Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency

Daniel P. Bednarik^{2,3}, Jennifer A. Cook and Paula M. Pitha¹

The Johns Hopkins University, and ¹Department of Molecular Biology and Genetics, School of Medicine Oncology Center, Baltimore, MD 21205, USA

²Present address: Centers for Disease Control, Retrovirus Diseases Branch, 1600 Clifton Road/Mail Stop G-19, Atlanta, GA, 30333, USA

³Corresponding author

Communicated by Walter Doerfler

Infection of cells by HIV can result in a period of quiescence or latency which may be obviated by treatment with inducing agents such as 5-azacytidine. Evidence from these experiments demonstrate the existence of two CpG sites in the HIV LTR which can silence transcription of both reporter genes (CAT) and infectious proviral DNA when enzymatically methylated. This transcriptional block was consistently overcome by the presence of the *trans*-activator *tat* without significant demethylation of the HIV LTR. These results suggest that DNA hypermethylation of the HIV LTR may change the binding characteristics between LTR sequences and cellular proteins, thereby suppressing HIV LTR transcription and modulating viral expression.

Key words: CpG methylation/HIV/latency/methyl binding protein/transactivation

Introduction

In humans, HIV infection is characterized by a period of latency followed by progression to AIDS or AIDS-related complex (ARC) (Lui *et al.*, 1986; Medley *et al.*, 1987). Factors that influence HIV latency are poorly understood. Several models have been proposed which might explain how HIV, when harbored in a latent form, can be induced by physiochemical stimuli and be expressed as infectious virus (Folks *et al.*, 1986, 1987; Fauci, 1988). These models include transcriptional repression of integrated proviral DNA by either DNA-binding proteins, chromatin conformation or DNA hypermethylation.

HIV-infected T-cells can be maintained *in vitro* for extended periods without release of virus particles (Folks *et al.*, 1987; Fauci, 1988). Only after stimulation by agents such as phytohemagglutinin or phorbol esters, cytokines, does virus replication and release of virus particles occur (Fauci, 1988). The expression of *tat* is believed to play a role in this escape from latency to lytic replication (Fauci, 1988).

Control of cellular and viral gene expression has been shown to be modulated by DNA methylation (Doerfler, 1983, 1984; Keshet *et al.*, 1986). Extensive evidence has implicated the enzymatic conversion of cytosine to 5-methylcytosine in the dinucleotide sequence CpG as a mechanism

for transcriptional inhibition of genes (Bird, 1986; Lindsay and Bird, 1988). Evidence from *in vitro* methylation/transfection experiments demonstrated that transcription of genes was inhibited when CpG islands were methylated (Keshet *et al.*, 1985; Bird, 1986). What determines whether a CpG island is methylated or unmethylated? Little is known about the molecular events leading to *de novo* methylation of DNA, but data imply that most cell types are capable of methylating accessible CpGs. Only methylation of sequences in the upstream promoter region render genes transcriptionally inactive (Keshet *et al.*, 1985).

Viruses are also susceptible to inactivation by methylation, since they contain sequences which are rich in CpG islands. Retroviral LTRs, adenovirus and Herpes viruses are G+C rich and moreover, the replication of both adenovirus and Moloney retrovirus can be inactivated by DNA methylation (Harbers *et al.*, 1981; Graessmann *et al.*, 1983; Kruczek and Doerfler, 1983; Keshet *et al.*, 1985). Reactivation of latent viruses has been demonstrated by experiments which employ the use of methylation antagonists, such as 5-azacytidine. Furthermore, promoter sequences which are hypermethylated showed a greatly reduced capacity to bind *trans*-acting nuclear proteins (Michalowsky and Jones, 1987), binding could be restored when the DNA was hypomethylated by 5-azacytidine treatment (Michalowsky and Jones, 1987). In contrast, hypermethylation of DNA increased the affinity of the DNA to bind a specific class of proteins (Zhang *et al.*, 1986; Meehan *et al.*, 1989) which interfered with transcription (Holliday, 1989; Meehan *et al.*, 1989). In addition, it was recently shown that *trans*-acting factors of adenovirus could overcome methylation-mediated transcriptional inhibition without changing the methylation pattern of the DNA (Weissbarr *et al.*, 1988).

In this report, we describe the role of two CpG sites within the HIV LTR which, upon enzymatic methylation *in vitro*, effectively incapacitates the ability of the LTR to direct expression of viral genes. This silencing effect could be obviated in *trans* by *tat* or by cellular factors. These observations are suggestive of a complex transcriptional regulatory mechanism, which may involve several cellular factors.

Results

Determination of methylation sensitive sites in the HIV LTR

Previous studies have tested the possibility that methylation of the HIV LTR was involved in suppression of LTR expression (Bednarik *et al.*, 1987). In cell lines permanently transfected with the HIV LTRCAT hybrid plasmids (pU3RIICAT), LTR sequences were demonstrated to be inactivated by hypermethylation, which could be reactivated by treatment with the DNA methylation antagonist 5-azacytidine (Bednarik *et al.*, 1987). As a logical extension

Fig. 3. Transcriptional analysis of proviral DNA expression after enzymatic, site-specific methylation. To determine the sensitivity of proviral, infectious clone DNA to methylation-dependent inhibition of transcription, 10 μ g of pNL4-3 infectious proviral clone DNA was subjected to enzymatic methylation as described in Materials and methods. Proviral DNA was subsequently transfected into SW480 (human) colonic adenocarcinoma cells and total RNA was isolated 24 h post-transfection. Transcripts were resolved on a 0.8% formaldehyde-agarose gel, followed by Northern transfer to a nylon membrane. Membranes were hybridized with a 2 kb riboprobe spanning the LTR-gag regions of the HIV provirus (pJM105), followed by subsequent autoradiography. **Lanes:** 1, unmethylated control; 2, *Bam*HI methylase; 3, *Hha*I methylase; 4, *Alu*I methylase; 5, *Hae*III methylase; 6, *Hpa*II methylase. Arrows denote (from top) 9.0 kb genomic mRNA, 4.2 kb envelope mRNA, 2.0 and 1.6 kb transcripts.

Inactivation HIV proviral DNA by site-specific *in vitro* methylation

Analysis of provirus expression following electroporation (10 μ g) of HIV proviral DNA (pHBCX2) methylated at either CpG or non-CpG sites is shown in Figure 3. Northern hybridization analysis conclusively demonstrated that loss of transcriptional activity was more profound when sites in the LTR were methylated (lanes 3–6) than when structural, non-LTR sequences were methylated (lane 2). This observation agrees with other findings indicating that gene promoters are sensitive to transcriptional inactivation via hypermethylation (Cedar, 1988) in contrast to minimal effects observed when structural gene sequences are methylated, with the exception of the thymidine kinase gene (Christy and Scangos, 1984). Inactivation of HIV expression by *in vitro* methylation is virtually identical to the *in vivo* inactivation of Moloney murine leukemia virus (M-MuLV) observed in undifferentiated mouse embryonal carcinoma cells (Cedar, 1988).

The *trans*-activator of *tat* can activate the methylated, transcriptionally inactive DNA

Previous studies by our laboratory have suggested that various *trans*-acting factors such as *tat* or the immediate-early proteins of herpes simplex virus infection activate the silent HIV LTR (Gendelman *et al.*, 1986; Mosca *et al.*, 1987a,b; Valerie *et al.*, 1988) with a corresponding change in DNA methylation patterns (Bednarik *et al.*, 1987, 1989). Studies by Weisshaar *et al.* (1988) also showed that *trans*-acting factors of the adenovirus system are capable of overcoming a transcriptional block introduced by *in vitro* methylation. We therefore studied the effect of the HIV *trans*-activator, *tat*, on LTR sequences which were methylated *in vitro*. Transient transfection assays were performed with Vero (simian) cell monolayers by transfection of *in vitro* methylated HIV LTRCAT (pU3RIICAT) plasmid DNA (10 μ g) either alone or in combination with 2 μ g LTR*tat* (pIIIextatIII). Induction of CAT protein synthesis was analyzed 24 h post-transfection in the presence or absence of *tat* is shown in Figure 4, panel A. The overall *cis*-level expression of the LTR was reduced over 3-fold when both *Hpa*II sites were methylated. In each case, *tat* was observed to obviate the transcriptional block. Analysis of CAT mRNA in transfected cells by S1 nuclease demonstrated the accumulation of correctly initiated transcripts after transfection with pIIIextatIII (panel B).

The function of host-cellular factors in the activation of methylated, transcriptionally inactive DNA

To determine whether cellular factors play a role in the activation of the methylated, transcriptionally silent LTR, methylated plasmid DNA was electroporated into A3.01 cells, followed by treatment with the phorbol ester TPA. It was shown by others that stimulation of T-cells by phorbol esters results in increased expression of HIV, mediated by the inducible transcription factor NF κ B (Nabel and Baltimore, 1987; Nabel *et al.*, 1988; Bielinska *et al.*, 1989). As demonstrated in Figures 2–4, *cis*-level LTR expression was abolished by methylation, but could be activated *in trans* by *tat*. Transactivation by *tat* could be eliminated when the LTR directing *tat* expression was methylated (Figure 2, lane 5). However, addition of TPA (50 ng/ml) followed by incubation for 6 h permitted complete recovery of *tat*-mediated transactivation (Figure 5, lanes 10–11).

These experiments suggest that host cellular nuclear protein(s) are activated by TPA which subsequently may circumvent the methylation-mediated transcriptional block thereby allowing the initial accumulation of *tat*. Since *tat* can *trans*-activate the HIV LTR regardless of the methylation state, a *trans*-activation cascade likely occurs in which TPA initially stimulates accumulation of low levels of *tat*, which in turn drives the expression of itself and CAT to high levels. This scenario is identical to the reactivation process of latent, integrated provirus during T-cell activation in response to the encounter with an inducing agent.

The effect of *tat* on the methylation profile of the HIV LTR

In order to determine whether *tat* mediates demethylation of the HIV LTR, *in vivo* methylated LTRCAT plasmid DNA (pU3RIICAT) was transiently transfected into SW480 cells lacking or containing the *tat* gene (Figure 6). Lanes 1 and

Fig. 4. Methylation-mediated inhibition of HIV LTR expression is overcome via *trans*-activation by *tat*. Transient transfection assays were performed on Vero (simian) cell monolayers by transfection of enzymatically methylated HIV LTRCAT (pU3RIICAT) plasmid DNA (10 μ g) either alone or in combination with LTR *tat* (pIIIextatIII) (2 μ g). **Panel A:** induction of CAT protein synthesis 24 h post-transfection in the presence or absence of *tat*. Lanes from left are: 1, mock transfection; 2, unmethylated control; 3, unmethylated control/*tat*; 4, *HpaII* methylated; 5, *HpaII* methylated/*tat*; 6, *AluI* methylated; 7, *AluI* methylated/*tat*; 8, *HphI* methylated; 9, *HphI* methylated/*tat*; 10, blank. **Panel B:** S1 nuclease transcriptional analysis of LTRCAT mRNA synthesized in response to *trans*-activation by *tat*. Lanes are identical to those depicted in panel A. The arrow indicates correctly initiated, 335 bp protected fragment detected 24 h post-transfection in total RNA extracts.

4 demonstrate the restriction pattern of unmethylated HIV LTRCAT (pU3RIICAT) DNA when digested with either *HpaII* or *MspI*, respectively. In either case, the endonuclease employed corresponded to the methylase used in the reaction.

In each case, plasmid DNA was transfected into either SW480 or SWN*tat* cells, and the DNA was subsequently recovered from isolated nuclei, followed by Southern hybridization analysis utilizing a 500 bp probe obtained by *BglII* restriction of pU3RIICAT. Recovery of methylated plasmid DNA from SW480 cells (Figure 6, lanes 2, 5) showed only the undigested forms of the plasmid DNA. Recovery of plasmid DNA from SW480 cells expressing *tat* protein (SWN*tat*) demonstrated the appearance of a band of approximately 1.6 kb in size suggesting partial digestion of the plasmid DNA had occurred. However, it is not known unequivocally whether demethylation occurred as a result of *tat* activity, or whether demethylation events involving CpG moieties outside the LTR were a contributing factor. These results suggest that *trans*-activation of the HIV LTR, like the adenovirus system (Weisshaar *et al.*, 1988), does not require complete demethylation.

The effect of site-specific LTR DNA methylation on binding of host transcriptional factors

In order to assess the effect of site-specific *in vitro* methylation on the binding of nuclear factors to the HIV LTR, we employed the mobility shift gel assay (Baldwin and Sharp, 1987), in which the mobility of a labeled DNA fragment is retarded on a non-denaturing polyacrylamide gel as a protein-DNA complex. A labeled [32 P]DNA fragment was incubated with a partially purified nuclear fraction obtained from SW480 cells or an SW480 cell line expressing the *tat* protein (SWN*tat*). Binding reactions were performed utilizing a 212 bp fragment, generated by *TaqI* restriction of the PCR-amplified DNA segment spanning both CpG sites

Fig. 5. Methylation-mediated inhibition of HIV LTR expression is influenced by cellular factors. A3.01 cells were electroporated with HIV LTRCAT (pU3RIICAT) (10 μ g) alone, or in combination with HIV LTR*tat* (pIIIextatIII); lanes 1,2). In each case, the LTRs were either individually methylated *in vitro*, or both were methylated simultaneously in order to differentiate the effect of methylation of one plasmid DNA on the expression of the other (lanes 5–8). Methylated plasmid DNA is denoted by superscript *M*. The effects of induced host cellular factors were determined by TPA treatment (50 ng/ml; lanes 3,4). Percent conversions to acetylated chloramphenicol (arrows) are illustrated below each lane.

(Figure 1, Probe I, –218/–140). Figure 7 demonstrates the electrophoretic mobility of nuclear proteins which retard the migration of labeled probe DNA as a protein:DNA complex. When LTR DNA fragments were methylated at positions –218 or –146 with either *HpaII* or *MspI* methylase (Figure 7A, lanes 2,4) the nuclear protein denoted NPI demonstrated a significantly lower binding affinity when

Fig. 6. Southern hybridization analysis of enzymatically methylated HIV LTRCAT plasmid DNA recaptured from transfected SW480 adenocarcinoma cells. HIV LTRCAT (pU3RIICAT) plasmid DNA was methylated enzymatically at either *HpaII* or *MspI* sites as described in Materials and methods. From left, lanes: 1, unmethylated plasmid DNA digested with *HpaII* endonuclease; 2, *HpaII* methylated plasmid DNA digested with *HpaII* endonuclease; 3, same as lane 2 except that the transfection was carried out in SW480 cells expressing *tat* (SWNtat); 4, same as lane 1; 5, same as lane 2, except that *MspI* methylase was employed and plasmid DNA was digested with *MspI* endonuclease; 6, same as lane 3, except that *MspI* methylase was employed and plasmid DNA was digested with *MspI* endonuclease. Southern hybridization analysis was carried out with an LTR probe obtained by *BglIII* digestion of pU3RIICAT.

compared with the unmethylated probe DNA (Figure 7A, lanes 1,3). The binding affinity of the methylated probe DNA was restored when incubated with nuclear extracts isolated from SW480 cells expressing *tat* protein (Figure 7A, lanes 6,7). Concomitant with this observation was the appearance of a second nuclear protein (NP2) which retarded the mobility of labeled probe.

To test the specificity of nuclear protein binding to CpG sites at -218/-146, mobility shift experiments were performed as above, however, a 5-fold excess of unlabeled competitor DNA was preincubated with nuclear extracts 5 min prior to incubation with unmethylated, labeled DNA probe. When *HpaII* or *MspI* methylated competitor DNA was employed, essentially no competition occurred (Figure 7B, lanes 3,4). When unmethylated competitor was co-incubated with labeled probe, no binding of the probe DNA was observed (Figure 7B, lane 2), thus confirming the specificity of protein binding.

Discussion

Infection of individuals by HIV-1 results in an incubation time averaging 2–5 years prior to the onset of severe clinical manifestations (Lui *et al.*, 1986; Medley *et al.*, 1987). Early after integration of proviral DNA into the host chromatin, virus expression can become quiescent, or latent (Gendelman *et al.*, 1986; Bednarik *et al.*, 1987; Mosca *et al.*, 1987a,b; Valerie *et al.*, 1988; Bednarik *et al.*, 1989; Folks *et al.*, 1989). Virus expression can be reactivated by exposure of

infected cells to a spectrum of agents or pathogens, and the regions of the HIV LTR sensitive to induction by each agent have been mapped (Bednarik *et al.*, 1989; Mosca, 1989). The physiological mechanism of latency is not unequivocally known, but is believed to involve a complex interplay between both viral and cellular host factors (Folks *et al.*, 1989). The physiological relevance of recent data implicating DNA methylation as a virus latency preservation mechanism is evident from studies in which inactive HIV proviral DNA was established in human T-cell lines (Bednarik *et al.*, 1989; Folks *et al.*, 1989).

In this study, we present evidence for the role of a host cellular function, DNA methylation, as a mechanism which may preserve the latent state of HIV. Prior studies have shown that methylation of cytosine residues in the controlling elements of murine retroviruses and adenoviruses can suppress expression of either virus (Harbers *et al.*, 1981; Graessmann *et al.*, 1983; Kruczek and Doerfler, 1983; Bird, 1986). Our earlier work also implicated HIV expression as being sensitive to methylation antagonistic agents (Bednarik *et al.*, 1987, 1989). The regions of the HIV LTR sequences sensitive to inactivation by methylation were mapped by deletion analysis to include upstream sequences containing both core enhancer/NF κ B binding sites and three Sp1 binding sites. Within this region we have identified two *HpaII/MspI* CpG sites located at -218 and -146 nucleotides upstream from the cap site which, when enzymatically methylated *in vitro*, renders the LTR transcriptionally inactive. *In vivo*, the presence of 5-methylcytosine in the integrated HIV LTR at these positions effectively preserved latency. Such a transcriptional block could prevent *cis*-level transcriptional initiation, thereby preventing the accumulation of downstream-encoded viral proteins such as *tat*. This scenario would eliminate the involvement of HIV viral products in the regulation of truly latent provirus assuming, of course, the infected cell does not become re-infected by a subsequent exposure to HIV, or to herpes class viruses (Mosca *et al.*, 1987a,b; Gendelman *et al.*, 1986).

If, indeed, secondary infection or exposure to other *trans*-acting factors were to occur, would the methylated LTR inhibit the action of the putative *trans*-activator? We have addressed this question by transient transfection of Vero cells with plasmid DNA (pU3RIICAT) methylated with CpG-specific (*HpaII*) or non-CpG-specific (*HphI*, *AluI*) methylase enzymes. It should be stressed that the CpG sequence is recognized as the physiological target of cytosine methylation in mammalian cells, and it is therefore important to consider non-CpG sequences in these experiments to determine whether it is the methylated CpG sequence, or simply the presence of a 5-methylcytosine moiety in a given region that is responsible for the transcriptional inhibition. Co-transfection of pIIIextatIII with methylated HIV LTRCAT (pU3RIICAT) DNA resulted in *trans*-activation of CAT expression, while *cis*-level expression was nearly eliminated in the absence of *tat*. This observation is in accord with the results described by Weisshaar *et al.* (1988) in which *trans*-acting factors of the adenovirus system overcome a similar transcriptional block introduced by *in vitro* methylation of adenovirus regulatory elements. The lack of effect of *HaeIII* methylation on *tat* expression (Figure 2) was due to the inefficiency of the enzymatic reaction in that particular experiment.

Recent evidence has implicated cellular factors, such as

Fig. 7. Panel A: effects of methylation of HIV LTR CpG sites at -146 and -218 on binding of nuclear factors. Labeled (32 P) DNA fragments were incubated with a partially purified nuclear fraction obtained from SW480 cells or SW480 cells expressing the *tat* protein. Binding reactions were performed utilizing a 212 bp, PCR-amplified DNA segment spanning both CpG sites. Protein-DNA complexes and uncomplexed probe DNA were resolved on a 32 cm 4% non-denaturing polyacrylamide gel by the mobility shift assay. Incubations were completed in a 1360-fold weight excess of polydI.dC relative to probe with 1 μ g nuclear protein extract at room temperature. Arrows denote the positions of nuclear binding proteins which retard the migration of labeled DNA probe (NPI, NPII). Lanes: 1, unmethylated control; 2, *HpaII* methylated; 3, unmethylated control; 4, *MspI* methylated; 5, unmethylated control; 6, *HpaII* methylated; 7, *MspI* methylated; 8, unmethylated-free probe; 9, *HpaII* methylated-free probe; 10, *MspI* methylated-free probe. Lanes 1-4 employ nuclear extracts obtained from SW480 cells; lanes 5-7 employ nuclear extracts obtained from SW480 *tat*-expressing cells (SWN_{tat}). **Panel B:** specificity of nuclear protein binding to probe DNA CpG sites at -218 and -146. Mobility shift assays were performed as described in Panel A, except that a 5-fold excess of unlabeled competitor DNA was preincubated with nuclear extracts for 5 min prior to incubation with labeled DNA probe. Competition was evaluated by the ability of unlabeled competitor DNA to effectively prevent binding to the labeled DNA probe. Lanes: 1, unmethylated probe lacking competitor; 2, unmethylated probe co-incubated with unmethylated competitor DNA; 3, unmethylated probe co-incubated with *HpaII* methylated competitor; 4, unmethylated probe co-incubated with *MspI* methylated competitor.

REGULATION OF HIV TRANSCRIPTION BY LTR METHYLATION

Fig. 8. Possible mechanisms of methylation-mediated transcriptional inhibition. Methylation of *HpaII* sites upstream from the HIV core enhancer region may (1) displace the binding of an essential transcription factor; (2) cause a change in DNA conformation which is unfavorable for transcription *in cis*; (3) may induce a DNA conformation which permits the binding of proteins that are negative regulators of transcription.

NF κ B, as important mediators in the activation of the HIV LTR (Nabel and Baltimore, 1987; Gimble *et al.*, 1988; Nabel *et al.*, 1988; Bielinska *et al.*, 1989). Activation of this

protein can be attained by treatment of cells with phorbol ester (Sen and Baltimore, 1986). Our results have shown that TPA was able to *trans*-activate the integrated LTR to

levels similar to that of LTR sequences which are fully hypomethylated. This evidence is suggestive of a cascade effect, whereby the action of cellular factors (i.e. NF κ B) can overcome the transcriptional block and permit the initial expression and accumulation of *tat* protein. Once low levels of *tat* accumulate, the latent LTRs are fully activated in *trans*, thereby restoring expression and obviating the transcriptional block. This process very closely resembles the observations of Langner *et al.* (1986), Weisshaar *et al.* (1988), and Knust *et al.* (1989) who demonstrated that adenovirus E1A protein could reactivate, in *trans*, the E1A promoter of adenovirus (Ad2 or Ad5) DNA without apparent demethylation of E2A sequences (Weisshaar *et al.*, 1988; Knust *et al.*, 1989). Southern hybridization analysis of methylated plasmid DNA (pU3RIICAT) recovered from nuclei of SW480 cells containing *tat* protein, showed only a partial demethylation while the majority of the plasmid remained largely methylated. The possibility that the transactivator *tat*, like E1A protein, may induce partial demethylation of one DNA strand to create a hemi-methylated state, is unlikely but cannot be completely ruled out (Weisshaar *et al.*, 1988). The hemi-methylated state has been described for the chicken vitellogenin II gene, which undergoes partial demethylation during the course of activation (Saluz *et al.*, 1986).

The affinity of nuclear binding proteins for methylated, or unmethylated probe DNA was determined largely by the region of the HIV LTR employed as the labeled probe. When nuclear extracts were incubated with a probe spanning -218 to -140 nucleotides, binding of a single protein (NPI) was observed which no longer bound DNA after CpG sites at -218 to -146 were methylated. Binding affinity was restored when the *tat* protein was present in nuclear extracts, and in addition binding of a second factor, NPII, was observed. It is tempting to speculate that a cooperative mechanism of protein binding may be involved in relieving the transcriptional block.

Others have also suggested that cytosine methylation may alter the conformation of neighboring DNA sequences which could increase the affinity of the DNA for one class of protein and/or simultaneously decrease the affinity of the same sequences for another class of proteins thereby selectively modulating gene expression (Caifa *et al.*, 1986; Antequera *et al.*, 1989; Meehan *et al.*, 1989). Indeed, the effect of cytosine methylation on gene expression is far more likely to be a global effect on surrounding DNA conformation rather than an inhibitory effect at a single site (Keshet *et al.*, 1986), and such methylation events have clearly been implicated in the increased binding of histone or other nuclear proteins (Huang *et al.*, 1984; Caifa *et al.*, 1986; Wang *et al.*, 1986; Zhang *et al.*, 1986; Khan *et al.*, 1988; Supakar *et al.*, 1988; Antequera *et al.*, 1989; Meehan *et al.*, 1989). Figure 8 illustrates a potential mechanism whereby binding of a transcriptional factor is inhibited by the modification of the two upstream CpG sites. Methylation at one or more sites may involve the displacement of downstream transcriptional factors by other proteins or by a change in the conformation of DNA introduced by the upstream methyl groups (Figure 8). A change in DNA conformation to a transcriptionally unfavorable state could be due to 5-methylcytosine alone or to the inhibition of transcription factor binding. One might speculate that *tat*, either alone or in concert with other factors, can orchestrate activation of transcription in *trans* regardless of the methylation state. In

the absence of *trans*-activation, *cis*-level expression would remain inactive as long as the LTR was methylated, thereby preserving latency. In such a model, the expression of virus would be minimal to non-existent until cellular activation occurred, or until a heterologous *trans*-acting factor was encountered. This model is similar to the mechanisms recently proposed by Meehan *et al.* (1989) whereby a methyl-CpG binding protein (MeCP) may interfere with binding of transcriptional factors such as Sp1, or may induce a DNA conformation inaccessible to Sp1.

In this report, we present evidence which demonstrates that the HIV LTR is sensitive to inactivation by site-specific DNA CpG methylation, a process which can be efficiently obviated by the HIV *trans* activator *tat*. We believe that the results described in this study support DNA methylation as a viable control mechanism for latency. The lack of information implicating DNA methylation in the latency of HIV infection *in vivo* stems largely from the difficulty of examining the methylation profiles of CpG sites which do not exist in a restriction site accessible to Southern hybridization analysis. Further studies, currently in progress, will elucidate the methylation profile of integrated, latent/active HIV by genomic sequencing techniques (Pfeifer *et al.*, 1989; Saluz and Jost, 1989) and will confirm *in vivo* physiological relevance of CpG methylation sites as controlling elements. This information will contribute to our understanding of the cellular control of retrovirus expression and to the mechanism of *trans*-activator function.

Materials and methods

Cells and viruses

Vero cells (simian), SW480 (human adenocarcinoma), and A3.01 (CD4⁺, human CEM T-cell line) were grown in either DMEM (Gibco) supplemented with 5% fetal bovine serum (Vero, SW480) or OPTI-MEM (Gibco) supplemented with 2% fetal bovine serum (A3.01). A3N92.2 cells, containing a single copy of HIVCATkB mutant and pSV2Neo, were constructed by electroporation of cells with each plasmid DNA (10 μ g and 1.0 μ g, respectively) followed by selection of colonies in soft agar containing G418 sulfate (1 mg/ml), and maintenance in OPTI-MEM media as described above (Bednarik *et al.*, 1989). Stocks of HIV were generated by electroporation of A3.01 cells with 10 μ g of infectious clone DNA (pHCXB2). HIV concentration was determined by p24 antigen capture (Abbott) (Higgins *et al.*, 1986). *De novo* infection of A3.01 cells was initiated by inoculating 5×10^6 cells with 1000 pg p24 antigen.

Plasmid DNA and transfection/electroporation of cells

The plasmids pU3RIICAT (HIVLTRCAT), pILextatIII (HIV LTR*tat*), pHCBX2, and pHIVkBCAT (courtesy Drs G.Nable and D.Baltimore) were constructed as previously described (Rosen *et al.*, 1985; Nabel and Baltimore, 1987). Fibroblast cell lines were transfected by either calcium phosphate coprecipitation (Mosca *et al.*, 1987a,b; Bednarik *et al.*, 1987) or lipofection (Felgner and Holm, 1989). Transfection of lymphoid cells was performed by either electroporation (Promega X-cell 450) or lipofection (Felgner and Holm, 1989).

Chloramphenicol acetyltransferase assay (CAT) and RNA analysis

Cell extracts were assayed for CAT enzyme activity as described (Mosca *et al.*, 1987a,b; Bednarik *et al.*, 1987). Isolation of total RNA, and analysis was performed by S1 nuclease and Northern hybridization as described previously (Mosca *et al.*, 1987a,b; Bednarik *et al.*, 1987).

In vitro methylation of plasmid DNA and preparation of DNA probes

Plasmid DNA was enzymatically methylated by protocols prescribed by the vendor (New England Biolabs), and was subsequently digested with the corresponding endonuclease and electrophoresed on a 0.8% agarose gel to determine the extent of the methylation reaction. Probe DNA was

generated by PCR amplification of a 418 bp segment of the HIV LTR depicted in Figure 1. Probe I, which was employed in mobility shift studies, extended from -352 to -140 nucleotides and was prepared by first ³²P end-labeling the 418 base pair DNA segment, followed by digestion at 65°C with *TaqI* endonuclease to yield a 212 bp probe [³²P-labeled at the 5' terminus of the coding strand. Alternatively, probe I could be generated by initially end-labeling the coding strand PCR primer and isolating the amplified DNA subsequent to the reaction. The PCR product could be digested with *TaqI* endonuclease as described above, and recovered after electrophoresis through a 0.8% low melting agarose gel.

Preparation of nuclear protein extracts, nuclei, and analysis of nuclear protein binding (mobility shift assay)

Isolation of nuclei and preparation of nuclear extracts was performed as described (Raj *et al.*, 1983). The DNA-binding assay and gel electrophoresis was performed by the methods of Baldwin and Sharp (1987). Nuclear protein extract (1 µg) was preincubated with a 1360-fold weight excess of poly(dI.dC) for 20 min at room temperature, followed by addition of 1 ng labeled probe DNA (probe I) and further incubation for 40 min. The incubation buffer consisted of 10 mM Tris-HCl (pH 7.5), 50–100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 12.5% glycerol, and 0.1% Triton X-100. The non-denaturing gel was composed of 4% acrylamide:bis acrylamide, 30:1, containing 1 mM EDTA, 3.3 mM sodium acetate, and 6.7 mM Tris-HCl (pH 7.5). Prior to loading, the gel was pre-run at 20 mA for 2 h at room temperature. Samples were loaded, and electrophoresed at 35 mA until the bromophenol blue dye reached the bottom of the gel. The gel was soaked for 10 min in 5% glycerol and transferred to Whatman 3MM filter paper and vacuum dried at 80°C for 1 h. Autoradiography (-70°C) was subsequently performed.

Polymerase chain reaction (PCR)

PCR was performed by employing a GeneAmp DNA amplification reagent kit (Perkin Elmer Cetus Corp., Norwalk, CT). Reactions were accomplished by protocols prescribed by the vendor. Oligonucleotide primers complementary to regions spanning the +46 to +66 nucleotides of the 3' coding strand and -352 to -332 nucleotides of the 5' non-coding strand were synthesized chemically (Genetic Designs, Inc., Houston, TX).

Plasmid DNA recovery and analysis

Plasmid DNA (10 µg), employed in methylation experiments was transfected into SW480 cells or SW480 cells expressing *tat* protein (SWN*tat*) via calcium phosphate co-precipitation. Incubation was allowed to proceed for 36 h, followed by isolation of nuclei (Saluz and Jost, 1989), and resuspension in 0.3 M sucrose/80 mM NaCl/1 mM EDTA/20 mM HEPES, pH 7.5, containing 0.15 mM spermine and 0.5 mM spermidine. An equal volume of 20 mM HEPES, pH 7.5/20 mM EDTA/1% SDS containing proteinase K (600 µg/ml) was added to the suspended nuclei and the lysate was incubated overnight at 37°C with shaking. After several phenol-chloroform extractions, the genomic DNA was precipitated with ethanol and removed. The unintegrated plasmid DNA was precipitated with 0.1 vol of 2 M ammonium acetate. The desiccated plasmid was reconstituted in 50 µl sterile water and quantitated. Restriction analysis was performed by incubating 0.5 µg DNA with *HpaII* by protocols described by the vendor (New England Biolabs). After electrophoresis on a 1% agarose gel, the DNA was transferred to a nylon membrane (Reed and Mann, 1985; Amersham Corp.), fixed by UV irradiation, and hybridized with ³²P-oligolabeled HIV LTR probe. Autoradiography was subsequently performed.

Acknowledgements

We acknowledge Drs H.P.Saluz and N.B.K.Raj for helpful discussions. We thank J.Simkins for technical assistance, and B.Schneider for excellent preparation of the manuscript. This work was supported by the American Foundation for AIDS Research to D.B. (grant no. 000639) by the The National Institutes of Health to P.M.P. (grant no. AI26123).

References

- Antequera, F., Macleod, D. and Bird, A.P. (1989) *Cell*, **58**, 509–517.
- Baldwin, A.S. and Sharp, P.A. (1987) *Mol. Cell. Biol.*, **7**, 305–313.
- Bednarik, D.P., Mosca, J.D. and Raj, N.B.K. (1987) *J. Virol.*, **61**, 1253–1257.
- Bednarik, D.P., Cook, A.J. and Pitha, P.M. (1989) In Clawson, G.A. *et al.*, (eds) *Nucleic Acid Methylation-UCLA Symposia on Molecular and Cellular Biology New Series*. Wiley-Liss, New York, Volume 128, pp. 153–169.
- Bielinska, A., Krasnow, S. and Nabel, G.J. (1989) *J. Virol.*, **63**, 4097–4100.
- Bird, A.P. (1986) *Nature*, **321**, 209–213.
- Caifa, P., Attina, M., Cacace, F., Tomassetti, A. and Strom, R. (1986) *Biochem. Biophys. Acta*, **867**, 195–200.
- Cedar, H. (1988) *Cell*, **53**, 3–4.
- Christy, B.A. and Scangos, G.A. (1984) *Mol. Cell. Biol.*, **4**, 611–617.
- Doerfler, W. (1983) *Annu. Rev. Biochem.*, **52**, 93–124.
- Doerfler, W. (1984) *Curr. Topics Microbiol. Immunol.*, **108**, 79–98.
- Fauci, A.S. (1988) *Science*, **239**, 617–622.
- Felgner, P.L. and Holm, M. (1989) *Focus*, **11**, 21–24.
- Folks, T.M., Powell, D.M., Lightfoote, M.M., Benn, S., Martin, M.A. and Fauci, A.S. (1986) *Science*, **231**, 600–602.
- Folks, T.M., Justement, J., Kinter, A., Dinarello, C.A. and Fauci, A.S. (1987) *Science*, **238**, 800–802.
- Folks, T.M., Clouse, K.A., Justement, J., Rabson, A., Duh, E., Kehrl, J.H. and Fauci, A.S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2365–2368.
- Gendelman, H.E., Phelps, W., Feigesbaum, L., Ostrove, J.M., Adachi, A., Howley, P.M., Khoury, G., Ginsberg, H.S. and Martin, M.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9759–9763.
- Gimble, J.E., Duh, E., Ostrove, J.M., Gendelman, H.E., Max, E.E. and Rabson, A.B. (1988) *J. Virol.*, **62**, 4104–4112.
- Graessmann, M., Graessmann, A., Wagner, H., Werner, E. and Simmon, D. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6470–6474.
- Harbers, K., Schieke, N., Stuhlmann, H., Jahner, D. and Jaenisch, R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7609–7613.
- Higgins, J.R., Pederson, N.C. and Carlson, J.R. (1986) *J. Clin. Microbiol.*, **24**, 424–430.
- Holliday, R. (1989) *Sci. Am.*, June, 60–73.
- Huang, L.-H., Wang, R., Gama-Sosa, M.A., Shenoy, A. and Ehrlich, M. (1984) *Nature*, **308**, 293–295.
- Keshet, I., Yisraeli, J. and Cedar, H. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2560–2564.
- Keshet, I., Leiman-Hurwitz, J. and Cedar, H. (1986) *Cell*, **44**, 535–543.
- Kahn, R., Zhang, X.-Y., Supakar, P.C., Ehrlich, K.C. and Ehrlich, M. (1988) *J. Biol. Chem.*, **263**, 14374–14383.
- Knust, B., Bruggemann, U. and Doerfler, W. (1989) *J. Virol.*, **63**, 3519–3524.
- Kruczek, J. and Doerfler, W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7586–7590.
- Langner, K.D., Weyer, U. and Doerfler, W. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1598–1602.
- Lindsay, S. and Bird, A.P. (1988) *Nature*, **327**, 336–338.
- Lui, K.-J., Lawrence, D.N., Morgan, W.M., Peterman, T.A., Haverkos, H.W. and Bregman, D.J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3051–3055.
- Medley, G.F., Anderson, R.M., Cox, D.R. and Billard, L. (1987) *Nature*, **328**, 719–721.
- Meehan, R.R., Lewis, J.D., McKay, S., Kleiner, E.L. and Bird, A.P. (1989) *Cell*, **58**, 499–507.
- Michalowsky, L.A. and Jones, P.A. (1987) *Mol. Cell. Biol.*, **7**, 3075–3083.
- Mosca, J.D. (1989) In preparation.
- Mosca, J.D., Bednarik, D.P., Raj, N.B.K., Rosen, C.A., Sodroski, J.G., Haseltine, W.A. and Pitha, P.M. (1987a) *Nature*, **325**, 67–70.
- Mosca, J.D., Bednarik, D.P., Raj, N.B.K., Rosen, C.A., Sodroski, J.G., Haseltine, W.A., Hayward, G.S. and Pitha, P.M. (1987b) *Proc. Natl. Acad. Sci. USA*, **84**, 7408–7412.
- Nabel, G.J. and Baltimore, D. (1987) *Nature*, **326**, 711–718.
- Nabel, G.J., Rice, S.A., Knipe, D.M. and Baltimore, D. (1988) *Science*, **239**, 1288–1302.
- Pfeifer, G.P., Steigerwald, S.D., Mueller, P.R., Wold, B. and Riggs, A.D. (1989) *Science*, **246**, 810–812.
- Raj, N.B.K. and Pitha, P.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3923–3927.
- Reed, K.C. and Mann, D.A. (1985) *Nucleic Acids Res.*, **13**, 7207–7221.
- Rosen, C.A., Sodroski, J.G. and Haseltine, W.A. (1985) *Cell*, **41**, 813–823.
- Saluz, H. and Jost, J.-P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2602–2606.
- Saluz, H., Jiricny, J. and Jost, J.-P. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 7167–7171.
- Sen, R. and Baltimore, D. (1986) *Cell*, **47**, 921–928.
- Supakar, P.C., Wiest, D., Zhang, D., Inamdar, N., Zhang, X.-Y., Khan, R., Ehrlich, K.C. and Ehrlich, M. (1988) *Nucleic Acids Res.*, **16**, 8029–8044.
- Valerie, K., Delers, A., Bruck, C., Thiriart, C. and Rosenberg, H. (1988) *Nature*, **333**, 78–81.
- Wang, R.Y.-H., Zhang, X.-Y., Kahn, R., Zhou, Y., Huang, L.-H. and Ehrlich, M. (1986) *Nucleic Acids Res.*, **14**, 1599–1614.
- Weisshaar, B., Langner, K.D., Juttermann, R., Muller, U., Zock, C., Klimkait, T. and Doerfler, W. (1988) *J. Mol. Biol.*, **202**, 255–270.
- Zhang, X.-Y., Ehrlich, K.C., Wang, R.Y.-H. and Ehrlich, M. (1986) *Nucleic Acids Res.*, **14**, 8387–8397.

Received November 27, 1989; revised January 26, 1990