particularly in lizards and birds, which commonly have derived nasal vestibules. The dearth of exceptions in the face of this diversity emphasizes the fundamental nature of the relationship.
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18. Why did anyone ever place the nostril caudally in dinosaurs? All leading early paleontologists regarded the largest dinosaurs (sauropods) as primarily "amphibious" (*32*–*35*). This view was clinched by the discovery in the sauropod *Diplodocus* of a large caudodorsal nasal opening (*36*) that was thought to serve as a snorkel, allowing the nearly submerged dinosaur to breathe (the dorsal opening in *Diplodocus* is actually just the caudal portion of the bony nostril, and the rostral portion extends far forward as a shallow narial fossa; vascular relationships confirm a rostral nostril). Although sauropods were later interpreted as terrestrial (*37*), the inferred caudal nostril stuck and somehow was transferred to other dinosaurs. Retracted nasal bones perhaps could be evidence, but nasal retraction is common in mammals in association with development of a proboscis, and mammals have been studied as extant analogs [e.g., (*1*, *38*)]. Despite retraction of the bony nostril, the fleshy nostril remains rostrally positioned; moreover, some mammals with retracted nasals (e.g., tapirs, elephants) enhance the fundamental amniote rostroventral position by extending the fleshy nostril out on a trunk. Thus, caudal expansion of the bony nostril apparently is not driven by caudal movement of the fleshy nostril, but rather by caudal expansion of the vestibular contents, that is, the narial apparatus.
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Methylation of Histone H4 at Arginine 3 Facilitating Transcriptional Activation by Nuclear Hormone Receptor

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Acetylation of core histone tails plays a fundamental role in transcription regulation. In addition to acetylation, other posttranslational modifications, such as phosphorylation and methylation, occur in core histone tails. Here, we report the purification, molecular identification, and functional characterization of a histone H4–specific methyltransferase PRMT1, a protein arginine methyltransferase. PRMT1 specifically methylates arginine 3 (Arg 3) of H4 in vitro and in vivo. Methylation of Arg 3 by PRMT1 facilitates subsequent acetylation of H4 tails by p300. However, acetylation of H4 inhibits its methylation by PRMT1. Most important, a mutation in the *S*-adenosyl-L-methionine–binding site of PRMT1 substantially crippled its nuclear receptor coactivator activity. Our finding reveals Arg 3 of H4 as a novel methylation site by PRMT1 and indicates that Arg 3 methylation plays an important role in transcriptional regulation.

Covalent modifications of core histone tails play important roles in chromatin function (*1*). One type of covalent histone modification is methylation (*2*), which has been observed in diverse organisms from yeast to human (*3*). However, the consequence of this posttranslational modification is not understood. One major obstacle in understanding the function of histone methylation is the lack of information about the responsible enzymes. The demonstrations that SUV39H1, the human homolog of the *Drosophila* heterochromatic protein Su(var)3-9, is an H3-specific methyltransferase (*4*) and that methylation of lysine 9 (Lys 9) on histone H3 serves as a binding site for the heterochromatin protein 1 (HP1) (*5*–*7*) underscore the importance of histone lysine methylation in heterochromatin function. Methylation of histones can occur on arginine residues, as well as lysine residues (*8*). The recent demonstrations that a nuclear receptor coactivator-associated protein, CARM1, is an H3-specific arginine methyltransferase suggests that histone arginine methylation may be involved in transcriptional activation (*9*).

To identify enzymes involved in core histone methylation, nuclear proteins from HeLa cells were separated into nuclear extract and nuclear pellet followed by further fractionation on DEAE52 and phosphate cellulose P11 columns. Fractions derived as above were assayed for methyltransferase activity by using core histone octamers as substrates (*10*). Multiple methyltransferase activities with distinctive specificity for histones H3 and H4 were seen (Fig. 1A). By following histone methyltransferase (HMT) activity (Fig. 1A), we purified an H4-specific HMT from the nuclear pellet fraction to homogeneity (*11*). Analysis of the column fractions derived from the hydroxyapatite column indicated that the peak of the enzymatic activity eluted in fraction 14 and trailed through fraction 26 (Fig. 1B, bottom panel). Silver staining of an SDS-polyacrylamide gel containing the column

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fractions revealed that a polypeptide of 42 kD coeluted with the enzymatic activity (Fig. 1B, top panel). To confirm this result, the same input was loaded onto a gel-filtration Superose-200 column. Analysis of the column fractions indicated that the peak of the enzymatic activity eluted around 330 kD between fractions 38 and 41 (Fig. 1C, bottom panel). Silver staining of an SDS-polyacrylamide gel containing the column fractions revealed again that a 42-kD polypeptide coeluted with the enzymatic activity. Mass spectrometry analysis (*11*) identified the 42-kD polypeptide as the human protein arginine *N*-methyltransferase 1, PRMT1 (*12*). Because the HMT activity eluted around 330 kD and only coeluted with PRMT1, it is likely that PRMT1 functions as a homo-oligomer. This was verified by the demonstration that recombinant PRMT1 fractionated in the same way as the endogenous PRMT1, as a 330-kD complex (*11*). Therefore, we conclude that PRMT1 functions as an H4-specific HMT in the form of a homo-oligomer.

The identification of PRMT1 as one of the most abundant H4-specific HMTs is surprising, because only Lys 20 of H4 has been reported to be methylated in vivo (*1*) and because PRMT1 is not known to be able to methylate lysine residues. Instead, PRMT1 and its yeast homolog have been reported to mainly methylate arginine of certain RNA binding proteins (*8*). To determine whether PRMT1 methylates H4 on Lys 20, core histone octamers were methylated with recombinant or native PRMT1 in the presence of *S*-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H]SAM). After separation by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), methylated H4 was recovered and mi-

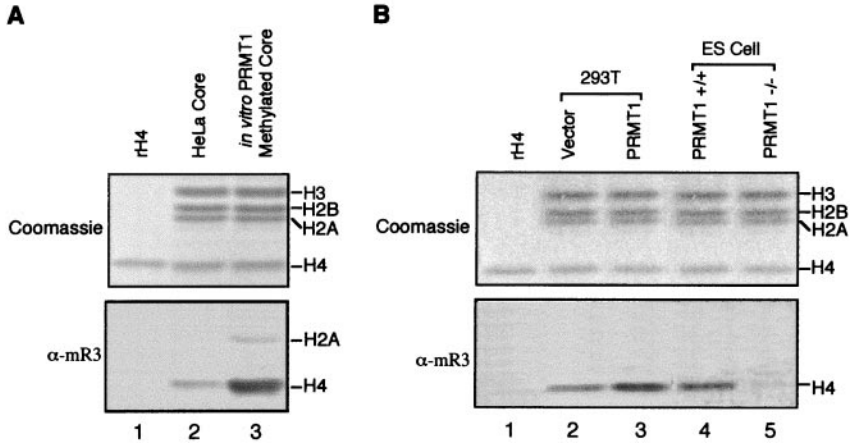

Fig. 2. PRMT1 methylates Arg 3 of H4 in vitro and in vivo. (**A**) Arg 3 methylation occurs in vivo. Recombinant histone H4 (200 ng) and equivalent amount of core histones from HeLa cells that were either subjected to mock (lane 2) or PRMT1 (lane 3) methylation before loading to SDS-polyacrylamide gel for Coomassie and Western blot analysis using the methyl-Arg 3–specific antibody. (**B**) PRMT1 is responsible for Arg 3 methylation in vivo. Recombinant histone H4 (200 ng) and equivalent amount of core histones purified from transiently transfected 293T cells (*23*) or ES cells were analyzed by Coomassie and Western blot as in (A).

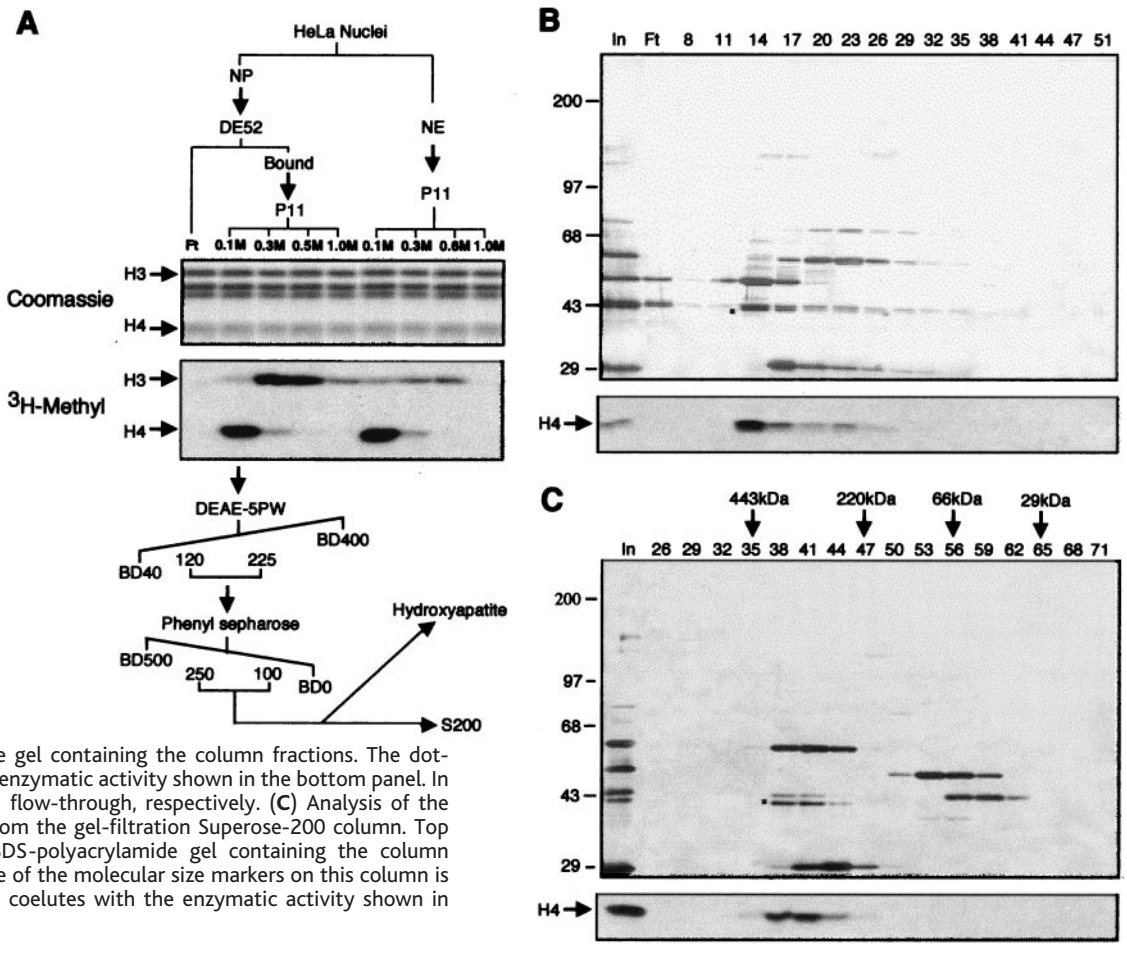

Fig. 1. Purification and molecular identification of an H4-specific methyltransferase. (**A**) HeLa nuclear proteins were fractionated as described (*11*) and fractions were assayed for histone methyltransferase activity (*10*). (Top) A Coomassie-stained gel indicating equal amounts of core histone substrate are used in each reaction. (Bottom) A fluorogram of the same gel indicating different fractions contain different HMT activities with specificity for H3 and/or H4. Equal volumes of the protein fractions were used in the enzymatic assay. Protein concentrations of the fractions from lanes 1 to 9 are 0.3, 0.3, 0.4, 0.4, 0.4, 1.2, 0.6, 0.3, and 1.2 mg/ml, respectively. (**B**) Analysis of the column fractions derived from the hydroxyapatite column. (Top) A silver-stained SDS-polyacrylamide gel containing the column fractions. The dotted-band coelutes with the enzymatic activity shown in the bottom panel. In and Ft represent input and flow-through, respectively. (**C**) Analysis of the column fractions derived from the gel-filtration Superose-200 column. Top panel is a silver-stained SDS-polyacrylamide gel containing the column fractions. The elution profile of the molecular size markers on this column is indicated. The dotted-band coelutes with the enzymatic activity shown in the bottom panel.

crosequenced by automated Edman chemical sequencing. Sequentially released amino acid derivatives were collected and counted by liquid scintillation, revealing that Arg 3, instead of Lys 20, was the major methylation site (*11*). Comparison of the ability of PRMT1 to methylate H4 tail peptides with or without a mutation on Lys 20 showed no difference, confirming that Lys 20 is not a site for PRMT1 methylation (*13*).

The identification of H4 Arg 3 as an in vitro methylation site for PRMT1 is intriguing. To determine whether Arg3 methylation occurs in vivo, antibodies against an Arg 3–methylated histone H4 NH_2-terminal peptide were generated (*14*). Although the antibody reacted strongly with PRMT1-methylated H4, it did not recognize equal amounts of recombinant H4 expressed in *Escherichia coli* (Fig. 2A, compare lanes 1 and 3), indicating that the antibody is methyl-Arg 3–specific. This same antibody also recognized histone H4 purified from HeLa cells (Fig. 2B, lane 2) indicating certain amount of Arg 3-methylation occurs in vivo. We note that H2A can also be weakly methylated by PRMT1 in vitro and that methylated H2A can be recognized by the methyl-Arg 3 antibody (Fig. 2A, compare lanes 2 and 3). The methylation site on H2A is likely to be Arg 3, because H2A has the same extreme NH_2-terminal sequence "SGRGK" as that of H4 (*14*). However, the amount of endogenous H2A methylation is undetectable under the same conditions (bottom panels of Fig. 2, A and B).

We next sought to determine whether PRMT1 is responsible for this site-specific Arg 3 methylation in vivo. If PRMT1 is responsible for Arg 3 methylation, overexpression of PRMT1 should increase the amount of Arg 3 methylation. The results shown in Fig. 2B indicate that overexpression of PRMT1 increases Arg 3 methylation (compare lanes 2 and 3). To confirm the above result, core histones from *PRMT1*$^{+/+}$ and *PRMT1*$^{-/-}$ embryonic stem (ES) cells (*15*) were purified and compared for their Arg 3 methylation. The results shown in Fig. 2B (compare lanes 4 and 5) demonstrated that inactivation of the *Prmt1* gene results in a dramatic decrease in the amount of Arg 3 methylation, indicating that histone H4 is likely an in vivo substrate for PRMT1. However, we could

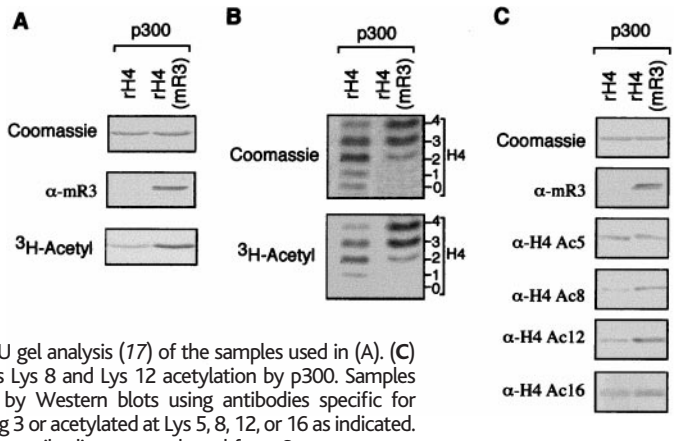

Fig. 3. Arg 3 methylation stimulates H4 acetylation by p300. (**A**) PRMT1-methylated H4 is a better substrate for p300 acetylation. Mock- and PRMT1-methylated recombinant H4 were subjected to p300 acetylation in the presence of [^{3}H]acetyl-CoA (*16*). Samples were analyzed by Coomassie, Western blot, and fluorogram. (**B**) TAU gel analysis (*17*) of the samples used in (A). (**C**) Arg 3 methylation facilitates Lys 8 and Lys 12 acetylation by p300. Samples used in (A) were analyzed by Western blots using antibodies specific for histone H4 methylated at Arg 3 or acetylated at Lys 5, 8, 12, or 16 as indicated. The site-specific acetyl-lysine antibodies are purchased from Serotec.

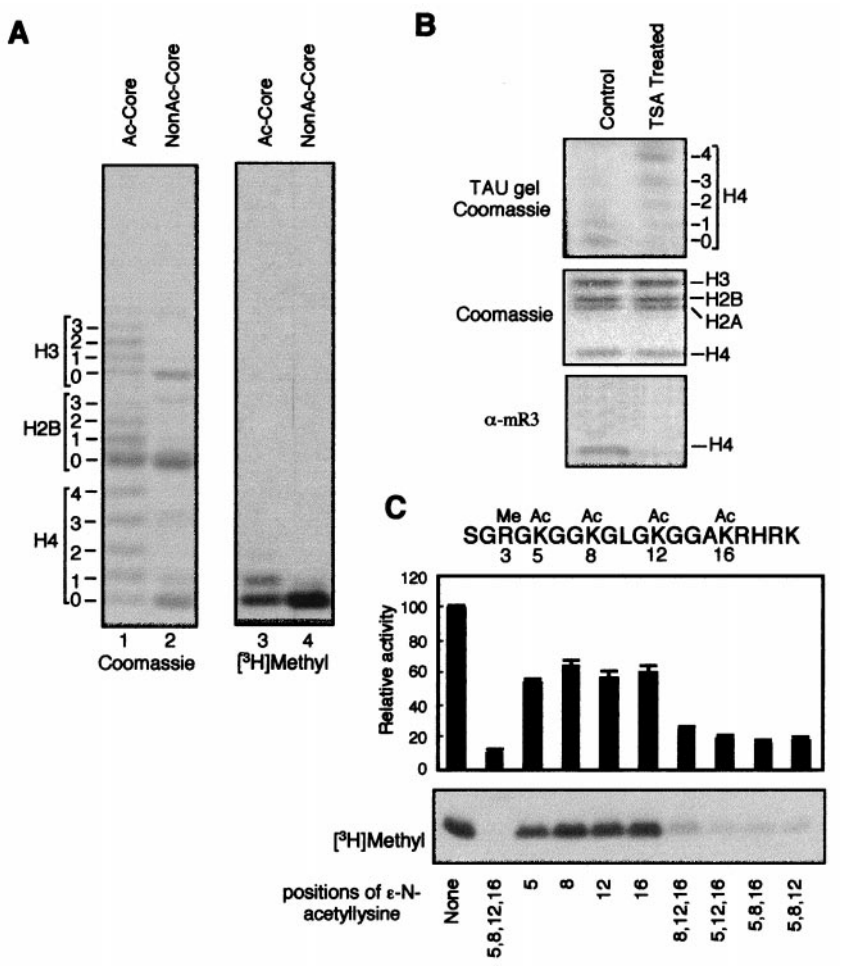

Fig. 4. Acetylation of H4 inhibits Arg 3 methylation by PRMT1. (**A**) Lysine acetylation inhibits H4-Arg 3 methylation in vitro. Hyper- (Ac) and hypoacetylated (Non-Ac) core histones purified from HeLa cells (*17*) were used as substrates for PRMT1 methylation (*10*). Different acetylated isoforms were resolved by a TAU gel and visualized by Coomassie staining. Methylation of different acetylated isoforms by PRMT1 was revealed by fluorogram. (**B**) Lysine acetylation inhibits H4-Arg 3 methylation in vivo. Core histones purified from untreated and TSA-treated (100 ng/ml final concentration) HeLa cells were analyzed by TAU gel (top panel), SDS-PAGE (middle panel), and Western blot (bottom panel). TAU gel reveals the acetylation state of H4, SDS-PAGE reveals equal loading, and Western blot reveals Arg 3 methylation state. (**C**) Comparison of the efficiency of PRMT1 to methylate different acetylated H4 peptides. Lysine residues that can be acetylated in vivo are indicated (top). Synthetic peptides that were not acetylated, monoed, triacetylated, and fully acetylated, respectively, were methylated with PRMT1 (*10*) and resolved by 20% SDS-PAGE before exposure to x-ray film (bottom). For quantification, the gel was cut and counted with scintillation counting. Results shown (middle) represent the average of two independent experiments with deviation.

not rule out the possibility that PRMT1 is an upstream regulator of an H4 Arg 3–specific HMT involved in H4 methylation through a methylation pathway similar to phosphorylation.

Having established that PRMT1 plays a critical role in Arg 3 methylation in vivo, we next sought to determine the function of this modification. Recent demonstration that methylation on Lys 9 of H3 inhibits Ser 10 phosphorylation (*4*) prompted us to ask whether Arg 3 methylation interferes with acetylation of lysine residues on H4 tails. To this end, we compared recombinant H4 that was either mock-methylated or PRMT1 methylated to serve as substrates for acetylation by p300 in the presence of [^{3}H]acetyl-CoA (*16*). Methylation of H4 by PRMT1 stimulated its subsequent acetylation by p300 (Fig. 3A). To confirm this result, samples equivalent to those analyzed in Fig. 3A were analyzed with a Triton-Acetic Acid-Urea (TAU) gel, which separates different acetylated histone isoforms. The results demonstrate that PRMT1-methylated H4 is a better substrate for p300 when compared with unmethylated H4, because all H4 molecules were acetylated (no 0 acetylated form) by p300 (Fig. 3B). However, under the same conditions, a fraction of the mock-methylated substrates still remains unacetylated (0 acetylated form). To determine which of the four acetylable lysine residues are affected by Arg 3 methylation, the acetylation status of samples analyzed above was examined by using acetylation site-specific antibodies. The results indicated that Arg 3 methylation facilitates K8 and K12 acetylation but has little affect on K5 or K16 acetylation (Fig. 3C).

To determine the effect of lysine acetylation on Arg 3 methylation, we purified both hyperacetylated and hypoacetylated core histones from HeLa cells (*17*) and used them as substrates for PRMT1 in the presence of [^{3}H]SAM. After methylation, samples were resolved in a TAU gel followed by Coomassie staining and autoradiography. Only un- and monoacetylated H4 isoforms were methylated to a detectable level, although nearly equal amounts of the different H4 isoforms were present in the methylation reaction (Fig. 4A, compare lanes 1 and 3). Because unacetylated H4 is the best substrate for PRMT1, when compared with different acetylated H4 isoforms (Fig. 4A), we concluded that acetylation on lysine residues inhibits H4 methylation by PRMT1. To determine whether this inhibition occurs in vivo, HeLa cells were treated with a histone deacetylase inhibitor, Tricostatin A (TSA), to induce hyperacetylation. Twelve hours after TSA treatment, core histones were isolated, and the methylation state of H4-Arg 3 was analyzed. Hypoacetylated H4 (untreated) had a higher amount of Arg 3 methylation when compared with hyperacetylated H4 (TSA treated), which had almost undetectable Arg 3 methylation (Fig. 4B). Therefore, hyperacetylation on lysine residues correlates with hypomethylation of H4 Arg 3. This result is consistent with the idea that acetylation on lysine residues inhibits subsequent Arg 3 methylation, and it is also consistent with earlier studies demonstrating that H4 methylation preferentially occurs on unacetylated histones, whereas H3 methylation occurs preferentially on acetylated histones (*18*). Because H4 contains four lysine residues that can be acetylated (Fig. 4C, top panel), we investigated whether acetylation on any of the four sites would have a similar effect on Arg 3 methylation. To this end, synthetic H4 tail peptides that were not acetylated or were monoacetylated, triacetylated and fully acetylated, were used as substrates for PRMT1. Acetylation on any of the four lysines inhibited Arg 3 methylation by PRMT1 (Fig. 4C). However, acetylation on Lys 5 had the most effect. In addition, acetylation on different lysines seemed to have an additive inhibition effect. Consistent with results shown in Fig. 4A, triacetylated and fully acetylated peptides were severely impaired in serving as substrates for PRMT1 (Fig. 4C).

That Arg3 methylation enhanced lysine acetylation (Fig. 3) predicts that PRMT1 is likely to be involved in transcriptional activation. Indeed, PRMT1 has been shown recently to function as a coactivator of nuclear hormone receptors (*19*). However, its coactivator activity has not been linked to its HMT activity. To directly address the function of Arg 3 methylation on transcription, we introduced a single amino acid mutation (G80R) in the conserved SAM binding domain of PRMT1, which has been previously shown to impair its enzymatic activity (*20*). The ability of the mutant and wild-type PRMT1 to facilitate activation by androgen receptor (AR), which is known to use CBP/p300 as coactivators, was compared in chromatin context by using *Xenopus* oocytes as a model system (*21*). A mouse mammary tumor virus (MMTV) long terminal repeat (LTR)-based reporter was injected into the nuclei of *Xenopus* oocytes, and successful assembly of the reporter into chromatin was confirmed by micrococcal nuclease digestion (Fig. 5A). Ectopic expression of AR in *Xenopus* oocytes led to an agonist-stimulated activation of the reporter (Fig. 5B, compare lanes 2 and 3). Co-expression of PRMT1 further augmented the activation by AR (Fig. 5B, compare lanes 3 and 5). Significantly, the PRMT1(G80R) mutant has little coactivator activity when compared with wild-type PRMT1 (Fig. 5B, compare lanes 4 and 5 with lanes 6 and 7). Western blot analysis revealed that the differences in transcription were not due to differential expression of PRMT1 and PRMT1(G80R) or their effect on AR expression (Fig. 5B). We thus conclude that the HMT activity of PRMT1 is critical for its coactivator activity.

Our studies demonstrating the interplay between Arg3 methylation and lysine acetylation support the "histone code" hypothesis (*1*). We provided evidence that H4 Arg 3 methylation plays an important role in transcriptional activation. An H3-specific arginine methyltransferase CARM1 was also shown to function as a nuclear hormone receptor coactivator (*9*, *22*). In contrast, the heterochromatin-associated protein SUV39H1 was found to be an H3-specific methyltransferase (*4*), and methylation of Lys 9 by SUV39H1 serves as a binding site for the recruitment of the heterochromatin protein 1 (HP1) (*5*–*7*), suggesting that methylation of Lys 9 on H3 is likely involved in heterochro-

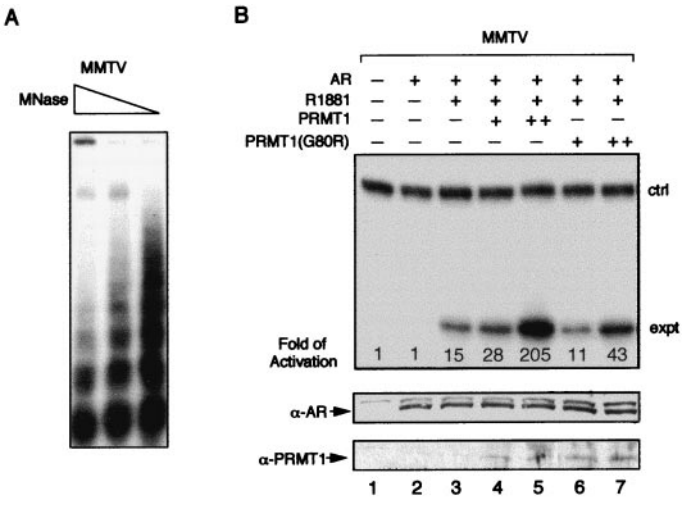

Fig. 5. The PRMT1 HMT activity is required for PRMT1 to function as a coactivator for AR. (**A**). The MMTV-LTR–based reporter injected into the nuclei of *Xenopus* oocytes was assembled into regularly spaced nucleosomes as revealed by Southern blot of a micrococcal nuclease digestion (MNase) assay (*11*). (**B**) Groups of *Xenopus* oocytes were injected with the MMTV-LTR reporter and the in vitro synthesized mRNAs encoding AR (100 ng/μl), PRMT1, or PRMT1(G80R) (100 ng/μl or 300 ng/μl) as indicated and were treated with or without the AR agonist R1881 (100 nM) overnight. The level of transcription from the reporter (expt) was analyzed by primer extension analysis of the total RNAs prepared from each group of oocytes and quantified by phosphor screen autoradiography (*11*). Folds of activation are shown below the primer extension product. The primer extension product from the endogenous histone H4 mRNA served as an internal control (ctrl). The expression levels of AR, PRMT1, and PRMT1(G80R) in each group of oocytes were analyzed by Western blot using an AR- or PRMT1-specific antibody, respectively.

matic gene silencing. Whether Arg 3 methylation helps the recruitment of specific histone acetyltransferases, such as p300, remains to be determined. As new HMTs responsible for the methylation of different histone arginine or lysine residues are identified, the functions of histone methylation on transcription and other processes involving chromatin will be revealed.

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10. Column fractions or recombinant PRMT1 was incubated with core histone octamers, recombinant H4, or H4 tail peptides in a total volume of 30 μl containing 20 mM Tris-HCl (pH 8.0), 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 1.5 μl [^{3}H]SAM (15 Ci/mmol; NEN Life Science Products) at 30°C for 1 hour. Reactions were stopped by the addition of SDS loading buffer followed by electrophoresis in an 18% SDS polyacrylamide gel. After Coomassie staining and destaining, gels were treated with Entensify (NEN Life Science Products) and dried before exposing to x-ray film.
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23. One 100-mm plate of 293T cells (about 1.5×10^6) were transfected with 4 μg of empty pCDNA vector or pCDNA-PRMT1 by using the Effectene transfection reagent (Qiagen). Forty-eight hours after transfection, nuclei were isolated and core histones were purified by acid extraction and trichloroacetic acid precipitation.
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Identification of a Gene Associated with Bt Resistance in *Heliothis virescens*

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Transgenic crops producing insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely used for pest control. Bt-resistant insect strains have been studied, but the molecular basis of resistance has remained elusive. Here, we show that disruption of a cadherin-superfamily gene by retrotransposon-mediated insertion was linked to high levels of resistance to the Bt toxin Cry1Ac in the cotton pest *Heliothis virescens*. Monitoring the early phases of Bt resistance evolution in the field has been viewed as crucial but extremely difficult, especially when resistance is recessive. Our findings enable efficient DNA-based screening for resistant heterozygotes by directly detecting the recessive allele.

Field populations of the tobacco budworm *H. virescens*, a key pest of cotton and other crops in the Americas, have developed resistance to most classes of chemical insecticides. This species is the primary target of recently commercialized transgenic Bt cotton, which protects itself from insect damage by producing the insecticidal Cry1Ac toxin from *B. thuringiensis*. Concerns about Bt resistance led the U.S. Environmental Protection Agency to mandate a management plan, the "high-dose/refuge strategy" (*1*). It assumes that Bt cotton produces enough toxin to kill heterozygotes (with just one resistance allele) as well as

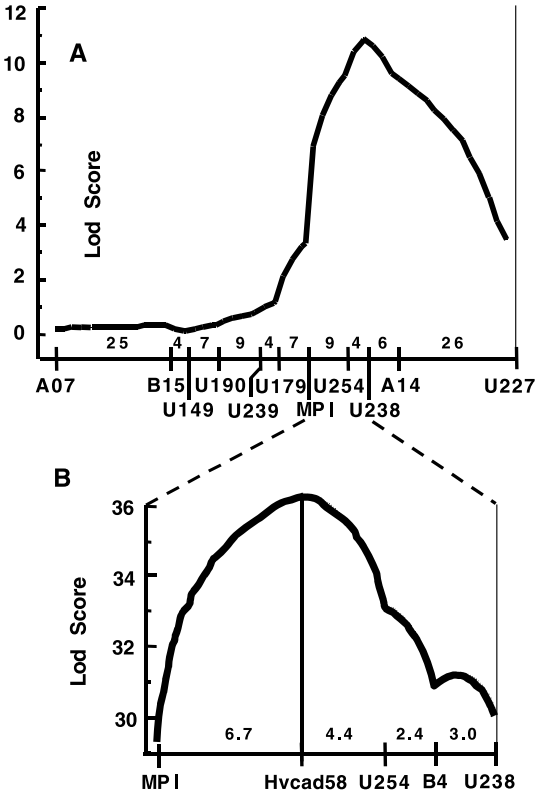

Fig. 1. QTL mapping of Cry1Ac resistance on linkage group 9 of *H. virescens*. (**A**) Resistance QTL lod (logarithm of the odds ratio for linkage) profile for initial scan of 105 cM on LG 9 spanned by 11 markers, based on 48 progeny of segregating backcross family D6. Marker order and spacing (in cM) was calculated by Mapmaker EXP 3.0 (*16*) and lod scores by Mapmaker QTL 1.9 (*17*). (**B**) Lod profile for fine-scale QTL mapping over the 16-cM region between MPI and U238, based on 268 progeny of nine segregating backcross families. The maximum lod score of 35.9 occurs at Hvcad58, which accounts for 46% of the trait variance. The resistance trait is the log of larval weight after 10 days of growth on 0.032 μg of Cry1Ac toxin per milliliter of diet (*3*).