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Methylation as a Modulator of Expression of Human Immunodeficiency Virus

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When Vero or murine cells were stably transfected with the human immunodeficiency virus (HIV) long terminal repeat (LTR) that directs the chloramphenicol acetyltransferase (CAT) gene (pU3R-III-CAT), expression was suppressed. Treatment with the nucleoside analog 5-azacytidine (5-azaC) restored CAT expression. S1 nuclease analysis and a nuclear run-on assay demonstrated that activation of the latent HIV LTR by 5-azacytidine occurred at the transcriptional level. Southern blot analysis demonstrated that this activation was due to the demethylation of cytosine residues in the LTR enhancer. Thus, the HIV LTR appears to be susceptible to transcriptional inactivation by methylation, a process that is proposed to play a modulatory role in viral latency.

The human immunodeficiency virus (HIV; also known as human T-cell lymphotropic virus type III [HTLV-III], lymphadenopathy-associated virus [LAV], and acquired immune deficiency syndrome [AIDS]-associated retrovirus [ARV]) is the etiologic agent associated with AIDS (3, 28). Infection by this virus leads to depletion of the $T4^+$ subset of lymphocytes and, ultimately, to suppression of the immune system (14, 23, 39). Expression of HIV has been attributed to virus-associated *trans*-acting factors that increase the level of gene expression directed by the HIV long terminal repeat (LTR). The *trans*-acting regulatory protein (*tat*III) has been located immediately 5′ to the envelope gene in the HIV genome (2, 35). Sequences in the LTR that respond to *tat*III have been localized to the mRNA start site, between nucleotides −17 and 80 (TAR region) (34). The positive feedback mechanism demonstrated for HIV gene expression is mediated by both transcriptional (9) and posttranscriptional (9, 15, 33) events.

In humans HIV infection is characterized by a period of latency followed by progression to AIDS or AIDS-related complex, in some cases (5). Factors that influence HIV latency are poorly understood. Several models have been proposed that might explain how HIV, when harbored in the latent form, can be induced by physiochemical stimuli and expressed as infectious virus particles (16). These models include transcriptional repression of integrated proviral DNA by DNA-binding proteins, chromatin conformation, or DNA hypermethylation. To understand these processes, permanent cell lines were constructed that contained the bacterial chloramphenicol acetyltransferase (CAT) gene directed by the HIV LTR (pU3R-III-CAT) or HTLV type I (HTLV-I) LTR (pU3R-I-CAT). We have previously demonstrated that the HIV LTR but not the HTLV-I LTR, suppressed CAT expression when it was permanently integrated into the host chromatin (25b). In this report we provide evidence that the suppression of HIV LTR expression is due to the methylation of LTR sequences.

Control of cellular and viral gene expression has been shown to be modulated by DNA methylation (7, 12, 13, 22). These processes have been shown to be affected by treatment with the nucleoside analog 5-azacytidine (5-azaC), a potent inhibitor of DNA methylation (21, 27). Therefore, we tested the possibility that methylation of the HIV LTR sequences was involved in suppression of its expression. In cell lines that were permanently transfected with the HIV CAT, expression of CAT activity was negligible (Fig. 1), whereas in cell lines containing the HTLV-I LTR CAT expression was constitutive (Fig. 1). Results of experiments on two independently pooled murine and simian cell lines demonstrated that expression of CAT from the HIV LTR could be restored by 5-azaC treatment (Fig. 1). Pooled cell lines were employed to average out any fluctuations that were observed with individual cell clones (25, 25b). When individual clones were treated, 11 of 12 tested cells lines expressed CAT activity after exposure to 5-azaC, whereas 5-azaC did not have any effect on the constitutive CAT expression directed by the HTLV-I LTR (Fig. 1). HIV and HTLV-I LTRs were expressed differently in fibroblast and lymphoid cells when they were analyzed by a transient transfection assay. Expression of CAT by these LTRs in fibroblast cells was high when compared with that in lymphoid cells (Table 1) (1). Expression in lymphoid cells is dependent on the presence of specific viral *trans*-acting factors (Table 1) (19, 36, 37). Treatment of lymphoid cells with 5-azaC in transient expression assays did not enhance HIV or HTLV-I LTR-directed CAT activity (data not shown).

Two lines of evidence suggest that reactivation of the HIV LTR in fibroblast cells occurs at the transcriptional level. First, S1 nuclease analysis showed that there were no constitutive, correctly initiated HIV LTR CAT mRNA transcripts present in our cell lines (Fig. 2, lanes −). Treatment with 5-azaC in both murine (Fig. 2A, lane +) and simian (Fig. 2B, lane +) permanent cell lines induced correctly initiated HIV LTR CAT mRNA. Second, run-on transcription in isolated nuclei showed no detectable HIV LTR CAT transcripts in these cells (Fig. 3, lane −). HIV LTR CAT nuclear transcripts were observed only after 5-azaC treatment (Fig. 3, lane +). Hybridization observed with the 3,000-base-pair (bp) band was probably due to run-on transcription through the pBR322 vector sequences in isolated nuclei.

To determine whether activation of CAT expression from the HIV LTR by 5-azaC is due to the hypomethylation of some key promoter sequences in the LTR enhancer region, methylation-specific restriction enzyme analysis of genomic

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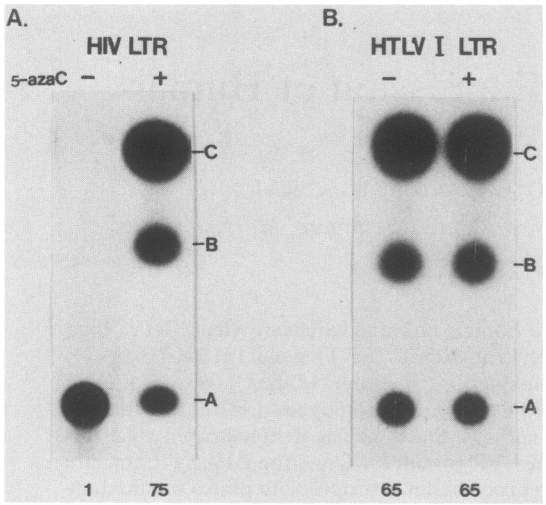

FIG. 1. Effect of 5-azaC on HIV (A) and HTLV-I (B) LTR-directed CAT protein synthesis in permanent simian (Vero) cell lines. Identical results were obtained for murine (Ltk$^-$) cells (data not shown). Preparation of cell lysates and CAT assays were as described previously (26). DNA was cotransfected with SV_2neo, and G418-resistant colonies were selected as described previously (26). Numbers at the bottom of each lane represent the percent conversion of [^{14}C]chloramphenicol (A) to the 3-acetylchloramphenicol product (C). Each cell line (pU3R-I [HTLV-I] and pU3R-III [HIV]) represents a pool of >100 colonies. Pooled cell lines contained approximately 1 copy of integrated DNA per cell, as determined by Southern blot analysis (38; data not shown). The 5-azaC stock solutions were prepared fresh and used immediately by direct addition to freshly supplied 10% fetal calf serum medium at a final concentration of 10 μM. Higher concentrations were cytotoxic. Incubations were terminated after 24 h. None of the HIV LTR-containing permanent cell lines showed any constitutive CAT activity, and 5-azaC consistently gave 50 to 85% conversion of [^{14}C]chloramphenicol to the 3-acetylchloramphenicol product. The letter B on the right side of the figure represents the position of the 1-acetylated chloramphenicol product. Lanes: −, mock treatment; +, 5-azaC treatment.

DNA was employed. Five potential methylation sites within the LTR were analyzed by restriction with *Msp*I-*Hpa*II, *Hae*III, *Ava*I, *Taq*I, or *Pvu*II. Each of the restriction endonucleases, with the exception of *Pvu*II, yielded the same restriction pattern, regardless of 5-azaC treatment (data not shown). Restriction with *Pvu*II of DNA from cell

TABLE 1. LTR expression in fibroblast and lymphoid cells

Plasmids	Percent conversion of[a]:		
	Fibroblast[b]	Lymphoid[c]	Lymphoid with *trans*-acting factors[d]
HIV LTR CAT	45	1.0	98
HTLV-I LTR CAT	55	1.0	95
SV_2 CAT	90	1.5	1.5

[a] Values are the percent conversion of [^{14}C]chloramphenicol to the 3-acetylchloramphenicol product. CAT activity is given as an average value from different cell lines in each group.

[b] Cells were transfected with 10 μg of plasmid DNA as described previously (26). Fibroblasts tested were Ltk$^-$, Vero, and SW480 cell lines.

[c] Lymphoid cells were transfected with 10 μg of plasmid DNA as described previously (29). Lymphoid cells tested were Raji (B cells) and Jurkatt (T cells) cells.

[d] Lymphoid cells containing *trans*-acting factors are Raji- (ZPtat-III), Jurkatt- (ZPtat-III) (32), and HTLV-I-infected MT-2 cells (17).

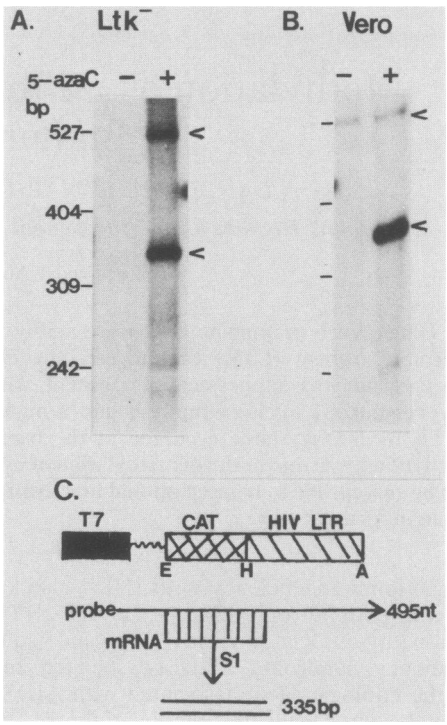

FIG. 2. S1 nuclease analysis of RNA isolated from the permanent Ltk$^-$ (A) and Vero (B) cell lines containing pU3R-III-CAT (HIV LTR CAT). The mock sample (lanes −) was incubated for 12 h in fresh 10% fetal calf serum-containing medium. The 5-azaC-treated sample (lanes +) was incubated for 12 h, as described in the legend to Fig. 1. Total cellular RNA was extracted from the cells by the guanidine isothiocyanate method (8), and 10 μg was hybridized to an excess (5×10^5 cpm) of a 495-nucleotide complementary strand probe transcribed by T7 RNA polymerase (25b). After S1 nuclease digestion, protected fragments were size separated on a 5% polyacrylamide urea sequencing gel at 30 mA. (C) Construction of the probe and the fragment generated after RNA hybridization and S1 nuclease treatment. Lengths are in nucleotides (nt). The letters E, H, and A represents the restriction enzymes *Eco*RI, *Hin*dIII, and *Ava*I, respectively. The wavy line indicates the 15-nucleotide region of the probe that is not complementary to the HIV CAT hybrid sequence. The hatched area represents the HIV LTR, and the cross-hatched area represents a portion of the CAT 5′-coding region. Arrows show the position of the full-length probe (top) and the correctly initiated transcript (bottom).

lines containing integrated HIV CAT and Southern hybridization with the LTR-specific probe yielded an expected 650-bp fragment only after 5-azaC treatment (Fig. 4A). When probed with CAT-specific sequences, a similar blot demonstrated no difference in restriction pattern for DNA obtained from untreated or 5-azaC-treated cultures (Fig. 4B). These data suggest that the *Pvu*II sites located at nucleotide −17 upstream from the cap site of HIV CAT and in cellular sequence about 650 bp upstream become hypomethylated after 5-azaC treatment. When genomic DNA from cell lines containing integrated pU3R-I-CAT was restricted with *Pvu*II or *Pvu*II-*Bam*HI and hybridized with LTR- or CAT-specific probes, respectively, we found no difference in the restriction pattern for DNA obtained from untreated or 5-azaC-treated cultures (Fig. 4A and B).

From the results of these experiments, we conclude that the HIV LTR can be inactivated by methylation of cytosine residues. Similar observations were described for a variant of the CEM T-cell line A3.01, which lacks the *leu*3 surface

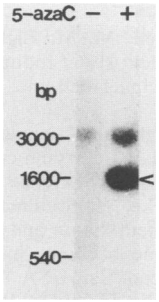

FIG. 3. In vitro labeling of HIV CAT RNA transcripts in nuclei isolated from Ltk$^-$ cells. Isolation of nuclei, conditions for labeling, and isolation and purification of RNA were as described previously (30). The pU3R-III-CAT plasmid DNA was cleaved with *Bgl*II and *Bam*HI (see the diagram in Fig. 4), size separated, denatured, transferred to nitrocellulose, baked, prehybridized, and hybridized to RNA labeled with [α-^{32}P]UTP in vitro. Hybridization was performed in a buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, and 10% dextran sulfate for 3 days at 37°C; nitrocellulose filters were washed and treated with RNase A (30). The indicated base pairs represent the size of the pU3R-III-CAT fragments after restriction with *Bgl*II and *Bam*HI. The 3,000-bp fragment (*Bam*HI to *Bgl*II in the cellular sequences) contains the pBR322 portion of the plasmid, whereas the 1,600-bp fragment (*Bgl*II at nucleotide +20 of the HIV LTR to the *Bam*HI) contains sequences from the HIV LTR cap site, the entire CAT coding region, and simian virus 40 polyadenylation site (arrowhead). The 540-bp fragment (*Bgl*II fragment) contains the HIV promoter. Mock (lane −) and 5-azaC (lane +) treatments were as described in the legend to Fig. 1.

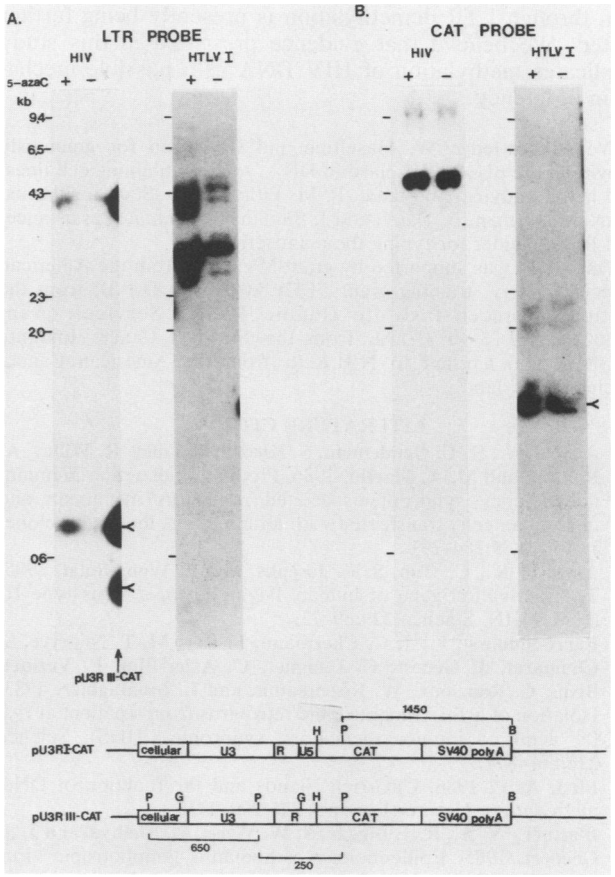

FIG. 4. Southern blot analysis of DNA from HIV CAT- or pU3R-I-CAT-containing Vero cell lines before and after treatment with 5-azaC. Genomic DNA was isolated (6); and 10 μg was restricted with either *Pvu*II or *Pvu*II and *Bam*HI (B, HTLV-I) for 24 h, followed by electrophoresis through a 0.8% agarose gel, and transferred to nitrocellulose by the method described by Southern (38). Filters were hybridized for 48 h at 37°C with either a [^{32}P]CTP-labeled, LTR (*Xho*I, located at the junction of pBR322 and cellular sequence (25a), and *Hin*dIII fragments from pU3R-I-CAT or -III-CAT; panel A) or a 1,600-bp CAT (*Hin*dIII and *Bam*HI; panel B). These were labeled by nick translation (31) to 10^8 cpm/μg of DNA. Lanes −, mock treatment; lanes +, 5-azaC treatment. HIV CAT plasmid DNA was restricted with *Pvu*II and run in parallel (panel A, pU3R-III-CAT). Numbers to the left of panel A are in kilobases (kb). A 250-bp fragment was also hybridized to the LTR probe, because this fragment had 100 bp complementary to the LTR. In 5-azaC-treated HIV CAT-containing genomic DNA, the hybridization to this 250-bp fragment was weak and is not shown in this exposure. A partial restriction map of the plasmids pU3R-I-CAT and pU3R-III-CAT is given below the figure. Abbreviations: H, *Hin*dIII; P, *Pvu*II; B, *Bam*HI; G, *Bgl*II; SV40, simian virus 40. Fragment sizes (in base pairs) are indicated below the brackets and also by arrowheads in panels A and B.

marker and does not produce virus (16). Infectious virus was produced when cells were treated with the nucleoside analog 5-iodo-2′-deoxyuridine, indicating that the virus was present in these cells in a latent form (16).

*Pvu*II is an unusual enzyme for identification of methylated sequences; however, 5′-^{m}CAGCTG-3′ is not restricted by this enzyme (11). This property of the *Pvu*II enzyme allowed us to "view" the methylation state within the HIV LTR enhancer-TAR region. Restriction of genomic DNA with the isoschizomers *Msp*I-*Hpa*II yielded no changes in the LTR methylation pattern. Recently, it has been recognized that transcription of genes with C+G-rich islands are inhibited when these regions are methylated (4). These C+G-rich islands are common to many viral sequences (retroviral LTR, adenovirus, herpesviruses), and some have been shown to be inactivated by DNA methylation (18, 24). Results of this study extend this modulatory concept of methylation to include the HIV LTR.

Infection of humans with HIV leads to the development of AIDS, which is preceded by a prolonged period of latency (5). HIV-infected T cells can be maintained in vitro for extended periods without the release of reverse transcriptase activity (40). Only after antigenic stimulation by agents such as phytohemagglutinin does virus replication and release of reverse transcriptase occur. The expression of *tat*III is believed to play a role in the escape from latency to lytic replication (2, 10, 37, 40). The release of HIV-infected cells from latency after antigenic activation must result first in the initial induction of viral mRNA synthesis from the viral LTR for the *tat*III gene to be expressed. If the viral LTR was inactivated by methylation early after integration into host chromatin, viral mRNA would not be synthesized and the *tat*III gene product would not accumulate. In this state latency would be preserved. An environmental stimulus, similar to exposure of cells to 5-azaC, may release the transcriptional block via demethylation of LTR sequences, thereby permitting induction of mRNA synthesis and escape from latency. Whether methylation is the primary event for HIV LTR suppression or one in a cascade of events is not known. In addition to 5-azaC treatment and herpesvirus infections (25b), we have observed other ways to reactivate LTR expression (J. D. Mosca, D. P. Bednarik, and N. B. K. Raj, unpublished data). The concept of reactiva-

tion through LTR demethylation is presently being further tested. We believe that evidence presented in this study implicates methylation of HIV DNA as a possible mechanism of latency.

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