

Methylation of Tat by PRMT6 Regulates Human Immunodeficiency Virus Type 1 Gene Expression

Marie-Chloé Boulanger,^{1,2} Chen Liang,³ Rodney S. Russell,^{3,4} Rongtuan Lin,^{1,5}
Mark T. Bedford,⁶ Mark A. Wainberg,^{3,4*} and Stéphane Richard^{1,2,5,7*}

Terry Fox Molecular Oncology Group,¹ Bloomfield Center for Research on Aging,² McGill University AIDS Center,³ Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, and Departments of Microbiology,⁴ Medicine,⁵ and Oncology,⁷ McGill University, Montréal, Québec, Canada, and M.D. Anderson Cancer Center, Department of Carcinogenesis, University of Texas, Smithville, Texas⁶

Received 13 May 2004/Accepted 27 August 2004

The human immunodeficiency virus (HIV) transactivator protein, Tat, stimulates transcription from the viral long terminal repeats via an arginine-rich transactivating domain. Since arginines are often known to be methylated, we investigated whether HIV type 1 (HIV-1) Tat was a substrate for known protein arginine methyltransferases (PRMTs). Here we identify Tat as a substrate for the arginine methyltransferase, PRMT6. Tat is specifically associated with and methylated by PRMT6 within cells. Overexpression of wild-type PRMT6, but not a methylase-inactive PRMT6 mutant, decreased Tat transactivation of an HIV-1 long terminal repeat luciferase reporter plasmid in a dose-dependent manner. Knocking down PRMT6 consistently increased HIV-1 production in HEK293T cells and also led to increased viral infectiousness as shown in multinuclear activation of a galactosidase indicator assays. Our study demonstrates that arginine methylation of Tat negatively regulates its transactivation activity and that PRMT6 acts as a restriction factor for HIV replication.

Human immunodeficiency virus type 1 (HIV-1) encodes a transactivator protein (termed Tat) that is transcribed from multiply spliced viral RNA molecules expressed at early stages of viral gene expression. Tat is a key player in HIV replication by virtue of its ability to dramatically increase gene transcription efficiency from the viral 5' long terminal repeat (LTR) (25). Tat exerts this activity through binding to a 57-nucleotide stem-loop RNA structure located at the 5' terminus of the nascent HIV RNA transcript, an element referred to as the Tat transactivation response region (TAR). To stimulate the elongation efficiency of RNA polymerase II that initiates RNA synthesis from the LTR, Tat interacts with cyclin T1 which, in turn, recruits cyclin-dependent kinase 9 (CDK9) proximity of the C-terminal domain of RNA polymerase II. Subsequently, CDK9 enacts the hyperphosphorylation of RNA polymerase II and, as a result, dramatically accelerates RNA transcription (39).

The transactivation activity of Tat is regulated posttranslationally by the acetylation of lysine residues (21, 31). One outcome of Tat acetylation is to trigger the dissociation of the Tat-cyclin T-CDK9 complex from TAR RNA and hence to transfer the latter protein complex to RNA polymerase II (19). In addition to lysine acetylation, arginine is frequently found to be methylated, particularly in the context of the GAR motif within RNA-binding proteins. Tat harbors an arginine-rich transactivation motif (ARM); however, it is unknown whether

Tat is a substrate of protein arginine methyltransferases (PRMTs).

Arginine methylation is a posttranslational modification that involves the addition of one or two methyl groups to the guanidino nitrogen atoms of arginine (14, 28). Arginine may be dimethylated asymmetrically, where both methyl groups are added to the same nitrogen atom, or symmetrically, where two methyl groups are added onto different nitrogen atoms. Type I PRMTs catalyze the formation of asymmetric dimethylarginines (ADMA), and type II PRMTs catalyze the formation of symmetric dimethylarginines (SDMA). PRMT1 (26), PRMT3 (37), coactivator-associated arginine methyltransferase 1 (CARM1) (8, 35), and PRMT6 (13) are type I enzymes, and PRMT5 is the only known type II enzyme (6, 33). Another class of PRMTs, including PRMT7 catalyze the formation of monomethylarginines (15, 29). No clear consensus sequence for CARM1 substrates or for the newly identified PRMT6 and PRMT7 are known. By using antibodies generated against methylated GAR, we have identified more than 200 putative arginine-methylated substrates involved in various aspects of cellular function (4). Our strategy, however, did not identify arginine-methylated substrates that do not contain GAR motifs nor did it identify any viral proteins.

Arginine methylation is known to influence gene expression (11, 36, 41, 42). PRMTs function as transcriptional coactivators by remodeling chromatin by modifying histone tails (1), and the methylation of histone H3 and H4 by CARM1 and PRMT1 is thought to contribute to the histone code (18). PRMTs also methylate other coactivators, including CBP to regulate their transcriptional activity (10, 40). Methylated arginines can negatively regulate protein-protein interactions (2), and it has been shown that PRMT1 activates transcription through methylation of STAT1 by preventing association with the STAT-

* Corresponding author. Mailing address: Lady Davis Institute, 3755 Côte Ste-Catherine Rd., Montréal, Québec, Canada H3T 1E2. Phone: (514) 340-8260. Fax: (514) 340-8295. E-mail for Mark A. Wainberg: mark.wainberg@mcgill.ca. E-mail for Stéphane Richard: stephane.richard@mcgill.ca.

inhibitor PIAS1 (30). PRMT5 is also involved in transcriptional repression of the cyclin E1 and Myc genes (12, 32). Arginine methylation has also been shown to regulate transcriptional elongation (23). The 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB)-sensitive inducing factor (DSIF) p160 or Spt5 was identified to be arginine methylated by PRMT1 and PRMT5. Methylation of the DRB-sensitive inducing factor DSIF p160 or Spt5 was shown to inhibit its association with RNA polymerase II and inhibit its transcriptional activity (23).

In this study, we demonstrate that Tat is methylated in the arginine-rich transactivating motif residing between amino acids 49 to 63 by PRMT6. The results of short interfering RNA (siRNA) treatment and PRMT6 overexpression experiments demonstrate that arginine methylation has a negative impact on the transactivation activity of Tat. It is noteworthy that HIV-1 Tat is the first substrate identified for PRMT6. Our results indicate that methylation of Tat is a novel mode of regulation for this RNA-binding protein.

MATERIALS AND METHODS

Reagents. Recombinant histidine-tagged Tat72 was purified as described previously (20). An expression vector (pHA-Tat) that encodes hemagglutinin (HA) epitope-tagged Tat was generated as follows: the expression vector pHA-VAX was generated by annealing two phosphorylated oligonucleotides (5'-CTA GCA CCA TGT ACC CAT ACG ATG TTC CAG ATT ACG CTA CG-3' and 5'-CTA GCG TAG CGT AAT CTG GAA CAT CGT ATG GGT ACA TGG TG-3') and subcloning the DNA fragment that encodes the HA tag into the NheI site of pVAX (Invitrogen, Carlsbad, Calif.). Tat86 was amplified by PCR with two oligonucleotides (5'-TTT GAA TTC TGA GCC AGT AGA TCC TAG ACT A-3' and 5'-TTT GAA TTC CTA TTC CTT CGG GCC TGT-3') from pCMV-Tat86 (Alan Frankel, University of California at San Francisco, San Francisco) and subcloned in frame into the EcoRI site pHA-VAX, resulting in pHA-Tat.

Recombinant glutathione-S-transferase (GST)-PRMT6 has been described previously (13). The myc epitope PRMT6 expression vector was generated by amplifying the PRMT6 cDNA fragment by PCR with two primers (5'-ATA GAA TTC GTC GCA GCC CAA GAA AAG A-3' and 5'-ATA GAA TTC TCA GTC CTC CAT GGC AAA GTC-3') and subcloned in the EcoRI site of myc-pVAX, resulting in pMyc-PRMT6. myc-pVAX was generated by annealing two oligonucleotides (5'-CGC GGA TCC ACC ATG GCG TCT ATG GAA CAA AAG CTG ATT AGC G-3' and 5'-GCG CTC GAG AAT TCC CGT TGT TCA GGT CCT CTT CGC TAA TCA GCT TTT-3') and filling in the ends with DNA polymerase I (Klenow fragment). This DNA fragment was subcloned into BamHI and XhoI sites of pVAX (Invitrogen). The PRMT6 (VLD-KLA) mutation was generated by inverse PCR with two oligonucleotides (5'-CTT GCG GTG GGC GCG GGC ACC GGC-3' and 5'-CTT CGT CTT GCC TCG CAG TGC T-3') using pMyc-PRMT6. The resulting plasmid was called pMyc-PRMT6:VLD-KLA.

Anti-PRMT5 and anti-Sam68 rabbit polyclonal antibodies were purchased from Upstate Biotech, Inc. (Upstate, N.Y.). Anti-PRMT6 antibodies were purchased from IMGEX (San Diego, Calif.). Control and PRMT6 double-stranded RNA oligonucleotides (siRNA) were purchased from Dharmacon Research (Lafayette, Colo.). PRMT6 siRNA was derived from the PRMT6 sequence (AY043278) that includes nucleotides 996 to 1114 (5'-GCA AGA CAC GCA CGU UUC A-3'). The sequence of the mock siRNA was 5'-AAU UGC CAC AAC AGG GUC GUG-3'. Tat peptides, HeLa-CD4-LTR- β -gal cells, BH10 proviral DNA, BH10 virus were acquired from the National Institutes of Health Research and Reference Reagent Program. GST-CARM1 was obtained from Michael Stallcup (8).

The sequence for PRMT7 was amplified by using two EcoRI oligonucleotides (5'-CCG GAA TTC CAA GAT CTT CTG CAG TCG GGC C-3' and 5'-CCG AAT TCT CAG TCT GGG GTA TCT GCA TGC C-3') and ligated into pVAX. The PRMT7 DNA fragment from PRMT7 pVAX was digested with BamHI and XhoI and ligated into pGEX 4T-2 to produce GST-PRMT7. The HIV long terminal repeat (LTR) luciferase reporter plasmid was described previously (34).

Methylation assays. Recombinant histidine-tagged Tat72 or Tat peptides (1 μ g) were incubated with mock siRNA (no methylase), 1 μ g of GST-CARM1,

GST-PRMT6, or GST-PRMT7 in the presence of 0.55 μ Ci of [*methyl*-³H]S-adenosyl-L-methionine (Amersham Biosciences, Piscataway, N.J.) and 25 mM Tris-HCl (pH 7.5) for 3 h at 37°C in a final volume of 30 μ l. Reactions were stopped by adding 20 μ l of 2 \times Laemmli buffer (24), followed by heating at 100°C for 5 min. Samples were loaded on 12 or 15% polyacrylamide gels containing sodium dodecyl sulfate (SDS) and a high level of *N,N,N',N'*-tetramethylethylenediamine (TEMED) and stained with Coomassie blue. The destained gels were visualized by fluorography with En³Hance (Perkin-Elmer, Boston, Mass.) according to the manufacturer's instructions.

For *in vivo* methylation, HEK293T cells were cotransfected with pHA-Tat and pVAX, pHA-Tat and pMyc-PRMT6, or pHA-Tat and pMyc-PRMT6:VLD-KLA. Twenty-four hours later, the cells were metabolically labeled with L-[*methyl*-³H]methionine for 3 h in the presence of cycloheximide (100 μ g/ml) and chloramphenicol (40 μ g/ml) (Sigma, St. Louis, Mo.). To ensure that protein translation was arrested and that incorporation of L-[*methyl*-³H]methionine was not due to methionine incorporation during protein synthesis, cells cotransfected with pHA-Tat and myc-PRMT6 were labeled with L-[³⁵S]methionine (Amersham Biosciences, Piscataway, N.J.) under the same conditions. The cells were lysed in lysis buffer and immunoprecipitated as described below. The bound proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by fluorography.

Immunoprecipitations. Cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl [pH 8.0], 50 mM NaF, 100 μ M sodium vanadate, 0.01% phenylmethylsulfonyl fluoride, 1 μ g of aprotinin per ml, 1 μ g of leupeptin per ml). The cell lysates were incubated with anti-HA antibody for 1 h on ice. Then 20 μ l of a 50% protein A-Sepharose slurry was added for 30 min with constant end-over-end mixing at 4°C. The beads were washed three times with lysis buffer and dissociated in 2 \times Laemmli buffer. The proteins were separated on SDS-12% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-myc (9E10; American Type Culture Collection, Rockville, Md.) or anti-HA (12CA5; BabCo, Berkeley, Calif.) antibody followed by goat anti-mouse antibodies conjugated to horseradish peroxidase (ICN Pharmaceuticals, Costa Mesa, Calif.) and detected by chemiluminescence (DuPont, Wilmington, Del.).

Gene expression assays. HEK293T cells (0.4×10^5 cells/well) were plated in 24-well plates. Cells were transfected with 100 ng of HIV-LTR-luciferase reporter plasmid (34) along with 50 ng of PRL-TK (Promega, Madison, Wis.) to control for transfection efficiency. Where indicated, HA-tagged Tat (HA-Tat) (100 ng) was transfected in the absence or presence of PRMT6 or PRMT6:VLD-KLA (0.5 and 1.0 μ g) or 90 pmol of PRMT6 siRNA with Lipofectamine (Invitrogen). The amount of DNA was adjusted to a total of 1.25 μ g in each well with pVAX. After 36 h, the transfected cells were lysed in passive lysis buffer (Promega), and luciferase and renilla activities were measured using the dual-luciferase reporter assay system (Promega). The luciferase activity was normalized to that of renilla. The luciferase activity in the absence of the Tat expression vector was normalized to 1, and the subsequent luciferase activity was expressed as fold induction.

RT-PCR. RNA was extracted from HEK293T and HeLa-CD4-LTR- β -gal cells by using Trizol (Invitrogen). One microgram of RNA was used in the reverse transcriptase reactions. The RNA was incubated with 1 μ l of a solution containing 100 pmol of oligo(dT) primer and 1 μ l of a solution containing 10 mM (total) of the four deoxynucleoside triphosphates in the presence or absence of Moloney murine leukemia virus reverse transcriptase (Promega) for 1 h at 42°C. Part (1/10) of the RT reaction mixture (2 μ l) was used as a template for the PCR mixture, which contained 1 μ l of a solution containing 100 pmol of primer per μ l (the primers for PRMT6 were 5'-ATA GAA TTC GTC GCA GCC CAA GAA AAG A-3' and 5'-ATA GAA TTC TCA GTC CTC CAT GGC AAA GTC-3', and the primers for glyceraldehyde-3-phosphate dehydrogenase were 5'-ATG GTG AAG GTC GGT GTC AAC-3' and 5'-TTA CTC CTT GGA GGC CAT G-3'), 2 μ l of a solution containing 10 mM (total) of the four deoxynucleoside triphosphates, 10 μ l of 10 \times Taq buffer, and 1 μ l of Taq polymerase (Pharmacia Pfizer, Kirkland, Québec, Canada). The mixture was heated at 98°C for 2 min. Twenty-five cycles of PCR were then performed, with 1 cycle consisting of 30 s at 98°C, 30 s at 58°C, and 1 min at 72°C. The mixture was then heated for 5 min at 72°C and cooled to 4°C. The amplified DNA fragment of 1.2 kb migrated on 1% agarose gel and was visualized using ethidium bromide.

MAGI assays. HeLa-CD4-LTR- β -gal cells were plated in 24-well plates at a density of 0.4×10^5 cells per well. On the next day, 90 pmol of siRNA was transfected into these cells by using Lipofectamine (Invitrogen). After 48 h, the cells were infected with 2 ng (multiplicity of infection of ~ 0.001 , as measured using HeLa cells with multinuclear activation of a galactosidase indicator [MAGI]) or 10 ng of BH10 virus for 4 h. Forty-eight hours after infection, the cells were fixed for 5 min in a solution containing 1 \times phosphate-buffered saline

(PBS), 1% paraformaldehyde, and 0.2% glutaraldehyde. The cells were washed with $1 \times$ PBS and incubated in staining solution (40 mM potassium ferrocyanide, 40 mM potassium ferricyanide, 2 mM Mg_2Cl , and 0.4 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside [X-Gal] per ml) for 50 min at 37°C. After the cells were washed with PBS, the blue cells were counted.

HIV-1 virus production assays. HEK293T cells seeded in 12-well plates were transfected with Lipofectamine (Invitrogen). Each well received 0.6 μ g of BH10 proviral DNA along with 90 pmol of mock siRNA or PRMT6 siRNA. Forty-eight hours after transfection, the culture supernatants were collected and assayed for HIV-1 p24 (capsid) by an enzyme-linked immunosorbent assay (ELISA) (Perkin-Elmer).

RESULTS

HIV-1 Tat is a substrate of PRMT6. The HIV Tat protein contains an arginine-rich transactivation motif that differs from the conventional GAR methylation site. Thus, we tested the abilities of CARM1 and two recently identified PRMTs (PRMT6 and PRMT7) with unknown specificity to methylate Tat. Histidine-tagged recombinant Tat was purified and incubated with the indicated recombinant PRMT in the presence of [*methyl*- 3H]S-adenosyl-L-methionine as a methyl donor. The products of the methylation reactions were separated by SDS-PAGE and visualized by using Coomassie blue, and 3H incorporation was observed by fluorography. Tat was methylated by PRMT6 (Fig. 1A, lane 7). The absence of a PRMT in the methylation reaction mixture (lane 5) or the addition of CARM1 or PRMT7 did not result in Tat methylation (Fig. 1A, lanes 6 and 8). The equal loading of His-tagged Tat and the presence of the GST-PRMTs was confirmed by Coomassie blue staining (Fig. 1A). Our findings demonstrate that Tat is a target of PRMT6.

Next, we examined whether Tat was methylated *in vivo* and whether the expression of PRMT6 increased Tat methylation. HA-tagged Tat was transfected alone in HEK293T cells and cotransfected with PRMT6 or PRMT6 harboring a methylase inactive mutation (VLD to KLA at amino acids 27 to 29), and methylation was assessed by metabolic labeling with [*methyl*- 3H]-L-methionine as previously described (3). The *in vivo* methylation assay is typically performed in the presence of translation inhibitors to ensure that the labeling is not due to incorporation of methionine into newly synthesized proteins. The immunoprecipitated proteins were resolved by SDS-PAGE and visualized by fluorography. HA epitope-tagged Tat was labeled with 3H in cells transfected with Tat alone (Fig. 1B, lane 1). These data demonstrate that an endogenous PRMT methylates Tat, which is consistent with the idea that PRMT6 is the methylase, as HEK293T cells are known to contain PRMT6 (see below). The cotransfection of PRMT6 increased the level of Tat incorporating *methyl*- 3H , and this increase was not observed with a methyltransferase-inactive PRMT6 (Fig. 1B, compare lanes 1 to 3). HA-Tat was not labeled with [^{35}S]methionine in the presence of translational inhibitors, demonstrating that translation was indeed inhibited (Fig. 1B, lane 4). These findings demonstrate that Tat is methylated *in vivo* and that PRMT6 increases this level of methylation.

To map the region of Tat that is methylated by PRMT6, we obtained three peptides that contained all the arginines of Tat and one control peptide. Tat peptide 1–14 contains arginine 7, Tat peptide 25–39 is devoid of arginines (negative control), peptide 49–63 contains the arginine-rich motif, and peptide 69–83 contains arginine 78. *In vitro* methylation reactions were

performed using the Tat peptides, recombinant GST-PRMT6, and [*methyl*- 3H]-S-adenosyl-L-methionine. The peptides were resolved on a 15% polyacrylamide gel containing SDS and a high level of TEMED and visualized by fluorography. The peptide containing the ARM was the only one that incorporated 3H (Fig. 1C, lane 4). Tat peptide 25–39 was used as a negative control, and histidine-tagged Tat was used as a positive control (Fig. 1C, lanes 1 and 3). His-tagged Tat often migrated as a broad band *in vitro* (Fig. 1C, lane 1) compared with *in vivo* expression (Fig. 1B), and we suspect it is caused by the presence of the histidine tag. In summary, our findings demonstrate that Tat is methylated between amino acids 49 and 63 in the ARM.

Tat and PRMT6 interact. PRMTs are known to interact with their substrates, and to determine whether Tat and PRMT6 interact, coimmunoprecipitation experiments were performed. HeLa cells transfected with myc epitope-tagged PRMT6 and HA epitope-tagged Tat were immunoprecipitated with anti-HA antibodies. The bound proteins were separated by SDS-PAGE and immunoblotted with anti-myc antibodies. Myc-PRMT6 was observed in the anti-HA immunoprecipitates but not in the control immunoglobulin G immunoprecipitates (Fig. 2, lanes 2 and 3). The reverse experiment was also true, as HA-Tat was obtained in anti-PRMT6 immunoprecipitates (Fig. 2, lanes 4 to 6). These findings confirm that Tat and PRMT6 interact, providing further support that PRMT6 is the Tat-specific methyltransferase.

Tat transcriptional activation is quenched by PRMT6 overexpression. The ability of PRMT6 to regulate the transcriptional activator function of Tat was examined by using a HIV LTR luciferase reporter plasmid. The HA-Tat expression vector was cotransfected with increasing amounts of pMyc-PRMT6 expression vector in HEK293T cells in the presence of the HIV LTR luciferase reporter plasmid. Transfection of Tat expression plasmid resulted in a ~ 10 -fold activation in luciferase activity in HEK293T cells (Fig. 3). Cotransfection of 0.5 and 1 μ g of the PRMT6 expression vector inhibited the ability of Tat to function as a transcriptional activator in a dose-dependent manner, as measured by luciferase activity (Fig. 3A). These results suggest that arginine methylation negatively regulates the ability of Tat to function as a transcriptional transactivator.

To confirm that the PRMT6 methylase domain was required, the same experiment was performed with the expression vector encoding the methylase-inactive pMyc-PRMT6: VLD-KLA. The methylase-inactive PRMT6 was unable to significantly inhibit HIV LTR gene expression (Fig. 3B). These findings suggest that PRMT6 methyltransferase activity is required for inhibition of HIV LTR gene expression. If the overexpression of PRMT6 inhibits Tat transactivation, knocking down PRMT6 should stimulate Tat transactivation. siRNA against PRMT6 was cotransfected with HA-Tat and the HIV LTR luciferase reporter plasmid in HEK293 cells. Indeed, the normalized luciferase activity was significantly increased after PRMT6 siRNA treatment (Fig. 3C).

PRMT6 reduction by siRNA stimulates HIV-1 transcription from an integrated HIV-1 promoter and viral production. The decrease in Tat transactivation observed with the overexpression of PRMT6 suggested that arginine methylation may be an inhibitory signal for HIV production. To test this idea, we

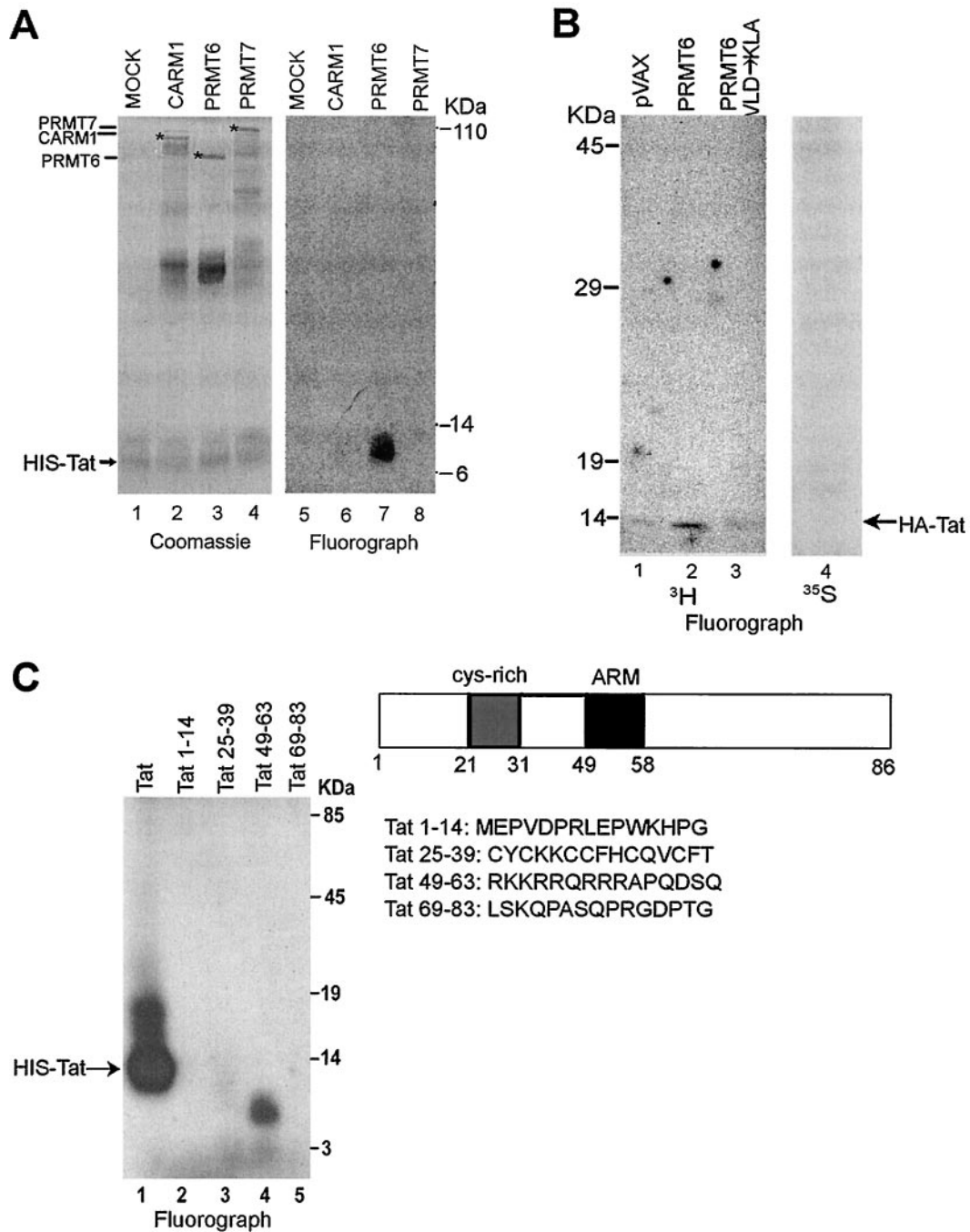


FIG. 1. (A) Arginine methylation of Tat by PRMT6. Histidine-tagged recombinant Tat (HIS-Tat) was incubated with [*methyl*-³H]S-adenosyl-L-methionine in the absence (lanes 1 and 5) and presence of recombinant GST-CARM1 (lanes 2 and 6), GST-PRMT6 (lanes 3 and 7), and GST-PRMT7 (lanes 4 and 8). The proteins were separated by SDS-PAGE, and the gels were stained with Coomassie blue. The incorporation of ³H label on Tat was visualized by fluorography. The migration positions of the GST-PRMTs and HIS-Tat are indicated to the left of the gel. (B) In vivo methylation assay of Tat. HA epitope-tagged Tat (HA-Tat) was transfected with either empty pVAX vector or vector expressing PRMT6 or methyltransferase-inactive PRMT6 (VLD-KLA). The transfected cells were metabolically labeled with L-[*methyl*-³H]methionine. HA-Tat was immunopurified, bound HA-Tat was resolved by SDS-PAGE, and labeled Tat was detected by fluorography. HA-Tat was also transfected alone, and the cells were incubated with L-[³⁵S]methionine to ensure that protein synthesis was indeed inhibited (lane 4). (C) PRMT6 methylates the ARM. A schematic diagram of Tat with its cysteine-rich core (Cys-rich peptide Tat 25-39 [negative control]) and ARM (peptide 49-63). The sequences of the TAT peptides used for methylation are shown. The peptides were incubated with [*methyl*-³H]S-adenosyl-L-methionine in the presence of recombinant GST-PRMT6, and ³H incorporation was visualized by SDS-PAGE followed by fluorography.

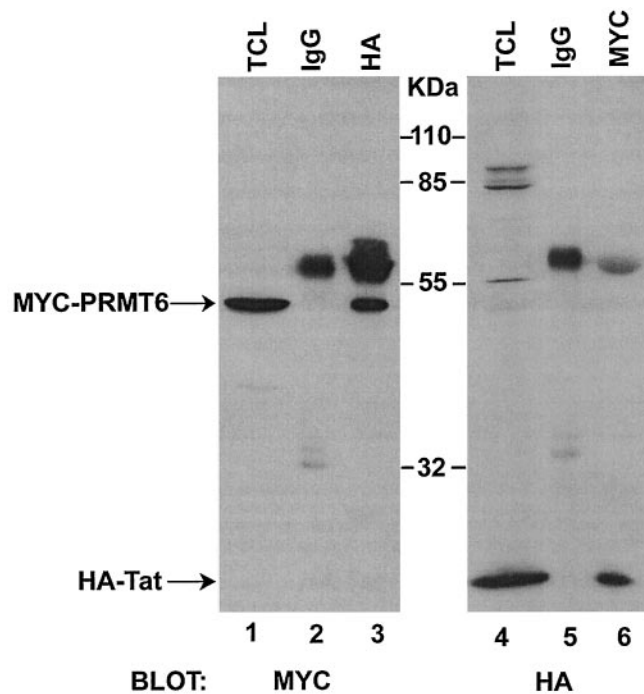


FIG. 2. Interaction of Tat with PRMT6. HeLa cells were transfected with expression vectors encoding HA-Tat and myc-PRMT6. The cells were lysed, and an aliquot was retained as total cell lysate (TCL). Cell extracts were incubated with either control immunoglobulin G (IgG) or with anti-HA or anti-Myc antibodies as indicated. The bound proteins were separated by SDS-PAGE and immunoblotted with anti-Myc (lanes 1 to 3) and anti-HA (lanes 4 to 6) antibodies. The migration of myc-PRMT6 and HA-Tat is shown. The bands at ~60 kDa are the heavy chains of the antibodies. The bands in lane 4 above 55 kDa are often observed with our anti-HA (12CA5) antibodies and are nonspecific.

examined whether reduced PRMT6 expression increased the replication of HIV-1 as measured by using a single HIV-1 replication assay. CD4⁺ HeLa cells stably transfected with HIV-1 LTR-*lacZ* (HeLa MAGI cells) were infected with BH10 virus (22). The production of β -galactosidase in this assay reflects the ability of BH10 virus to undergo one round of replication leading to the production of Tat, generated by the HIV-1 infection, which then activates the stably integrated HIV-1 LTR.

To examine the effect of PRMT6 on HIV-1 transcription, we performed RNA interference to knock down the expression of PRMT6. HeLa MAGI cells were transfected with either mock siRNA or siRNA against PRMT6 (si6). The knockdown of PRMT6 mRNA was confirmed by semiquantitative RT-PCR. A PRMT6 cDNA fragment was amplified in mock siRNA-treated samples, not in PRMT6 siRNA-treated samples, suggesting that the PRMT6 transcript is degraded by the siRNA. cDNA fragments were not amplified in RT-PCR assays without reverse transcriptase, confirming that the DNA fragment is not the result of contaminating DNA (Fig. 4A, lanes 1 and 3). Moreover, the same RT-PCR mixtures contained equal amounts of glyceraldehyde-3-phosphate dehydrogenase mRNA (Fig. 4A, bottom gel) confirming the semiquantitative nature of the assay.

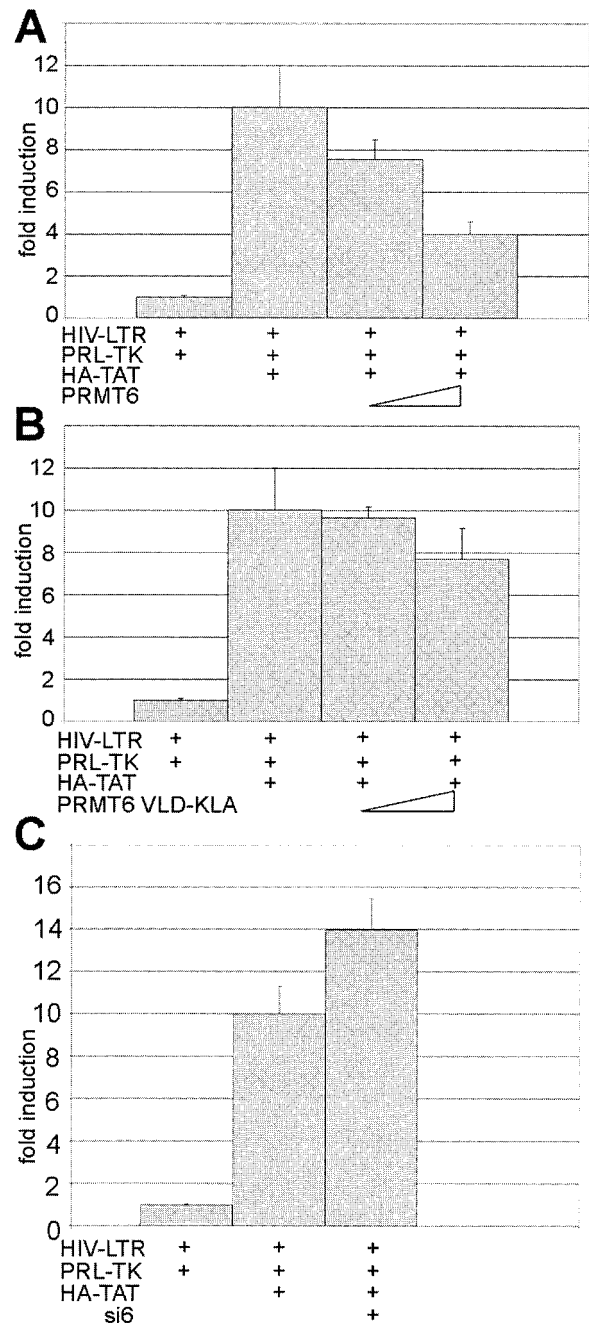


FIG. 3. PRMT6 expression inhibits Tat-mediated transactivation of the HIV-LTR promoter. HEK293T cells were transfected with the HIV LTR luciferase reporter plasmid along with expression vectors for HA-Tat, PRMT6 (A), and methylase-inactive PRMT6:VLD-KLA (B) or PRMT6 siRNA (C) as indicated. In panels A and B, the amount (0.5 and 1.0 μ g) of PRMT6 or PRMT6:VLD-KLA is indicated by the thickness of the triangle below the bar. PRL-TK was included to control for transfection efficiency. The transfected cells were lysed 48 h after DNA transfection and assayed for luciferase activity, which was normalized to the activity of renilla. The luciferase activity without Tat was normalized to 1, and Tat induction is shown as fold induction. The results shown represent the mean \pm standard error of the mean from three separate experiments for each bar ($n > 9$).

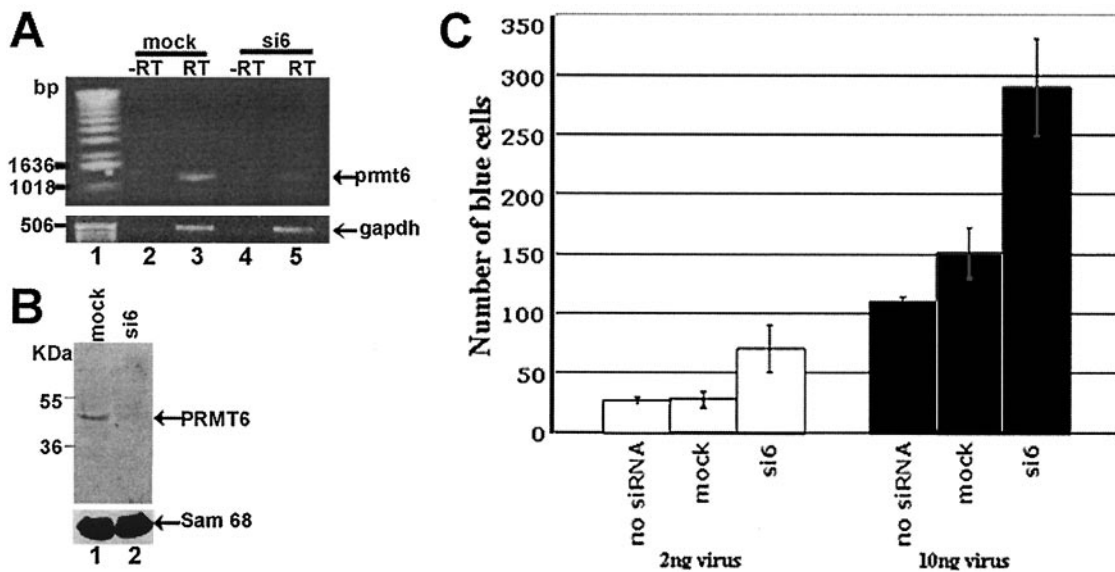


FIG. 4. Reduced PRMT6 expression enhances HIV-1 LTR expression. HeLa MAGI cells were transfected with mock siRNA or PRMT6 siRNA. After 48 h, the cells were lysed and the mRNA (A) and protein (B) extracts were analyzed by RT-PCR and immunoblotting with anti-PRMT6 or anti-Sam68 antibodies. The presence (+RT) or absence (-RT) of reverse transcriptase is indicated above the lanes. gapdh, glyceraldehyde-3-phosphate dehydrogenase. (C) HeLa MAGI cells containing a stably integrated copy of HIV-1 LTR- β -galactosidase and expressing the CD4 receptor were transfected with mock siRNA or PRMT6 siRNA. At 24 h posttransfection, the cells were infected with 2 or 10 ng of T-tropic HIV-1. The cells were incubated for 48 h, fixed, and stained with X-Gal. The bars represent the number of blue cells counted in three or four nonoverlapping fields.

We then examined whether PRMT6 siRNA led to a reduction of the PRMT6 protein. HeLa MAGI cells were lysed, and the proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-PRMT6 antibodies. The amount of PRMT6, with a molecular mass of ~40 kDa, was considerably lower in cells treated with PRMT6 siRNA and not mock siRNA (Fig. 4B, lane 2). The same membrane was immunoblotted with anti-Sam68 antibody to confirm equivalent loading (Fig. 4B, bottom gel). These data show that PRMT6 mRNA and protein are down-regulated (knocked down) with the PRMT6 siRNA in HeLa MAGI cells. Moreover, no phenotypic changes in the cells were observed with PRMT6 knock-down (data not shown).

HeLa MAGI cells treated with mock siRNA or PRMT6 siRNA were infected with either 2 or 10 ng of BH10 virus. Forty-eight hours later, the cells were fixed, and β -galactosidase expression was assayed by X-Gal staining. With 2 ng of BH10 virus, ~25 cells expressed β -galactosidase, and this increased to ~70 cells for PRMT6 siRNA-treated cells. A similar 2.5-fold induction was also observed with 10 ng of HIV-1 (Fig. 4C). These results suggest that the absence of PRMT6 increases viral infectiousness by up-regulating the Tat transactivation activity.

We also provide evidence that Tat methylation by PRMT6 plays an important role in the regulation of HIV-1 production. HEK293T cells were transfected with HIV-1 BH10 proviral DNA in combination with 90 pmol of mock siRNA or PRMT6 siRNA, and the presence of p24 (capsid) was measured as a marker of viral production. Forty-eight hours after transfection, an aliquot of the supernatant was collected for p24 measurement, and cell extracts were prepared to detect the levels of endogenous PRMT6 protein. A decrease in PRMT6 expres-

sion was detected in PRMT6 siRNA-transfected cells as examined by immunoblotting cell extracts with anti-PRMT6 antibodies (Fig. 5A). Equivalent loading was achieved as noted by immunoblotting with anti-Sam68 antibodies (Fig. 5A). The supernatant was collected 48 h after transfection, and the presence of p24 (capsid) protein was quantitated by an ELISA. Transfection of PRMT6 siRNA led to a sixfold-higher production of HIV particles compared to that of control siRNA-treated cells (Fig. 5C). Our findings suggest that PRMT6 would impede HIV-1 production, and overexpression of PRMT6 did indeed lead to a significant decrease in p24 production (Fig. 5C). The expression of myc epitope-tagged PRMT6 was verified by immunoblotting with Myc antibodies (Fig. 5B). Our results demonstrate that PRMT6 protein levels regulate HIV production.

DISCUSSION

We have identified HIV-1 Tat protein as a substrate for PRMT6. This is not only the first report that a HIV-1 protein is subject to arginine methylation but also the first time that Tat was identified as the first functional substrate for PRMT6. Proteins may undergo various types of posttranslational modification. Arginine methylation often takes place on RNA-binding proteins that contain a GAR motif that is recognized by PRMT1 and PRMT5. HIV-1 Tat does not harbor such a consensus sequence and is consistently not modified by either PRMT1 or PRMT5 (23). Interestingly, the results of our study show that the ARM of Tat is methylated by PRMT6, an arginine methyltransferase that was recently identified by database screening and its substrates had hitherto been unclear. Our work not only helps resolve this issue but also reveals a novel

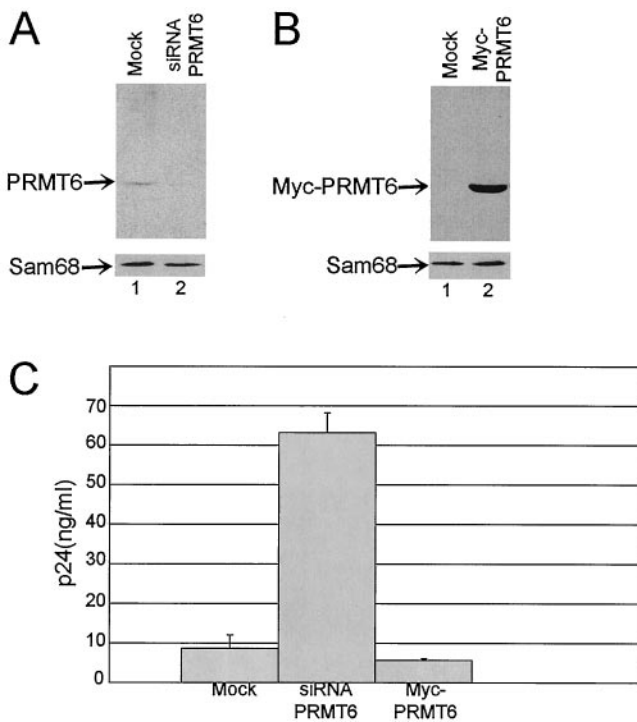


FIG. 5. HIVp24 production is increased with the knockdown of PRMT6 and decreased with PRMT6 overexpression. HEK293T cells were transfected with the BH10 proviral HIV-1 plasmid along with mock siRNA, PRMT6 siRNA, or myc-PRMT6 expression vector. Forty-eight hours after transfection, the knockdown of PRMT6 was examined by immunoblotting with anti-PRMT6 antibodies (A) or anti-Myc antibodies (B). The same membranes were also immunoblotted with anti-Sam68 antibodies to verify equivalent loading. (C) HIV-1 production was assessed by measuring the level of HIV p24 present in the supernatant of the transfected cells by an ELISA after transfection with PRMT6 siRNA or expression vectors. The results shown represent the mean \pm standard error of the mean from three separate experiments ($n > 6$).

pathway that may regulate the activity of Tat during HIV replication.

In contrast to the positive effect of lysine acetylation on Tat activity, the results of this study show that arginine methylation diminishes the transactivation capacity of Tat. For instance, overexpression of PRMT6 inhibited HIV-1 LTR-directed expression of luciferase in the presence of Tat, while the down-regulation of PRMT6 by siRNA treatment significantly augmented Tat function and led to the production of higher levels of viral protein. This newly discovered modification of Tat has various implications. It is possible that Tat activity needs to be fine-tuned by both positive and negative regulatory mechanisms within cells in order to achieve optimal viral gene expression. On the other hand, arginine methylation may be required by Tat in order for the latter protein to perform functions other than the stimulation of gene transcription, such as controlling viral reverse transcription (16, 20). Last, Tat methylation may reflect one of the many mechanisms that cells exploit to inhibit HIV replication, such as the HIV-antagonizing activity of APOBEC3G (27, 43) and the restriction factor Ref-1 (17, 38).

Arginine methylation of Tat may regulate its transactivation

activity in a number of ways. The ARM of Tat is essential for Tat-TAR binding and localization. Methylation of the arginine residues within this region may diminish the affinity between Tat and viral RNA or prevent the proper localization of Tat. It is also possible that Tat methylation affects the association of Tat with cellular factors, such as cyclin T1. We have previously demonstrated that arginine methylation modulates protein-protein interactions (2). A precedent supporting this hypothesis is the finding that acetylation of lysine 50 within Tat helps generate a new site that directs the formation of the Tat-PCAF-P-TEFb ternary complex (7). Last, methylation of Tat by PRMT6 may impede the acetylation of lysine 50 by p300, thus blocking Tat induction (21). The fact that lysine 50 is located within the arginine-rich motif of Tat makes this hypothesis attractive.

The arginine methylation pathway can also affect HIV gene expression through cellular factors. For instance, Spt5 can function together with Tat to promote transcriptional activation by preventing the premature release of mRNA (5). It has recently been demonstrated that Spt5 contains methylated arginines, a process that regulates its interaction with RNA polymerase II, hence affecting its transcriptional elongation properties (23). Overexpression of methyltransferases can inhibit transactivation, and methyltransferase inhibitors can also increase transactivation (23).

In summary, we have identified the first known substrate of PRMT6 and have shown that the HIV-1 Tat protein can be regulated in regard to its methylation status. This is also the first such demonstration for any HIV-1 protein. Further research on the effect of PRMT6 and Tat methylation on HIV-1 production may lead to new insights in the regulation of HIV-1 gene expression and reveal new potential targets for HIV-1 therapeutics. Indeed, both positive and negative small-molecule regulators of PRMT activity have recently been identified (9). Thus, compounds that activate PRMT6 could function as a brake on TAT coactivator properties. Also, it remains to be examined whether diets rich in vitamin B₁₂ that increase the levels of biological methylation offer protection against HIV.

ACKNOWLEDGMENTS

We thank Maureen Oliveira for the p24 ELISAs.

This work was supported in part by grants to S.R. (MOP-67070), C.L. and M.A.W. from the Canadian Institutes of Health Research (CIHR). S.R. is an investigator of the CIHR. M.T.B. is supported by the Damon Runyon Cancer Research Foundation Scholar award DRS-28-02.

REFERENCES

1. An, W., J. Kim, and R. G. Roeder. 2004. Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. *Cell* **117**: 735–748.
2. Bedford, M. T., A. Frankel, M. B. Yaffe, S. Clarke, P. Leder, and S. Richard. 2000. Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains. *J. Biol. Chem.* **275**:16030–16036.
3. Boisvert, F., J. Côté, M. C. Boulanger, P. Cléroux, F. Bachand, C. Autexier, and S. Richard. 2002. Symmetrical dimethylarginine methylation is required for the localization of SMN in Cajal bodies and pre-mRNA splicing. *J. Cell Biol.* **159**:957–969.
4. Boisvert, F. M., J. Cote, M. C. Boulanger, and S. Richard. 2003. A proteomic analysis of arginine-methylated protein complexes. *Mol. Cell Proteomics* **2**:1319–1330.
5. Bourgeois, C. F., Y. K. Kim, M. J. Churcher, M. J. West, and J. Karn. 2002. Spt5 cooperates with human immunodeficiency virus type 1 Tat by preventing premature RNA release at terminator sequences. *Mol. Cell. Biol.* **22**: 1079–1093.

6. Branscombe, T. L., A. Frankel, J. H. Lee, J. R. Cook, Z. Yang, S. Pestka, and S. Clarke. 2001. PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. *J. Biol. Chem.* **276**:32971–32976.
7. Bres, V., H. Tagami, J. M. Peloponese, E. Loret, K. T. Jeang, Y. Nakatani, S. Emiliani, M. Benkirane, and R. E. Kiernan. 2002. Differential acetylation of Tat coordinates its interaction with the co-activators cyclin T1 and PCAF. *EMBO J.* **21**:6811–6819.
8. Chen, D., H. Ma, H. Hong, S. S. Koh, S. M. Huang, B. T. Schurter, D. W. Aswad, and M. R. Stallcup. 1999. Regulation of transcription by a protein arginine methyltransferase. *Science* **284**:2174–2177.
9. Cheng, D., N. Yadav, R. W. King, M. S. Swanson, E. J. Weinstein, and M. T. Bedford. 2004. Small molecule regulators of protein arginine methyltransferases. *J. Biol. Chem.* **279**:23892–23899.
10. Chevillard-Briet, M., D. Trouche, and L. Vandell. 2002. Control of CBP co-activating activity by arginine methylation. *EMBO J.* **15**:5457–5466.
11. Daujat, S., U. Bauer, V. Shah, B. Turner, S. Berger, and T. Kouzarides. 2002. Crosstalk between CARM1 methylation and CBP acetylation on histone H3. *Curr. Biol.* **12**:2090–2097.
12. Fabbriozio, E., S. El Messaoudi, J. Polanowska, C. Paul, J. R. Cook, J.-H. Lee, V. Nègre, M. Rousset, S. Pestka, A. LeCam, and C. Sardet. 2002. Negative regulation of transcription by the type II arginine methyltransferase PRMT5. *EMBO Rep.* **3**:641–645.
13. Frankel, A., N. Yadav, J. Lee, T. Branscombe, S. Clarke, and M. T. Bedford. 2002. The novel human arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *J. Biol. Chem.* **277**:3537–3543.
14. Gary, J. D., and S. Clarke. 1998. RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucleic Acid Res. Mol. Biol.* **61**:65–131.
15. Gros, L., C. Delaporte, S. Frey, J. Decesse, B. R. de Saint-Vincent, L. Cavarec, A. Dubart, A. V. Gudkov, and A. Jacquemin-Sablon. 2003. Identification of new drug sensitivity genes using genetic suppressor elements: protein arginine N-methyltransferase mediates cell sensitivity to DNA-damaging agents. *Cancer Res.* **63**:164–171.
16. Harrich, D., C. Ulich, L. F. Garcia-Martinez, and R. B. Gaynor. 1997. Tat is required for efficient HIV-1 reverse transcription. *EMBO J.* **16**:1224–1235.
17. Hatzioannou, T., S. Cowan, S. P. Goff, P. D. Bieniasz, and G. J. Towers. 2003. Restriction of multiple divergent retroviruses by Lvl1 and Ref1. *EMBO J.* **22**:385–394.
18. Jenuwein, T., and C. D. Allis. 2001. Translating the histone code. *Science* **293**:1074–1080.
19. Kaehlecke, K., A. Dorr, C. Hetzer-Egger, V. Kiermer, P. Henklein, M. Schnoelzer, E. Loret, P. A. Cole, E. Verdin, and M. Ott. 2003. Acetylation of Tat defines a cyclinT1-independent step in HIV transactivation. *Mol. Cell* **12**:167–176.
20. Kameoka, M., L. Rong, M. Gotte, C. Liang, R. S. Russell, and M. A. Wainberg. 2001. Role for human immunodeficiency virus type 1 Tat protein in suppression of viral reverse transcriptase activity during late stages of viral replication. *J. Virol.* **75**:2675–2683.
21. Kiernan, R. E., C. Vanhulle, L. Schiltz, E. Adam, H. Xiao, F. Maudoux, C. Calomme, A. Burny, Y. Nakatani, K. T. Jeang, M. Benkirane, and C. Van Lint. 1999. HIV-1 tat transcriptional activity is regulated by acetylation. *EMBO J.* **18**:6106–6118.
22. Kimpton, J., and M. Emerman. 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β -galactosidase gene. *J. Virol.* **66**:2232–2239.
23. Kwak, Y. T., J. Guo, S. Prajapati, K. J. Park, R. M. Surabhi, B. Miller, P. Gehrig, and R. B. Gaynor. 2003. Methylation of SPT5 regulates its interaction with RNA polymerase II and transcriptional elongation properties. *Mol. Cell* **11**:1055–1066.
24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
25. Liang, C., and M. A. Wainberg. 2002. The role of Tat in HIV-1 replication: an activator and/or a suppressor? *AIDS Rev.* **4**:41–49.
26. Lin, W. J., J. Gary, M. C. Yang, S. Clarke, and H. R. Herschman. 1996. The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. *J. Biol. Chem.* **271**:15034–15044.
27. Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono. 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* **424**:99–103.
28. McBride, A. E., and P. A. Silver. 2001. State of the arg: protein methylation at arginine comes of age. *Cell* **106**:5–8.
29. Miranda, T. B., M. Miranda, A. Frankel, and S. Clarke. 2004. PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity. *J. Biol. Chem.* **279**:22902–22907.
30. Mowen, K. A., J. Tang, W. Zhu, B. T. Schurter, K. Shuai, H. R. Herschman, and M. David. 2001. Arginine methylation of STAT1 modulates IFN α / β -induced transcription. *Cell* **104**:731–741.
31. Ott, M., M. Schnolzer, J. Garnica, W. Fischle, S. Emiliani, H. R. Rackwitz, and E. Verdin. 1999. Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity. *Curr. Biol.* **9**:1489–1492.
32. Pal, S., R. Yun, A. Datta, L. Lacomis, H. Erdjument-Bromage, J. Kumar, P. Tempst, and S. Sif. 2003. mSin3A/histone deacetylase 2- and PRMT5-containing Brg1 complex is involved in transcriptional repression of the Myc target gene *cad*. *Mol. Cell. Biol.* **23**:7475–7487.
33. Pollack, B. P., S. V. Kotenko, W. He, L. S. Izotova, B. L. Barnoski, and S. Pestka. 1999. The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity. *J. Biol. Chem.* **274**:31531–31542.
34. Roof, P., M. Ricci, P. Genin, M. A. Montano, M. Essex, M. A. Wainberg, A. Gatignol, and J. Hiscott. 2002. Differential regulation of HIV-1 clade-specific B, C and E long terminal repeats of NF- κ B and the Tat transactivator. *Virology* **296**:77–83.
35. Schurter, B. T., S. S. Koh, D. Chen, G. J. Bunick, J. M. Harp, B. L. Hanson, A. Henschel-Edman, D. R. Mackay, M. R. Stallcup, and D. W. Aswad. 2001. Methylation of histone H3 by co-activator arginine methyltransferase 1. *Biochemistry* **40**:5747–5756.
36. Stallcup, M. R., J. H. Kim, C. Teyssier, Y. H. Lee, H. Ma, and D. Chen. 2003. The roles of protein-protein interactions and protein methylation in transcriptional activation by nuclear receptors and their coactivators. *J. Steroid Biochem. Mol. Biol.* **85**:139–145.
37. Tang, J., J. D. Gary, S. Clarke, and H. R. Herschman. 1998. PRMT 3, a type I protein arginine N-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation. *J. Biol. Chem.* **273**:16935–16945.
38. Towers, G. J., T. Hatzioannou, S. Cowan, S. P. Goff, J. Luban, and P. D. Bieniasz. 2003. Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. *Nat. Med.* **9**:1138–1143.
39. Wei, P., M. E. Garber, S. M. Fang, W. H. Fischer, and K. A. Jones. 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* **92**:451–462.
40. Xu, W., H. Chen, K. Du, H. Asahara, M. Tini, B. M. Emerson, M. Montminy, and R. M. Evans. 2001. A transcriptional switch mediated by cofactor methylation. *Science* **294**:2507–2511.
41. Xu, W., H. Cho, and R. M. Evans. 2003. Acetylation and methylation in nuclear receptor gene activation. *Methods Enzymol.* **364**:205–223.
42. Yadav, N., J. Lee, J. Kim, J. Shen, M. C.-T. Hu, C. M. Aldez, and M. T. Bedford. 2003. Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. *Proc. Natl. Acad. Sci. USA* **100**:6464–6468.
43. Zhang, H., B. Yang, R. J. Pomerantz, C. Zhang, S. C. Arunachalam, and L. Gao. 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* **424**:94–98.