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Differential Epigenetic Regulation Within an Arabidopsis Retroposon Family

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ABSTRACT

We previously reported a novel family of *Arabidopsis thaliana* nonautonomous retroposons, *Sadhu*, showing epigenetic variation in natural populations. Here, we show that transcripts corresponding to *Sadhu* elements accumulate in a subset of mutants carrying disruptions in genes encoding chromatin modification enzymes, but are not significantly expressed in mutants defective in RNA silencing pathways, indicating that RNA-directed processes are not necessary to maintain transcriptional suppression of this class of retroelements. We focused our analysis on three representative elements showing differential responses to *ddm1*, *met1*, and *hda6* mutations. These mutations had differing effects on cytosine methylation depending on the element and the sequence context. Curiously, the *Sadhu6-1* element with the strongest CpHpG methylation is expressed in a *met1* CpG methyltransferase mutant, but is not expressed in *ddm1* or *cmt3* mutants. Regardless of the mutant background, H3meK9 was found at silenced loci, while H3meK4 was restricted to expressed alleles. We discuss the different modes of regulation within this family and the potential impact of this regulation on the stability of silencing in natural populations.

EUKARYOTIC genomes are crowded with a diverse array of transposable elements. Effective control of transposons is essential to maintain the integrity of the genome. This control is exerted initially at the epigenetic level by interfering with element expression and movement. One level of control is exerted by post-transcriptional turnover of element transcripts through RNA interference (RNAi) (Sijen and Plasterk 2003; Almeida and Allshire 2005). Transposons are also controlled epigenetically by altering the accessibility of the elements to the transcriptional machinery and transposases through cytosine hypermethylation and differential chromatin modification and packaging (Hirochika *et al.* 2000; Miura *et al.* 2001; Singer *et al.* 2001; Lippman *et al.* 2003; Kato *et al.* 2004). Subsequent accumulation of mutations within epigenetically silenced elements leads to their irreversible inactivation. In some cases, this genetic decay is accelerated by epigenetic modification as methylated cytosines mutate at a higher rate than unmodified cytosines (Bird 1980; Poole *et al.* 2001).

Transposable elements are particularly prevalent in plant genomes. For instance, ~55% of the sequences in characterized "gene-rich" regions of the wheat genome are composed of transposable elements and it is estimated that >80% of the wheat genome corresponds to transposons (Sabot *et al.* 2005). Even the small genome of Arabidopsis has >2300 transposable element sequences, excluding the elements that reside in the unsequenced gaps corresponding to the centromeres (Haas *et al.* 2005).

The abundance of transposons in plant genomes is associated with the apparent elaboration of cytosine methylation systems. In plants, cytosine methylation is found in three different nucleotide contexts: CpG, CpHpG, and CpHpH (where H is A, C, or T). This expansive DNA methylation results from the interaction of three different methyltransferase systems—MET1 (a Dnmt1-class CpG methyltransferase) (Finnegan and Dennis 1993; Kankel *et al.* 2003), CMT3 (Chromomethyltransferase 3, targeting primarily CpHpG methylation) (Bartee *et al.* 2001; Lindroth *et al.* 2001), and DRM2 (the CpHpH *de novo* methyltransferase) (Cao and Jacobsen 2002b). Examination of the effects of mutations in these methyltransferase genes suggests that cytosine methylation at CpG and CpHpG contributes to silencing of transposable elements (Tompa *et al.* 2002; Kato *et al.* 2003; Lippman *et al.* 2003; Tran *et al.* 2005).

Cytosine methylation interacts with chromatin modifiers and small RNA processing enzymes to promote epigenetic silencing. The CMT3 protein can recognize histone H3 methylated at both lysine 9 and lysine 27, and the effects of *cmt3* mutations resemble those of the histone H3 lysine 9 methyltransferase *SUVH4/KYP*

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. DY230445, DY230446, EC268296, EC613982, EC613983, EC613984TH, EC613985, and EC613986.

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(Jackson *et al.* 2002; Lindroth *et al.* 2004). Therefore, it is likely that CMT3 provides a link between chromatin level and DNA methylation silencing. Other chromatin modifying enzymes that act in transposon silencing include Decrease in DNA Methylation 1 (DDM1) (Miura *et al.* 2001; Singer *et al.* 2001; Gendrel *et al.* 2002; Lippman *et al.* 2003), a SWI2/SNF2 chromatin remodeling protein, and HDA6, an RPD3-class histone deacetylase (Lippman *et al.* 2003). Furthermore, because DRM2-directed *de novo* methylation is thought to be directed by the RNA silencing pathway (Cao *et al.* 2003), Tran *et al.* (2005) proposed that an RNA-directed pathway might silence dispersed transposable elements in otherwise unsilenced regions of the genome. This model is supported by the considerable overlap between targets of DRM2 and AGO4 (Tran *et al.* 2005), an Argonaute family protein implicated in RNA-directed DNA methylation (Zilberman *et al.* 2003). Work in maize has also highlighted the importance of the RNA-directed RNA polymerase, *MOP1*, in maintaining methylation of *Mutator* elements (Lisch *et al.* 2002; Alleman *et al.* 2006; Woodhouse *et al.* 2006a,b). Notably, these elements become reactivated only after several generations of propagation in a *mop1* mutant background. Some repetitive sequences are also regulated by the plant-specific RNA polymerase IV involved in small RNA-directed chromatin silencing (Herr *et al.* 2005; Kanno *et al.* 2005; Onodera *et al.* 2005; Huettel *et al.* 2006).

We recently discovered the Sadhu family of small nonautonomous non-LTR retroposons in Arabidopsis in a screen for DNA sequences showing differential epigenetic modification among different natural strains (Rangwala *et al.* 2006). These sequences are nonprotein coding with an average pairwise nucleotide identity of ~70%. Since there are no clear RNA polymerase II or RNA polymerase III promoter consensus sequences in the vicinity of the elements, the mode of transcriptional regulation is unclear, as is the identity of the mobilizing autonomous element. However, Sadhu elements can be transcribed, often at high levels, and the transcripts are polyadenylated. We previously observed a large degree of variability in natural populations in expression and cytosine methylation of members of this retroposon family. Here, we report that different members of this transposon family are silenced with varying contributions from overlapping epigenetic modification pathways. These results suggest that the type of epigenetic regulation targeted at transposable elements is locus specific and that plant genomes do not use a single strategy for epigenetically silencing transposons, even for different members of a single transposon family.

MATERIALS AND METHODS

Plant materials: The *ddm1-1* and *met1-1* mutations were generated using EMS in a Col strain background. The original mutant isolates were backcrossed at least six times to Col wild type to remove unlinked mutations (Vongs *et al.* 1993; Kankel *et al.* 2003). *ddm1-1* homozygotes were examined in the first generation of self-fertilization to minimize the progressive accumulation of genetic and epigenetic alleles, as previously reported in this background (Kakutani *et al.* 1996). The *met1-1* allele is hypomorphic but not null. The *suvh4* allele corresponds to T-DNA line SALK_044606 (ABRC), the *drm2* allele corresponds to T-DNA SAIL_70_E12 (ABRC), and the *cmt3-11* allele corresponds to SALK_148381 (ABRC) (Chan *et al.* 2006). The *rdr2-1* (GARLIC_1227) and *dcl3-1* (SALK_005512) mutants were obtained from J. Carrington (Xie *et al.* 2004). The *hda6-5/axe1-5* mutant was obtained from T. Guilfoyle (Murfett *et al.* 2001) while the *nrpd2a/nrpd2b* double mutant was obtained from C. S. Pikaard (Onodera *et al.* 2005). Natural wild-type accessions were obtained from ABRC or Lehle Seed Company; stock numbers are indicated in Table 3.

Plant material was grown on soil or 1× MS + 1% sucrose plates for 2–3 weeks before preparing for RT–PCR, cytosine methylation, or ChIP analysis. Samples from each mutant background were grown in parallel to minimize environmental effects.

RNA and DNA analysis: DNA was isolated from rosette leaves or whole 3-week-old seedlings as previously described (Rangwala *et al.* 2006). RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) followed by DNAseI treatment (Invitrogen). First-strand cDNA was primed with oligo(dT) (15) primer using Superscript II reverse transcriptase (Invitrogen). Reactions excluding the reverse transcriptase were carried out in parallel to monitor DNA contamination. PCR was conducted with *Taq* DNA polymerase (QIAGEN, Valencia, CA) under standard cycling conditions. Primers within the transcribed region of cyclophilin (*At4g38740*) (Henikoff and Comai 1998) were used as PCR amplification controls. Data in Figure 1 are representative of two independent biological replicates of all samples. RT–PCR for the *Sadhu* elements used primers X1 + Y1 indicated in Figure 2. All primer sequences in this study are provided in supplemental Table 1 at http://www.genetics.org/supplemental/.

The 5′ ends of *Sadhu3-1*, *Sadhu7-2*, and *Sadhu6-1* were determined using SMART RACE cDNA amplification (BD Biosciences) reagents and protocols and primers Sadhu3-1Y1, Sadhu7-2Y1, and Sadhu6-1X1; 3′ RACE of *Sadhu3-1* used primer Sadhu3-1X3. The RACE PCR products were cloned into pGEMT-EASY (Promega, Madison, WI) and transformed into heat-shock-competent *Escherichia coli* using standard protocols. Plasmid from individual colonies was isolated using QIAprep spin columns (QIAGEN) and sequenced using T7 and Sp6 primers and Big-Dye Terminator cycle sequencing (Perkin-Elmer, Norwalk, CT) reagents.

DNA methylation analysis: Genomic DNA from Col wild-type, *ddm1-1*, *met1-1*, and *hda6-5* was modified by sodium bisulfite using the CpGenome DNA modification kit (Chemicon) or the EpiTect Bisulfite Kit (QIAGEN). For COBRA assays, modified DNA was first amplified by PCR (36–40 cycles) with Klentaq (CLONTECH, Palo Alto, CA) and the resulting products were digested with the endonucleases (New England Biolabs, Beverly, MA) indicated in Figure 3. Converted DNA is susceptible to *Pac*I and *Hph*I cleavage and resistant to *Taq*I, *Mbo*I, and *Hpa*II cleavage. We controlled for efficient bisulfite conversion using a *Pac*I COBRA assay in an amplicon of *At1g01010* (primer At1g01010 F + R), which was previously determined to be unmethylated (H. Kuo, unpublished data) (Figure 3A). PCR for COBRA at the *Sadhu* elements used primers Bt1 + Bt2. Data in Figure 3 are representative of COBRA assays conducted on at least two independent conversions for each mutant background.

PCR products from bisulfite-modified genomic DNA templates were cloned and sequenced as described above for

RACE. Primers Bt1 + Bt2 and Bb1 + Bb2 amplify "top" and "bottom" strand-specific bisulfite-modified products. Between 11 and 15 clones were sequenced per strand from Col wild type for each of *Sadhu3-1*, *Sadhu7-2*, and *Sadhu6-1*. Between 11 and 27 clones were sequenced per locus using either the Bt1 + Bt2 amplicon (*Sadhu7-2* and *Sadhu6-1*) or the Bb1 + Bb2 amplicon (*Sadhu3-1*) generated from *ddm1-1*, *met1-1*, *hda6-5*, or *cmt3-11* genomic templates. The sequence data were derived from at least two independent PCR amplifications per genetic background per locus. Complete bisulfite data along with DNA sequence context are presented in graphical form in supplemental Figure 2 at http://www.genetics.org/supplemental/. McrBC (New England Biolabs) digests in Table 3 were carried out at 37° overnight using the supplier's recommended conditions as described previously (Rangwala *et al.* 2006).

Chromatin immunoprecipitation: Chromatin immunoprecipitation (ChIP) assays were carried out using the protocol described in Lawrence *et al.* (2004). Immunoprecipitation was carried out overnight using no antibody, antibody to trimethylated histone H3 lysine 4 (Abcam AB-8580, Cambridge, UK), or antibody to dimethylated histone H3 lysine 9 (Upstate 07-441, Temecula, CA). Precipitated DNA was resuspended and PCR was performed using Klentaq (CLONTECH) and standard cycling conditions for 35–40 cycles. Three independent biological replicates of chromatin immunoprecipitation were conducted. ChIP PCR used primers Sadhu3-1X1 + Y2, Sadhu7-2X2 + Y2, and Sadhu6-1X2 + Y2 for the *Sadhu* elements, as well as primers At4g04040 F + R (Gendrel *et al.* 2002) and cinful F + R as controls for H3meK4 and H3meK9 antibodies, respectively.

RESULTS

Derepression of *Sadhu* elements in different chromatin mutant backgrounds: We recently reported a family of previously uncharacterized *Arabidopsis thaliana* retroelements, named *Sadhu* (Rangwala *et al.* 2006). These elements are dispersed, do not encode proteins, exist at low copy numbers, and share only moderate sequence similarity (typical pairwise nucleotide identity ~70%). We developed an ontological scheme on the basis of sequence similarity to rename each family member (supplemental Figure 1 at http://www.genetics.org/supplemental/). One member of this family, *Sadhu1-1* (*At2g10410*), exists in different epigenetic states in different natural accessions, despite the fact that this element in different accessions is inserted into the same chromosomal location and is nearly identical in nucleotide sequence (Rangwala *et al.* 2006). The silenced epigenetic state of the *Sadhu1-1* allele from the Ler strain contains dense CpG methylation, and both silencing and DNA methylation can be reversed by a mutation in the *DDM1* gene (Rangwala *et al.* 2006).

We were interested in determining whether other *Sadhu* elements were regulated epigenetically, as well as understanding the epigenetic mechanisms operating on the elements. One possibility is that all elements in the same transposon family are regulated using the same epigenetic mechanisms. Alternatively, different *Sadhu* elements might be regulated independently on the basis of their genomic environment, DNA sequence, or evolutionary history. We chose six elements that were single copy and not expressed in the Col strain (Figure 1) and examined their expression by RT–PCR in eight mutants in the Col background carrying loss-of-function or hypomorphic mutations in genes previously implicated in chromatin level gene regulation (*ddm1*, *met1*, *hda6/axe1*, and *suvh4/kyp*) or RNA-directed silencing processes (*rdr2*, *drm2*, *nrpd2*, and *dcl3*) (Figure 1). All six elements were expressed in at least one of the mutants defective in chromatin regulation. *Sadhu3-1*, *Sadhu3-2*, *Sadhu5-2*, and *Sadhu8-1* were expressed in the *ddm1*, *met1*, and *hda6* mutants. These four elements together form a group that is susceptible to multiple mutations that perturb maintenance of transcriptionally silent chromatin. In addition, *Sadhu3-2* showed increased expression in the *suvh4* mutant. *Sadhu5-2* showed slight expression in the *suvh4* mutant, while *Sadhu3-1* showed slight expression in the *drm2* and *rdr2* mutants. By contrast, *Sadhu7-2* was expressed at high levels only in the *ddm1* mutant, with relatively little expression in *met1*, while *Sadhu6-1* was expressed exclusively in the *met1* mutant. Notably, none of the six *Sadhu* elements were strongly expressed in any of the lines carrying mutations implicated in RNA-directed *de novo* silencing.

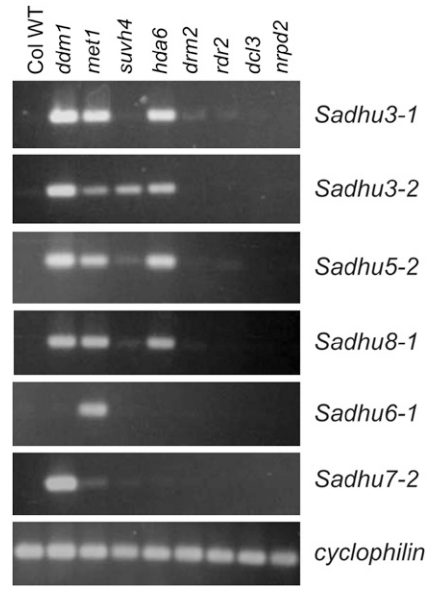


Figure 1.—RT–PCR expression analysis of six *Sadhu* elements in Col wild-type and various chromatin or RNA silencing mutants. Cyclophilin amplification is shown as an input control.

The observation that the six *Sadhu* elements fell into three distinct classes with respect to chromatin mutation susceptibility (*ddm1/met1/hda6*, *ddm1* ≫ *met1*, *met1* only) points to the diversity of strategies used to silence these elements. We focused on representative elements from these three classes—*Sadhu3-1*, *Sadhu6-1*, and *Sadhu7-2*—as models to study different modes of *Sadhu* retroelement regulation in Arabidopsis. As a foundation for these studies, we mapped the 5′ ends of the

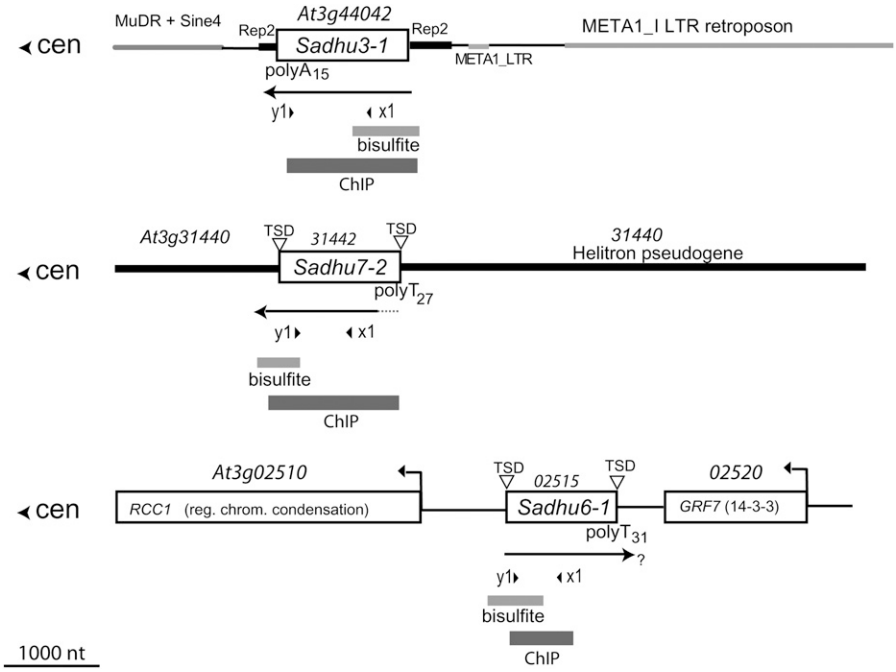
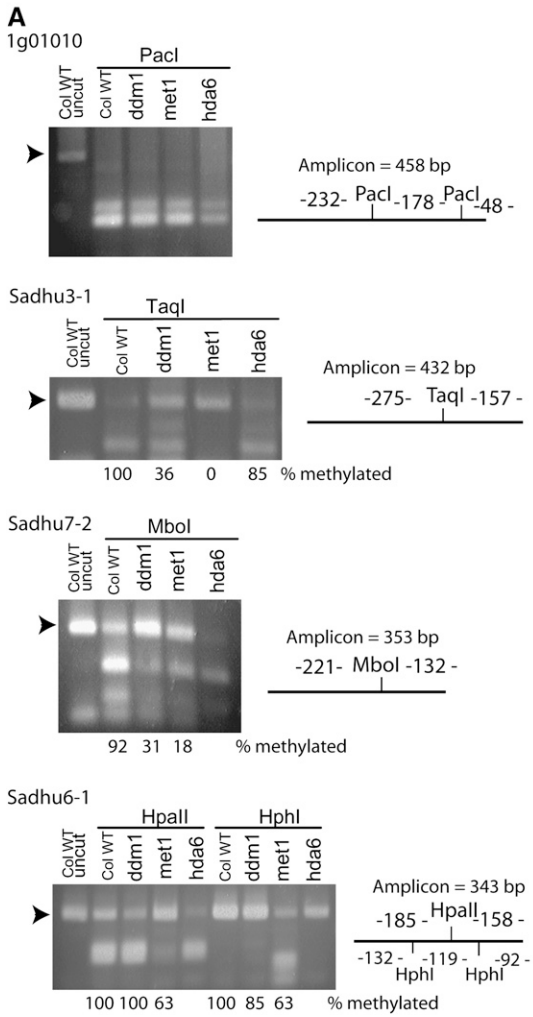


FIGURE 2.—Detailed maps of ~6-kb regions encompassing *Sadhu3-1*, *Sadhu7-2*, and *Sadhu6-1*. The direction of the centromere and the scale are indicated. The horizontal arrow below each element denotes the direction and length of the transcript, as determined by RACE–PCR. The dotted region of the arrow under *Sadhu7-2* indicates a lack of a firm 5′ boundary of transcription. The 3′ end of the *Sadhu6-1* transcript has not been empirically determined, as denoted by the question mark. Poly(A) or poly(T) tracts are marked on the lower strand. Target site duplications (TSD), where present, are indicated. MuDR, SINE4, REP2, and META1 are repetitive elements in the vicinity of *Sadhu3-1* (JURKA *et al.* 2005). The location of PCR regions assayed by RT–PCR (X1 + Y1), bisulfite sequencing, and ChIP are marked for each element.

transcripts using RACE–PCR in genetic backgrounds that expressed the element (*ddm1* for *Sadhu3-1* and *Sadhu7-2*; *met1* for *Sadhu6-1*). In all three cases, the start of transcription mapped close to or within the sequence of the element, indicating that ectopic transcription in these mutants is not due to readthrough from a promoter far upstream of the element (Figure 2). In the case of *Sadhu7-2*, transcription was antisense to the direction of the retroposon and 5′ RACE analysis identified several alternate 5′ ends. The 3′ end of the *Sadhu3-1* transcript was determined by 3′ RACE and shown to be polyadenylated 3′ to the poly(A) tract. *Sadhu3-1* and *Sadhu7-2* are embedded within DNA transposon remnants in the repeat-rich pericentromere, whereas *Sadhu6-1* is found on the chromosome arm in an intergenic region between two protein-coding genes with no nearby repetitive elements (within 100 kb) (Figure 2).

Effects of mutations in chromatin regulators on cytosine methylation at different *Sadhu* elements: Next, we examined cytosine methylation at the representative *Sadhu* elements—*Sadhu3-1*, *Sadhu6-1*, and *Sadhu7-2*—both in Col wild-type and in selected mutant backgrounds. We focused our analysis on the mutations that had the most striking effect on transcription of the *Sadhu* elements: *ddm1*, *met1*, and *hda6*. We used *co*mbined *b*isulfite *r*estriction *a*nalysis (COBRA) (XIONG and LAIRD 1997) to examine loss or gain of a restriction enzyme recognition site after genomic amplification from templates treated with sodium bisulfite, which converts unmethylated cytosines to uracil. For *Sadhu3-1*, which was ectopically expressed in *ddm1*, *met1*, and *hda6* mutants (Figure 1), loss of *Taq*I cleavage indicates hypomethylation of the CpG within the *Taq*I restriction site. While we observed substantial *Taq*I cleavage in the Col wild-type sample, we noted decreased cleavage of the amplified product derived from the *ddm1* mutant and little or no cleavage of the *met1* mutant product (Figure 3A). However, the *hda6* mutant sample showed the same amount of cleavage as Col wild type, suggesting that no change in cytosine methylation occurred in this mutant at this particular CpG site despite the loss of transcriptional silencing. We also obtained DNA sequence from within the *Sadhu3-1* element from cloned products derived from Col wild-type, *ddm1*, *met1*, and *hda6* bisulfite converted templates (Figure 3B; Tables 1 and 2). The *ddm1* mutant samples showed a dramatic loss of CpG methylation relative to Col wild type from 89 to ~17%, as well as a change in CpHpG methylation from 31 to 3%. The *met1* mutant, consistent with the COBRA assay, showed an even greater reduction of methylation in this region, with <3.5% of cytosines methylated regardless of sequence context. The *hda6* mutant also showed a dramatic decrease in CpHpG and CpHpH methylation. However, the loss of CpG methylation was more modest. Consistent with the COBRA result suggesting retention of methylation, the CpG present in the assayed *Taq*I site was methylated in 85% of sequenced clones. Therefore, although all three mutations—*ddm1*, *met1*, and *hda6*—were able to derepress the *Sadhu3-1* element (Figure 1), each mutation had a unique effect on cytosine methylation at this locus.

We next examined cytosine methylation of the *Sadhu7-2* element using a COBRA assay reporting on CpG methylation at an *Mbo*I site. This site was cleaved in a majority of the products derived from Col wild-type and the *hda6* mutant templates, but hypomethylated in the *ddm1* and *met1* mutants (Figure 3A), consistent



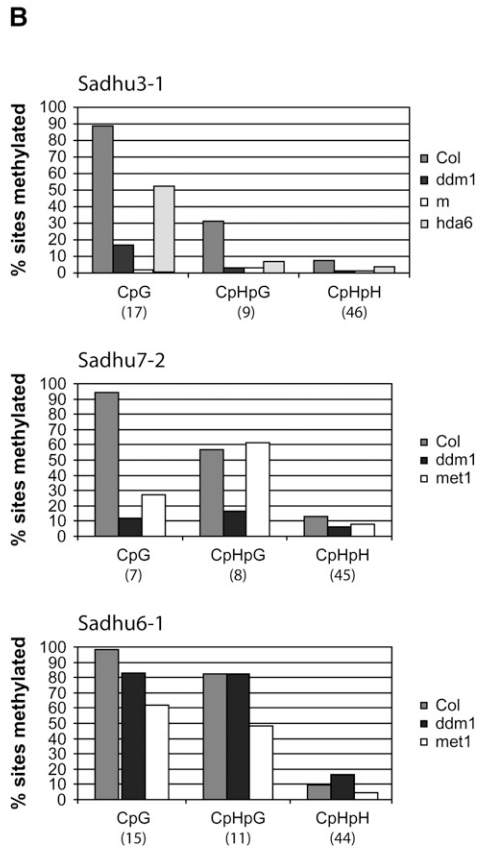


FIGURE 3.—DNA cytosine methylation of *Sadhu* elements in Col wild-type, *ddm1*, *met1*, and *hda6* mutants. (A) COBRA analysis of an unmethylated control locus, *At1g01010* (cleavage confirms efficient conversion of bisulfite-modified templates), and *Sadhu3-1*, *Sadhu7-2*, and *Sadhu6-1* elements. Restriction maps of assayed regions are shown to the right of the gel images. Arrowheads indicate positions of uncleaved PCR product. Numbers below lanes indicate percentage of methylation at the cytosines assayed by COBRA, as determined by sequencing of independent bisulfite-modified amplicons. (B) Percentage of methylated sites in Col wild-type, *ddm1*, *met1*, and *hda6* (*Sadhu3-1*) genetic backgrounds by sequence context, as determined by sequencing of bisulfite-modified amplicons. Numbers in parentheses below graphs indicate the number of cytosines in each sequence context for each locus. H is C, T, or A. A complete graphical representation of bisulfite information at each individual cytosine residue is available in supplemental Figure 2 at http://www.genetics.org/supplemental/.

with the accumulation of transcripts in these mutants. Sequencing of amplification products from bisulfite-treated templates revealed that CpG methylation was reduced from 95% in Col wild type to 27% in the *met1* mutant and 12% in the *ddm1* mutant (Figure 3B; Tables 1 and 2). CpHpG methylation was not decreased in the *met1* mutant, but was greatly reduced from 57% in Col wild type to 16% in the *ddm1* mutant. This more extreme hypomethylation in the *ddm1* mutant correlates well with the RT–PCR data that indicated striking expression of *Sadhu7-2* in the *ddm1* mutant but little to no expression in the *met1* or *hda6* mutants (Figure 1).

TABLE 1

Proportion of cytosines methylated in Col wild type in *Sadhu3-1*, *Sadhu7-2*, and *Sadhu6-1*

		Sadhu3-1			*Sadhu7-2*			*Sadhu6-1*		
Strand	No. of sites	CpG	CpHpG	CpHpH	CpG	CpHpG	CpHpH	CpG	CpHpG	CpHpH
Top	Assayed	266	210	1120	91	104	585	210	154	616
	Methylated (%)	230 (86.5)	39 (18.6)	60 (5.4)	86 (94.5)	59 (56.7)	77 (13.2)	207 (98.6)	127 (82.5)	60 (9.7)
Bottom	Assayed	255	135	675	91	91	416	77	77	352
	Methylated (%)	227 (89.0)	42 (31.1)	53 (7.9)	76 (83.5)	28 (30.8)	31 (7.5)	73 (94.8)	56 (72.7)	34 (9.7)
Combined	Assayed	521	345	1795	182	195	1001	287	231	968
	Methylated (%)	457 (87.7)	81 (23.5)	113 (6.3)	162 (89.0)	87 (44.6)	108 (10.8)	280 (97.6)	183 (79.2)	94 (9.7)

TABLE 2

Proportion of cytosines methylated in *ddm1*, *met1*, and *hda6* mutants

		Sadhu3-1			*Sadhu7-2*			*Sadhu6-1*		
Mutant	No. of sites	CpG	CpHpG	CpHpH	CpG	CpHpG	CpHpH	CpG	CpHpG	CpHpH
ddm1-1	Assayed	238	126	644	91	104	585	195	143	572
	Methylated (%)	40 (16.8)	4 (3.2)	10 (1.6)	11 (12.1)	17 (16.3)	35 (6.0)	162 (83.1)	118 (82.5)	94 (16.4)
met1-1	Assayed	238	126	644	77	88	495	405	297	1188
	Methylated (%)	5 (2.1)	4 (3.2)	10 (1.6)	21 (27.3)	54 (61.4)	40 (8.1)	250 (61.7)	144 (48.5)	54 (4.5)
hda6-5	Assayed	221	117	598						
	Methylated (%)	114 (51.6)	8 (6.8)	22 (3.7)						

The *Sadhu6-1* element was also assayed for changes in cytosine methylation in the mutant backgrounds by both COBRA and bisulfite-mediated genomic sequencing. In this case, the COBRA assays monitored loss of CpG methylation at an *Hpa*II site, as well as the creation of an *Hph*I site upon bisulfite conversion of unmethylated DNA. Our COBRA results indicated that cytosine methylation was present in Col wild-type plants, persisted in the *ddm1* and *hda6* mutants, but was lost in the *met1* mutant (Figure 3A). These results were corroborated by sequencing of PCR products amplified from bisulfite-treated templates (Figure 3B; Tables 1 and 2). Products derived from the *ddm1* mutant showed a slight decrease in CpG methylation (99 → 83%) and a modest increase in CpHpH methylation (10 →16%) relative to Col wild type. Consistent with the COBRA result, the *met1* mutant showed a stronger decrease in methylation in both the CpG (99 → 62%) and CpHpG (83 → 49%) sequence contexts relative to Col wild type. A significant reduction of methylation in the *met1* but not the *ddm1* mutant mirrors the expression data showing expression of the *Sadhu6-1* element solely in the *met1* mutant (Figure 1).

Although all three elements showed a high degree of CpG methylation (~90%) and low levels of methylation at asymmetric CpHpH sites (~10%), each element showed a different degree of methylation at CpHpG sites in Col wild type (Table 1). CpHpG methylation was moderate at *Sadhu3-1* (24%), greater at *Sadhu7-2* (45%), and highest at *Sadhu6-1* (79%). Because the CMT3 chromomethylase enzyme is responsible for methylation at CpHpG sites, we examined a Col strain carrying a previously characterized hypomorphic T-DNA insertion allele in the *CMT3* gene (Chan *et al.* 2006). We did not see any increase in expression of the *Sadhu3-1*, *Sadhu7-2*, or *Sadhu6-1* loci in this Col *cmt3* mutant (Figure 4A). Bisulfite sequencing of the *Sadhu6-1* element in the *cmt3* mutant indicated a nearly 50% reduction of CpHpG methylation, with no change in CpG methylation (Figure 4B). Therefore, the partial loss of CpHpG methylation in this particular *cmt3* mutant was not sufficient to reverse silencing at this locus.

Distribution of H3meK4 and H3dimeK9 at different *Sadhu* elements correlates with expression state: Next we examined histone modification in nucleosomes associated with the three representative *Sadhu* elements using ChIP. Histone H3 trimethylated at lysine 4 (H3meK4) has been shown to be correlated with active genes in Arabidopsis and other eukaryotes, while H3 dimethylated at lysine 9 (H3meK9) is associated with silent loci (Gendrel *et al.* 2002; Lippman *et al.* 2003).

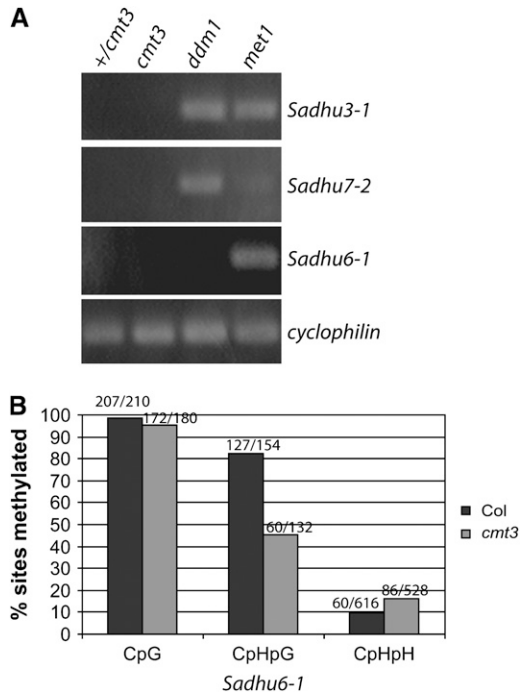


Figure 4.—*Sadhu* elements are not expressed in a mutant that decreases CpHpG methylation. (A) RT–PCR analysis of *Sadhu3-1*, *Sadhu7-2*, and *Sadhu6-1* in a *cmt3-11* mutant compared to heterozygous siblings (+/*cmt3*). *ddm1* and *met1* samples are included as controls. Cyclophilin is shown as an amplification control. (B) Percentage of methylated sites in Col wild type *vs. cmt3-11*, by sequence context, as determined by bisulfite-mediated genomic sequencing of *Sadhu6-1*. Ratios above bars are the number of methylated sites/total number of sites assayed.

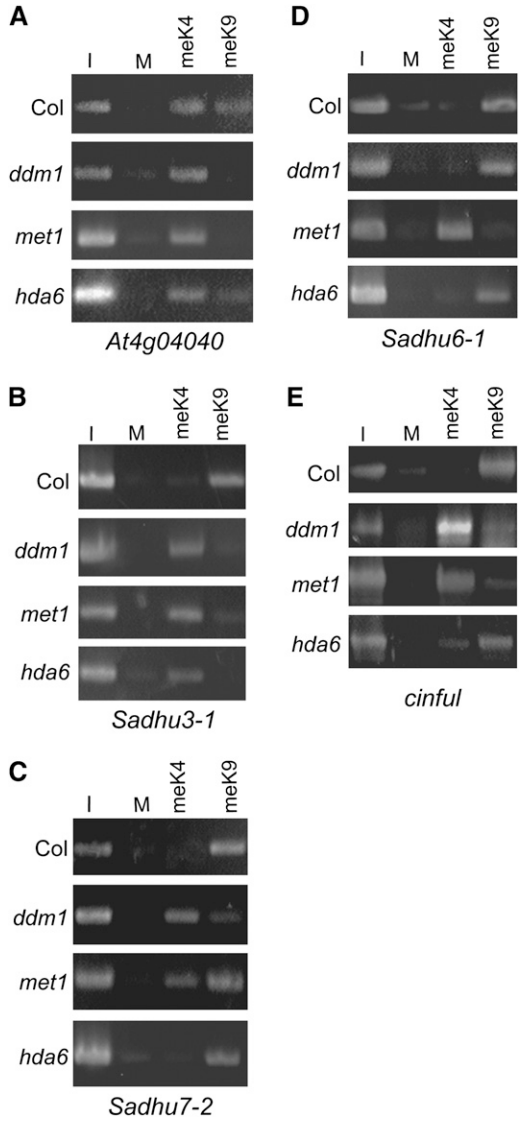


FIGURE 5.—Chromatin immunoprecipitation assay of histone H3 methylation at lysine 4 and lysine 9. (A) *At4g04040* represents a constitutively expressed locus; (B) *Sadhu3-1*; (C) *Sadhu7-2*; (D) *Sadhu6-1*; and (E) *cinful* retroelement. I, 10% input; M, mock (no antibody); meK4, H3 trimethyl K4 antibody; meK9, H3 dimethyl K9 antibody.

The three *Sadhu* elements showed differential histone modifications in different mutant backgrounds. We examined a constitutively active gene, *At4g04040*, as a control for the H3meK4 antibody (Figure 5A), and a *cinful* retroelement as a control for a heterochromatic sequence (GENDREL *et al.* 2002) (Figure 5E). *Sadhu3-1*, *Sadhu6-1*, and *Sadhu7-2* and the *cinful* element were associated primarily with H3meK9 in Col wild-type plants, consistent with their epigenetically suppressed states (Figure 5, B–E).

The *ddm1*, *met1*, and *hda6* mutations caused a shift in association from H3meK9 to H3meK4 at *Sadhu3-1* (Figure 5B), accompanying the ectopic expression of this locus (Figure 1). *Sadhu7-2* was expressed at high levels (Figure 1) and showed increased levels of H3meK4 relative to H3meK9 in the *ddm1* mutant (Figure 5C). The strong effect of a mutation in *DDM1* on histone modification state also correlated well with the cytosine methylation data showing the greatest hypomethylation at this locus in the *ddm1* background (Figure 3). In the *met1* mutant, which showed only slight expression of *Sadhu7-2*, H3meK9 remained associated with this locus. *Sadhu6-1* showed a marked shift in association of H3meK4 relative to H3meK9 only in the *met1* mutant (Figure 5D), consistent with expression of this locus in a *met1* background (Figure 1). In the nonexpressing mutants (*ddm1*, *hda6*) H3meK9 was still associated with *Sadhu6-1*. Therefore, the silenced state at all three elements correlated well with the presence of H3meK9, while expression was associated with H3meK4, regardless of the genomic location, genetic background, or DNA methylation state.

Variation in expression and methylation of *Sadhu* elements in natural wild-type accessions: We examined *Sadhu3-1*, *Sadhu7-2*, and *Sadhu6-1* in 20 natural *A. thaliana* accessions for the presence of the element (using internal PCR primers), cytosine methylation (assayed by McrBC PCR), and expression (assayed by RT–PCR) (Table 3). We found a large degree of variation in all three parameters at each of the three elements. For both *Sadhu6-1* and *Sadhu3-1*, several accessions expressed the element and silencing was not always perfectly correlated with cytosine methylation. In contrast, the *Sadhu7-2* locus was not expressed in any of the wild-type accessions and was methylated in every accession in which it was present. These results suggest that *Sadhu7-2*, which is derepressed only in a *ddm1* mutant, is more stably silenced in natural populations than the other *Sadhu* elements.

DISCUSSION

We investigated the epigenetic regulation of individual *Sadhu* non-LTR retroelements in the Arabidopsis genome. *Sadhu* elements that are not appreciably expressed in the Col wild-type background are expressed in a subset of mutants defective in chromatin silencing. We investigated DNA cytosine methylation and histone methylation at three representative *Sadhu* elements—*Sadhu3-1*, *Sadhu6-1*, and *Sadhu7-2*. Histone H3 dimethylation at lysine 9 was predictive of silenced states at these three elements, while expressed states correlated with H3 methylation at lysine 4. All three elements showed high levels of CpG methylation and low levels of asymmetric cytosine methylation when silenced. However, the three elements showed different levels of CpHpG methylation. *ddm1*, *met1*, and *hda6* mutations had differing effects on gene expression, cytosine methylation, and histone modification at the different loci. Therefore, members of this retroelement family are regulated differentially by various mutations that perturb chromatin. Previous studies have highlighted the complexity and diversity of

TABLE 3

Genomic DNA amplification (DNA), DNA methylation (5mC), and RNA levels (RNA) in *Sadhu* elements in a set of Arabidopsis accessions

		Sadhu3-1[a]			*Sadhu7-2*			*Sadhu6-1*		
Accession	Stock no.	DNA	5mC	RNA	DNA	5mC	RNA	DNA	5mC	RNA
Br-0	CS22628	Yes	Yes	+	No	NT	NT	Yes	Yes	−
Bur-0	CS22656	Yes	Yes	−	No	NT	NT	Yes	Yes	−
Col	LehleWT-2	Yes	Yes	−	Yes	Yes	−	Yes	Yes	−
Cvi	LehleWT-18	Yes	Yes	++	No	NT	NT	No	NT	−
Cvi-0	CS22614	No	NT	NT	No	NT	NT	No	NT	−
Fei-0	CS22645	Yes	Yes	−	No	NT	NT	Yes	Yes	−
Kn-0	CS6762	No	NT	NT	Yes	Yes	−	Yes	Yes	−
Kondara	CS22651	Yes	Yes	−	No	NT	NT	Yes	No	+++
Kz-1	CS22606	No	NT	NT	No	NT	NT	Yes	No	+++
N13	CS22491	Yes	Yes	−	Yes	Yes	−	Yes	Yes	−
No-0	CS6805	No	NT	NT	Yes	Yes	−	No	NT	−
Po-0	CS6839	Yes	Yes	++	No	NT	NT	Yes	No	+++
Pro-0	CS22649	Yes	Yes	−	No	NT	NT	Yes	No	+++
Pu2-7	CS22592	Yes	Yes	−	Yes	Yes	−	Yes	Yes	−
Ra-0	CS22632	Yes	No	+++	Yes	Yes	−	Yes	Yes	−
Tamm-27	CS22605	No	NT	NT	No	NT	NT	Yes	No	−
Ts-1	CS22647	No	NT	NT	No	NT	NT	Yes	No	−
Tsu-1	CS22641	Yes	Yes	−	Yes	Yes	−	Yes	Yes	++
Van-0	CS22627	Yes	Yes	−	No	NT	NT	Yes	Yes	−
Wei-0	CS22622	Yes	Yes	−	Yes	Yes	−	Yes	Yes	−
Ws-2	CS22659	Yes	Yes	−	Yes	Yes	−	Yes	Yes	−

NT, not tested. Genomic DNA amplification uses X1 and Y1 primers (supplemental Table 1 at http://www.genetics.org/supplemental/). DNA methylation is determined by McrBC–PCR using X1 and Y1 primers. RNA levels are detected by RT–PCR: −, none detected; +, low levels; ++, moderate levels; +++, high levels.

[a] Data are from Rangwala *et al.* (2006).

chromatin-based epigenetic silencing mechanisms, targeting different types of Arabidopsis transposable elements (Lippman *et al.* 2003; Ebbs and Bender 2006). Our studies, as well as the recent work by Huettel *et al.* (2006), emphasize that this diversity extends to individual members of the same transposable element family.

We hypothesized that *Sadhu* elements might be regulated differentially due to genomic position effects, DNA sequence, or a more complex combination dictated by the evolutionary history of a particular insertion. Several of the elements in this study—*Sadhu3-1*, *Sadhu3-2*, *Sadhu5-2*, and *Sadhu7-2*—are present in a similar repetitive pericentromeric environment. Notably, all four elements show slightly different responses to mutations in chromatin modifiers (Figure 1), suggesting that genomic environment is not solely responsible for epigenetic regulation in this family. Because *Sadhu* elements are divergent in sequence, we could not examine the role of DNA sequence directly. However, both *Sadhu3-1* and *Sadhu3-2* are members of the same subclade (Rangwala *et al.* 2006), present in different locations, yet they show slightly differing modes of regulation (Figure 1). In addition, *Sadhu7-1* and *Sadhu7-2* share ~85% nucleotide identity. While *Sadhu7-2* is pericentromeric and expressed at high levels only in a *ddm1* mutant background, *Sadhu7-1* is present in a more euchromatic region and is constitutively active in Col wild type (data not shown). Therefore, neither genomic environment nor phylogenetic sequence grouping alone can account for the regulation of *Sadhu* elements.

Tran *et al.* (2005) found that the targets of DRM2 included small isolated repeats in gene-rich regions and proposed a model in which RNA-directed *de novo* cytosine methylation may act to suppress repetitive DNA found outside of larger silenced domains (*e.g.*, pericentromere or knob). *Sadhu* elements are relatively short (<1000 bases) and some of the elements examined (*Sadhu8-1* and *Sadhu6-1*) are present outside of repeat-rich genomic regions, making them candidates for RNA-dependent regulation of isolated repeats under this model. The relatively minor effect of mutations in genes involved in RNA-directed silencing (*nrpd2*, *dcl3*, *rdr2*) provides an exception to previous studies indicating regulation of repetitive sequences through RNA-directed DNA methylation and RNA polymerase IV (Xie *et al.* 2004; Onodera *et al.* 2005; Huettel *et al.* 2006). We have not been able to detect small RNAs specific to *Sadhu3-1*, *Sadhu7-2*, or *Sadhu6-1* on RNA gel blots (data not shown), although the small RNA massively parallel signature sequencing (MPSS) database does contain a unique sequence match to *Sadhu3-1*. Because we have not examined DNA methylation or histone modification in the RNA

silencing mutants, we cannot rule out the possibility that RNA silencing pathways influence the chromatin state at this locus. It is also possible that derepression in mutants affecting RNA silencing may take several generations to become manifest, as is the case with derepression of certain single-copy loci in Arabidopsis *ddm1* and maize *mop1* mutants (Kakutani *et al.* 1996; Lisch *et al.* 2002; Woodhouse *et al.* 2006b). Nevertheless, the lack of an effect of the *drm2* mutation and the low levels of CpHpH DNA methylation suggest that silencing by RNA is not necessary for maintenance of suppression of these elements.

By contrast, the importance of *DDM1*, *MET1*, and *HDA6* in silencing *Sadhu* elements, regardless of genomic location, is in keeping with previous results suggesting that these genes may act coordinately at the chromatin level to maintain silent chromatin states (Gendrel *et al.* 2002; Lippman *et al.* 2003). In addition, *Sadhu3-2* was derepressed in a *suvh4* mutant background, indicating a critical contribution of histone H3 lysine 9 methylation in silencing at least some of these elements. However, not all *Sadhu* elements were derepressed equally in all mutants affecting chromatin-level silencing. The *met1-1* mutation abolished methylation at *Sadhu3-1*, but had a lesser effect on DNA methylation and no influence on histone H3 methylation at *Sadhu7-2*. Interestingly, ectopic expression of *Sadhu7-2* occurred in the antisense orientation. A *met1* or an *hda6* mutation might not be able to reactivate sense transcription of this element. However, the *ddm1* mutation, which caused a loss of both CpG and CpHpG methylation at *Sadhu7-2*, might have compromised integrity of the chromatin in this region enough to allow activation of a cryptic antisense promoter within the element. *Sadhu7-2* was also not expressed in any of the eight wild-type accessions in which it is present, consistent with the view that silencing at this locus is more stable than at other *Sadhu* elements. Therefore, although both loci are located in a repeat-rich pericentromere, the nature of silencing at *Sadhu3-1* and *Sadhu7-2* may be fundamentally different.

The regulation of *Sadhu6-1* is also unexpected and provides the first example where *DDM1* activity is not necessary to maintain transposable element silencing. In contrast to its effect at the other *Sadhu* elements, the *ddm1* mutant showed little effect on cytosine methylation and no loss of H3 lysine 9 at *Sadhu6-1*, indicating that no significant change in the nature of the chromatin occurred in this mutant. This difference in effect might result from the nonrepetitive genomic environment of *Sadhu6-1*. *ddm1* mutations often do not cause significant hypomethylation at single-copy euchromatic sites until the mutant plants have been self-fertilized for several generations (Kakutani *et al.* 1996), and the *ddm1-1* mutant used in this study had only been inbred one generation. However, *Sadhu8-1*, another element in a repeat-poor region of the genome, is derepressed in this *ddm1-1* sample. Therefore, the lack of effect of the *ddm1* mutation at *Sadhu6-1* is likely to be more complex, perhaps influenced by both the DNA sequence and the immediate microenvironment of this locus.

Sadhu6-1 is also unique for its high level of methylation in the CpHpG sequence context. CpHpG methylation is thought to be regulated by the CMT3 or DRM2 methyltransferases and to be associated with methylation at H3 lysine 9 and lysine 27 (Jackson *et al.* 2002; Cao *et al.* 2003; Lindroth *et al.* 2004). However, neither the *drm2*, *cmt3*, nor the H3 lysine 9 methyltransferase mutation *suvh4* showed derepression of this locus, possibly due to redundancy between DRM2 and CMT3 in regulating non-CpG methylation (Cao and Jacobsen 2002a). Because the *cmt3* mutation showed a decrease in CpHpG but not CpG methylation at this element, it is possible that retention of CpG methylation may be sufficient to maintain silencing at *Sadhu6-1*. In contrast, the *met1* mutation derepressed *Sadhu6-1* and reduced cytosine methylation in both the CpG and the CpHpG contexts. This result corroborates previous evidence that MET1, thought to be a CpG methyltransferase, can also be important in the maintenance of CpHpG methylation (Bartee and Bender 2001; Cao and Jacobsen 2002a). We note that the regulation at *Sadhu6-1* contrasts with the case at *Sadhu7-2*, where the *met1* mutation had no effect on CpHpG methylation. Therefore, silencing of the different representative *Sadhu* elements appears to involve a combination of both locus and gene-specific effects.

Sadhu elements, as a divergent family of retroelements, are a model to study epigenetic repression of middle repetitive DNA. This study reveals three distinct strategies to silence *Sadhu* elements in the absence of RNA level silencing. These strategies use an overlapping set of DDM1, MET1, and HDA6 activities, suggesting that while these proteins may act together at some loci, they have separate effects at others. In addition, our study reveals that not all members of a repeat family are silenced coordinately or by the same pathway. These findings suggest that caution must be applied when interpreting results from elements gained by experiments based on degenerate PCR primers or hybridization probes. Instead, different copies of elements within the genome are targeted independently, perhaps depending on a combination of different parameters, including the epigenetic history of the element, its genomic environment, or its own unique nucleotide sequence microenvironment.

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