Both transgenes are on chromosome 3. In Figures 4 and 5, controls refer to strain-31 homozygotes, whereas MATH20-expressing larvae were recovered from a cross between strain-31 and strain-53 homozygotes.

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