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Epigenetic analysis of HIV-1 proviral genomes from infected individuals: Predominance of unmethylated CpG's

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Abstract

Efforts to cure HIV-1 infections aim at eliminating proviral DNA. Integrated DNA from various viruses often becomes methylated de novo and transcriptionally inactivated. We therefore investigated CpG methylation profiles of 55 of 94 CpG's (58.5%) in HIV-1 proviral genomes including ten CpG's in each LTR and additional CpG's in portions of *gag*, *env*, *nef*, *rev*, and *tat* genes. We analyzed 33 DNA samples from PBMC's of 23 subjects representing a broad spectrum of HIV-1 disease. In 22 of 23 HIV-1-infected individuals, there were only unmethylated CpG's regardless of infection status. In one long term nonprogressor, however, methylation of proviral DNA varied between 0 and 75% over an 11-year period although the CD4+ counts remained stable. Hence levels of proviral DNA methylation can fluctuate. The preponderance of unmethylated CpG's suggests that proviral methylation is not a major factor in regulating HIV-1 proviral activity in PBMC's. Unmethylated CpG's may play a role in HIV-1 immunopathogenesis.

Keywords

Epigenetics of HIV-1 proviral DNA; Integrated HIV-1 DNA in PBMC's from infected individuals; Wide spectrum of infection outcome; Bisulfite sequencing; Methylation analysis of integrated HIV-1 genomes; Predominance of unmethylated CpG's in PBMC's; Escape from proviral DNA methylation; Fluctuation of CpG methylation in one LTNP individual

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Author contributions

S.W., W.D., H.B, and B.W. designed research; S.W. performed the experiments; K.S.K., K.A., K.K. R.K., and C.K. contributed participant recruitment and new specimen reagents; S.W., C.R., W.D., H.B., and B.W. analyzed data; W.D. and B.W. wrote the paper.

Introduction

Treatment with highly active antiretroviral therapy (HAART) has not only led to suppression of plasma viremia in many patients but also to a dramatic reduction in the rates of illness and death in HIV-1-infected individuals (Anastos et al., 2004; Ledergerber et al., 1999). HIV-1 cure, however, has not been attained despite HAART due to the persistence of proviral DNA integrated into the host genome (Persaud et al., 2000; Trono et al., 2010). Strategies to cure HIV-1 need to aim at modifying or eliminating proviral DNA from the cellular genomes. Recent work, however, has revealed that individuals chronically infected with HIV-1 harbor a reservoir of integrated proviral DNA many times larger than previously estimated (Ho et al., 2013). Elimination of these proviruses presents a major challenge, making the characterization of integrated proviruses across the genome a high priority.

To better understand the state of proviral DNA in infected individuals, we characterized epigenetic modifications including uniquely located CpG methylation in a number of HIV-1 proviral DNA genes obtained from HIV-1-infected individuals; these subjects represented a spectrum of HIV-1 disease progression. Latent HIV-1 proviruses are thought to reside in a proportion of resting CD4+ cells (Persaud et al., 2000; Trono et al., 2010). As a first step in analyzing methylation patterns, we chose to limit our approach to the analysis of methylation patterns in integrated HIV-1 proviral DNA from PBMC's. The selection of cells in culture requires the application of methods that might fundamentally alter the actual profiles of proviral CpG methylation.

Foreign DNA integrated into mammalian genomes can become methylated de novo, as was recognized early on for the genomes of DNA viruses (Desrosiers et al., 1979; Hochstein et al., 2007; Kalantari et al., 2008; Sutter et al., 1978; Sutter and Doerfler, 1980; Takacs et al., 2010) and retroviruses (Christman et al., 1980; Hoffmann et al., 1982; Stuhlmann et al., 1981). Specific viral genome segments can be permanently silenced in this way. HIV-1 proviral genomes in human cells have also been studied for their patterns of DNA methylation to gain insight into the mechanisms underlying the long-term fate of HIV-1 infections. Methylation studies of HIV-1 initially analyzed infected cells in cell culture and demonstrated that methylation of CpG residues in vitro can lead to the transcriptional inactivation of HIV-1 LTR's. These results suggested that methylation might play a role in HIV-1 latency (Bednarik et al., 1987, 1990; Blazkova et al., 2009; Chavez et al., 2011; Fang et al., 2001; Gutekunst et al., 1993; Ishida et al., 2006; Jeeninga et al., 2008; Kauder et al., 2009; Mok and Lever, 2007; Pion et al., 2003; Schulze-Forster et al., 1990; Tanaka et al., 2003; Terme et al., 2009). Proviral HIV-1 genomes can be reactivated by treating cultured cells with 5-azacytidine, a well-known inhibitor of DNA methyltransferase systems (Tanaka et al., 2003). Modifications of the histone code in the HIV-1 LTR regions can also be involved in establishing HIV-1 latency (Duverger et al., 2009; Friedman et al., 2011; Suzuki et al., 2005; Van Duyne et al., 2008). The complex problem of latency with multifactorial causation, however, continues to pose a major challenge for HIV-1 cure in infected individuals (Cohen, 2011).

Studies on DNA methylation in HIV-1 genomes from infected individuals are far fewer, and the patient populations and published results differ. In PBMC's taken directly from HIV-1-infected patients, one report has found 20% to 100% methylated CpG's in proviral DNA (Blazkova et al., 2009). A later study has described that the HIV-1 5′LTR's are scarcely methylated in latently infected, resting CD4(+) T cells from aviremic HIV-1 infected individuals on HAART (Blazkova et al., 2012). By contrast, Palacios et al. (2012) report that in long term nonprogressors (LTNP's) and elite controllers, there might be a low level of methylation, whereas in patients with suppressed viremia due to HAART, methylation is virtually absent. These previous studies of HIV-1 obtained from infected subjects focused mainly on the 5′LTRs of the proviral genomes. In PBMCs, monocytes, and CD4+ T cells from feline immune deficiency virus (FIV)-infected cats, no evidence has been obtained for hypermethylation in the 5′- and 3′-LTR's of the FIV proviral genomes (Murphy et al., 2012).

The present investigation extends previous studies of HIV-1 methylation in infected individuals in several ways. We examined a large group of patients with a broad spectrum of HIV-1 disease progression and serial samples obtained over a period of years. Furthermore, we analyzed the integration status of HIV-1 DNA and the methylation patterns in several genes encompassing 55 of the 94 CpG (58.5%) dinucleotides in the total HIV-1 proviral genome, a significantly larger portion of the viral genome than studied previously.

Results

Study population

We studied 23 individuals (10 women and 13 men) with a broad range of HIV-1 infection. Six individuals were documented to be elite controllers (Okulicz et al., 2009), 4 were LTNP's (Okulicz et al., 2009), and 13 had progressive disease (Table 1). Subjects with identification codes starting with "*W*" were participants in the Bronx, NY site of the Women's Interagency HIV Study (WIHS), a natural history investigation of HIV-1 infection in women (Anastos et al., 2004). Subjects whose codes start with "*T*" were members of the Maple Leaf Clinic Cohort in Toronto, Canada, and those with code numbers starting with "9" or "13" were from the Department of Virology, Erlangen University Medical School.

Proviral genome segments investigated

The HXB2-K03455 sequence (http://www.ncbi.nlm.nih.gov) used as the reference for HIV-1 proviral DNA throughout this study contains a total of 94 CpG's. The map in Fig. 1A describes the distribution of the CpG's in the 5′ and 3′ regions of the HIV-1 proviral genomes. The locations of the HIV-1 genes are also indicated. Of a total of 94 CpG dinucleotides in the HIV genome, we screened 55 for their methylation status. Thirty-one CpG's in the 5′ region were studied; these were numbered 2 to 32 and included 10 of 11 CpG's in the 5′-LTR and 21 CpG's in the *gag* gene. In the 3′-region, we analyzed 24 CpG's, 10 of 11 in the 3′-LTR, and 15 CpG's representing portions of the *nef*, *rev*, *tat*, and *env* (gp41) genes. The CpG very close to the terminus of either LTR could not be analyzed due to insufficient sequence lengths for primer binding.

Intracellular forms of HIV-1 DNA in PBMC's

Two sets of primers were selected to characterize the intracellular forms of HIV-1 genomes in PBMC's. To access the integrated HIV-1 genomes, one primer was placed inside the LTR, the other one in adjacent cellular AluI sequences. The possible occurrence of 2-LTR HIV circles (Graf et al., 2011; Sloan and Wainberg, 2011) was assessed by using a PCR primer pair placed inside either LTR sequence. Except for the DNA sample from one elite controller (TR15), which contained small amounts of 2-LTR circles, the HIV-1 DNA sequences of the HIV-1 infected individuals analyzed here were exclusively in the integrated proviral form (Table 2). The data thus confirmed the integrated state of almost all HIV-1 proviral genomes in the DNA samples analyzed in this study. Circular 2-LTR HIV-1 DNA was not found in most samples, although the reconstruction experiments described under Methods documented that the procedure employed for DNA extraction permitted the ready isolation and subsequent detection of small circular DNA of about 5.6 kbp or of 14.8 kbp, a size range within that of the 9.7 kbp of 2-LTR circles.

Methylation profiles of HIV-1 genomes in PBMC's from infected individuals

By using the bisulfite sequencing technique (Frommer et al., 1992; Clark et al., 1994), we determined methylation profiles in PBMC-derived proviral DNA from HIV-1-infected individuals (Table 1). The following HIV-1 proviral genome segments were targeted by appropriate primer selection: 10 of 11 CpGs in the 5′-LTR, portions of the *gag*, *env*, and auxiliary genes *nef, rev, tat*, as well as 10 of 11 CpGs in the 3′-LTR (Fig. 1B and C, map in Fig. 1A). The number of bisulfite-sequenced clones analyzed in different DNA samples ranged from 2 to 84. Out of 88 samples tested, we were able to amplify the selected regions of the HIV-1 genome in 33 DNA samples (37.5%) that were derived from 23 HIV-1 infected individuals (Table 3).

An exemplary selection of the results is shown in Fig. 1B–F. The CpG's analyzed from chronically infected patient 901271 (Fig. 1B) were unmethylated in the 5′ and 3′-regions, as graphically depicted by open squares representing unmethylated CpG's. For chronically infected subject 901251 (Fig. 1C), the proportion of unmethylated CpG's was again close to 100% in all regions investigated in the proviral genome. In individual TR19 03/97, a long term nonprogressor (Fig. 1D), close to 100% of the CpG's in the regions analyzed were unmethylated as well. Similarly in individual W-350 05/2002, an elite controller, all CpG's were unmethylated in all regions studied in the proviral genome (Fig. 1E). In these four methylation profiles, both the 5′- and 3′-LTR were included in the analyses and found to be unmethylated. Because the 5′-terminal region of HIV-1 proviral genomes carries functionally important signals, we determined the methylation profiles in this HIV-1 genome segment in an additional 3 DNA samples from 3 HIV-1 infected individuals (Table 1). Again, the CpG's in the 5′-terminal segments were unmethylated in all samples analyzed (Fig. 1F). Similar results were obtained for an additional 22 DNA samples from 15 patients (Tables 1 and 3). This finding underscores the lack of CpG methylation in the majority of HIV-1 infected individuals studied. The few seemingly methylated CpG's (black squares in Fig. 1B–E) were due to rare incomplete conversions of cytosine residues during the bisulfite reaction, because repeated analyses of the same region did not reveal methylation of the same CpG's. It is important to emphasize that regardless of infection outcome, whether

subjects under study were chronically infected, LTNP's or elite controllers; both 5′- and 3′-LTR's plus the additional viral genome segments analyzed proved completely unmethylated.

The bisulfite analyses of proviral DNA from subject W-1, an LTNP who had been HIV-1 infected for >8 years when her first PBMC sample was obtained (Tables 1 and 3), revealed a much more complex situation. HIV-1 genomes from this person were studied longitudinally over an eleven-year period, and the patterns of methylation differed markedly with time (Table 3, lines 1–4; Fig. 2). In 06/1995, the CpG's investigated in the 3′ region of the proviral DNA were all unmethylated (Fig. 2A; Table 3, line 1). At later times, however, variable patterns of CpG methylation were detected. In 05/1996, there were 65% methylated CpG's in the 5′ region and 72% methylated CpG's in the 3′region (Fig. 2B; Table 3, line 2). Seven years later, 04/2003, 32% methylated CpG's were observed in the 3′region (Fig. 2C; Table 3, line 3). Finally, in 04/2006, the CpG's in the 3′region of individual W-1 were again found unmethylated (Fig. 2D; Table 3, line 4). A forensic analysis of the specimens analyzed confirmed that all DNA samples tested in this series were derived from individual W-1. Thus a longitudinal survey of proviral methylation profiles encompassing a period of 11 years revealed considerable fluctuations in the presence of this epigenetic (defense) signal. The CD4+ counts of LTNP individual W-1 were examined over the same time period. We did not find a significant difference among the CD4+ counts, including the 04/2006 time point (Table 1). Dichotomizing the values before and after this time point also did not yield any significant differences. In addition, the subject was clinically well and did not receive antiretroviral therapy during the entire period. These findings indicated that the HIV-1 infection of individual W-1 had remained stable between 1995 and 2006.

No 5-hydroxymethylcytosine (5-hmC) in the HIV-1 proviral DNA analyzed

In the demethylation pathway of DNA, which converts 5-mC to unmodified C residues, 5-hmC is generated as the first product (Jin et al., 2010; Krais et al., 2011; Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). Because bisulfite sequencing does not permit the discrimination between 5-mC and 5-hmC, we analyzed the DNA samples with a high degree of CpG methylation (Fig. 2B and C; Table 3) for the presence of 5-hmC. An immunological colorimetric quantification assay (Methods section) was used. This technique did not facilitate interrogation of individual CpG dinucleotides, but yielded cumulative results over a region. The data indicated that the DNA from sample W-1 05/1996 (Fig. 2B; Table 3) contained <0.1% of 5-hmC. In two additional samples W-1 04/2003 and W-1 04/2006, no evidence for the presence of 5-hmC was obtained. We conclude that 5-hmC is not a significant C modification in HIV-1 proviral genomes in the few samples in which 5-mC had been found.

Discussion

DNA methylation has been recognized as one of the first epigenetic signals with an essential function in long-term gene regulation in eukaryotic biology including genomically integrated viral genomes (for reviews, Doerfler, 1983, 2011). By intent and experimental design, we chose to study CpG methylation profiles in HIV-1 proviral genomes in human

PBMC's and, for the time being, avoided to bias the results by the use of cell selection or of cell culture systems.

New findings

The present study breaks new ground and differs in a number of significant aspects from those communicated previously (Blazkova et al., 2009, 2012; Palacios et al., 2012). We investigated the methylation profiles of proviral DNA in 23 HIV-1 infected individuals including several studied longitudinally over time (Tables 1 and 3). Although previous studies focused mainly on the 5′-LTR, we extended this investigation to examine more than 58%, i.e. 55 out of the total of 94 CpG's in the entire HIV-1 genome for their methylation status. We included in the analysis not only the 5′ and 3′ LTR's, but also portions of the gag and *env* genes, and the auxiliary genes *nef*, rev, and tat. Moreover, single HIV-1 proviral genomes were analyzed by bisulfite sequencing, and these sequences were derived exclusively from integrated HIV-1 DNA. We detected a small fraction of 2-LTR circles in only one DNA sample analyzed here.

There is an underrepresentation of CpG dinucleotides in the HIV-1 genome (Karlin et al., 1994); nevertheless there are CpG clusters in parts of the HIV-1 genome (Fig. 1). Unmethylated CpG's have the capability to induce a range of host immune responses (Hartmann and Krieg, 2000; Krieg, 1996). Oligonucleotides which contain unmethylated CpGs serve as agonists for Toll-like receptor 9 (TLR9) (Jiang et al., 2008; Malaspina et al., 2008). TLR9 polymorphisms have been found to play a role in HIV-1 disease progression, illustrating the contribution of TLR9 in HIV-1 immunopathogenesis (Pine et al., 2009). We hypothesize that the unmethylated CpG's in the HIV-1 proviral genomes may serve as agonists for TLR9 and thereby influence HIV-1 immunopathogenesis.

Methylation profiles of HIV-1 genomes in PBMC's from infected individuals

We have now analyzed PBMC derived DNA samples from 23 HIV-1 infected individuals with a wide range of infection outcomes. In some samples, we analyzed up to 84 single HIV-1 proviral DNA molecules to address the possibility of variations in their epigenetic characteristics. DNA samples from 13 patients with HIV-1 disease progression and a range of viral loads; 6 elite controllers; and 3 of 4 LTNP's were analyzed for methylation status in 55 of a total of 94 CpG's. Regardless of clinical status, the 10 of 11 CpG's in the 5′ and 3′-LTR's and 35 CpG's in parts of the *gag*, *env*, *nef*, *rev*, and *tat* genes in HIV-1 proviral DNA remained unmethylated in 22 of the 23 individuals studied here (Table 3; Fig. 1A–F). By unknown means, HIV-1 proviral genomes are capable of escaping the cellular defence mechanism of de novo methylation. This conclusion holds for the proviral genomes in PBMC's examined here but cannot be extrapolated to viral genomes in other cell types of HIV-1 infected individuals.

In contrast, we observed that LTNP W-1 (Table 1), who had harbored integrated proviruses for many years without developing AIDS, presented with a time-dependent profile of proviral methylation that was much more complex (Fig. 2). During 11 years of follow-up, subject W-1 had viral loads that were consistently <500 copies/mL, stable CD4 counts in the normal range, and an asymptomatic untreated HIV-1 infection (Table 1). Yet the proviral

genomes obtained from her PBMC's during this period were unmethylated at two time points (1995 and 2006) and partly methylated at two other times (1996 and 2003) (Fig. 2A–D; Tables 1 and 3). For unknown reasons, proviral DNA methylation can fluctuate in some HIV-1 infected individuals without apparent effects on viral replication. Investigation of the DNA samples exhibiting methylated HIV-1 proviruses (Fig. 2B and C) revealed no evidence for appreciable 5-hmC levels. At three time points during HIV-1 infection (Table 2), our analyses of DNA from subject W-1 detected exclusively integrated proviral HIV-1 genomes, but no 2-LTR circles.

The temporal fluctuation in proviral DNA methylation at a given time in the same individual W-1 might be a highly significant observation and may in part explain different findings by different laboratories. Multifactorial parameters such as intercurrent infections, drug use, hormonal influences, or fluctuating viral reservoirs and cell populations could have played a role. Moreover, HIV-1 proviruses may have been integrated at different sites in the cellular genomes at various times after infection, leading to variable patterns of CpG methylation. In a different biological system, we have found earlier that the de novo methylation patterns of integrated plasmid constructs in mouse ES cells appeared to depend on the site of plasmid DNA insertion (Hertz et al., 1999). The documented resistance to de novo methylation might also be explained by the finding that DNA methyltransferases cannot modify CpG's in certain sequence or conformational environments (Jurkowska et al., 2011). HIV-1 has developed multiple strategies to escape host defense mechanisms, among them that of avoiding de novo methylation upon integration. It will be of considerable interest to further evaluate this presumptive escape mechanism.

Conclusions

Several laboratories have reported that HIV-1 proviral CpG methylation plays a role in HIV-1 infected cells in culture (Bednarik et al., 1987,1990; Blazkova et al., 2009; Chavez et al., 2011; Fang et al., 2001; Gutekunst et al., 1993; Ishida et al., 2006; Jeeninga et al., 2008; Kauder et al., 2009; Mok and Lever, 2007; Pion et al., 2003; Schulze-Forster et al., 1990; Tanaka et al., 2003; Terme et al., 2009). Our data examining multiple sites across the HIV-1 proviral genome in PBMC-derived DNA from individuals with a wide range of HIV-1 disease, as well as the results of recently published studies (Blazkova et al., 2009, 2012; Murphy et al., 2012; Palacios et al., 2012), are at variance with this view. The unmethylated state of all CpG's in major segments of the HIV-1 proviral genome in 22 of 23 subjects suggests that proviral DNA methylation is unlikely to serve as the major regulator of HIV-1 gene silencing in PBMC's. The paucity of methylation in vivo may facilitate HIV-1 expression by avoiding transcriptional silencing. The apparent difference between infected individuals and cells in culture can be explained by surmising that the specific CpG methylation profiles in HIV-1 genomes arose under the pressure of "natural" selection in HIV-1-infected individuals. In contrast, under cell culture conditions, HIV-1 proviral genomes are not subject to the selective pressure exerted by defense systems operative in the human host. Studies in the future will aim to examine the genetic and epigenetic characteristics of HIV-1 proviral genomes in humans and in animal models, focusing on particular cell types including resting CD4+ T cells, dendritic cells, and viral reservoirs.

Methods

Detection of 5-mC by bisulfite sequencing of DNA

In a cohort of HIV-1 infected individuals at different stages of infection, we studied the methylation status of HIV-1 proviruses in PBMC's by bisulfite sequencing. This technique permits the unequivocal differentiation between cytidine (C) and 5-methylde-oxycytidine (5-mC) residues in a DNA sequence (Frommer et al., 1992; Clark et al., 1994). Technical details of the method were described previously (Hochstein et al., 2007; Naumann et al., 2009). In Table 4, all primers and the experiments in which they were used were listed.

Detection of 5-hmC

For the detection of 5-hmC in the few DNA samples which had been identified to contain 5-mC, the MethylFlash Hydroxymethylated DNA Quantification Kit (Epigentek) was used. The percentage of hydroxymethylated DNA was estimated in a reaction of the matrix-bound DNA with capture and detection antibodies and by quantifying the reaction colorimetrically.

Detection of small circular DNA

DNA from PBMC′s was extracted with the MagnaPure DNA large volume kit (Roche Diagnostics, Mannheim, Germany). In a reconstruction experiment, we added DNA of the 5.65 kbp bacterial plasmid pEGFP-FMR1 or of the 14.8 kbp plasmid pNL4-3-dE-EGFP directly to the PBMC's immediately prior to DNA extraction and purification by methods as recommended by the manufacturer. In the final DNA preparation, the two different plasmid DNAs could be readily detected by PCR using primers inside the plasmid DNA sequence. The results of this reconstruction experiment demonstrated that HIV-1 2-LTR circles of about 9.7 kbp length would have been co-extracted with the proviral HIV-1 DNA and would have been readily detectable by PCR if at all present.

Methods of statistical analyses

The longitudinal data on CD4+ counts were analyzed by using linear mixed models. Tests between groups were performed using a Wilcoxon Rank Sum test, a non-parametric test.

The data described in this report were previously presented at the XIX International AIDS Conference in Washington, DC in July 2012 (Weber et al., 2012).

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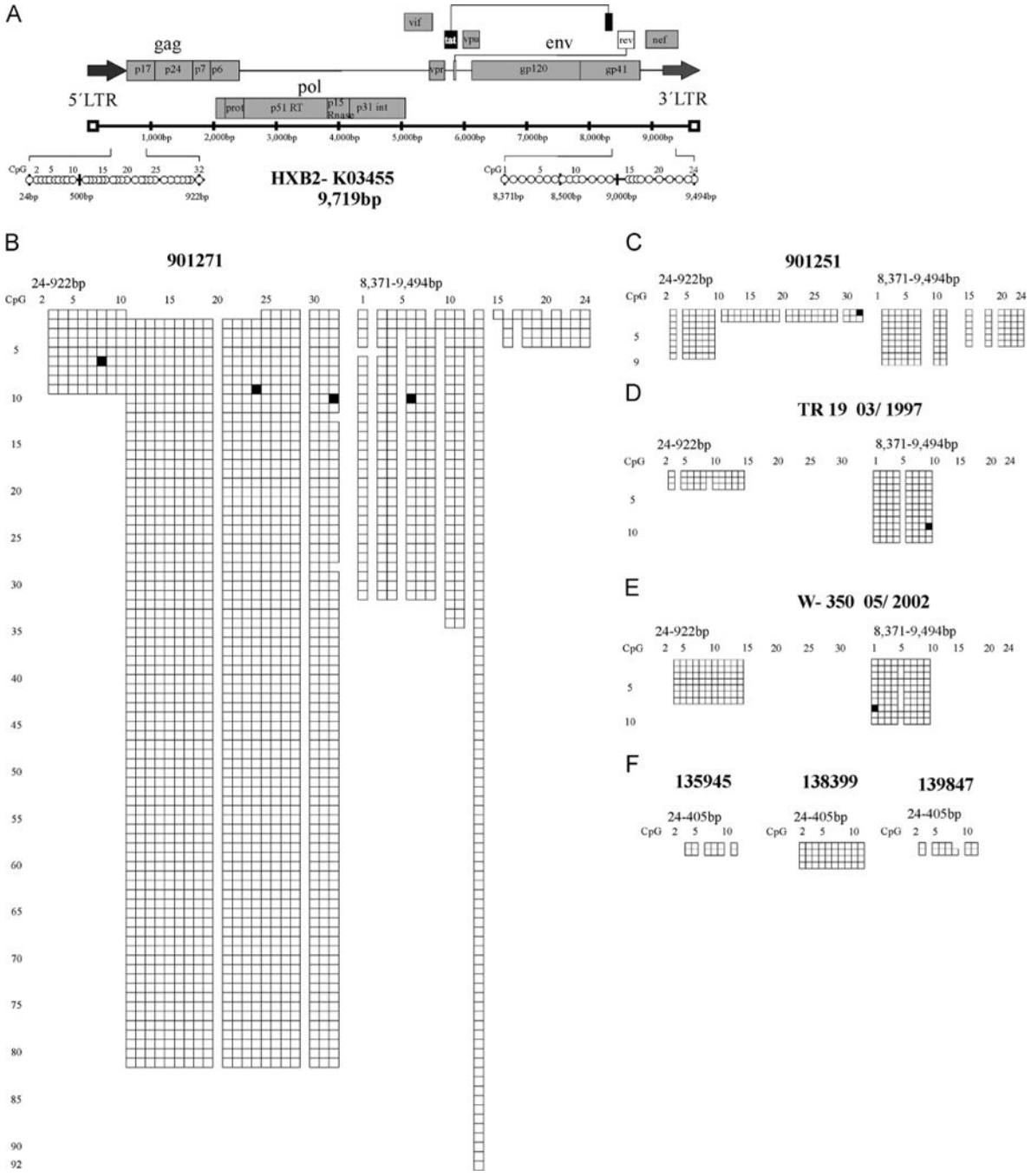

Fig. 1.

DNA methylation profiles in the HIV-1 proviruses in PBMC's from seven HIV-1 infected individuals. (A) This map of the HIV-1 genome is based on the HXB2-K03455 reference sequence (http://www.ncbi.nlm.nih.gov) and locates important viral genes and 32 CpG's in 922 bp in the 5′-region as well as 24 CpG's in 1124 bp in the 3′- region of the HIV-1 proviral genome. The 31 CpG's in the 5′-region and the 24 CpG's in the 3′- region were interrogated for their methylation status. In the 5′ and 3′ LTR's, 10 of 11 CpG's were analyzed for methylation. One CpG immediately close to the terminus of the HIV-1 genome

in either LTR could not be accessed because of limitations in primer binding at the termini. Twenty-one CpG's in the 5′-region fall within the CpG-rich part of the *gag* segment. In addition to the 10 CpG's in the 3′ LTR, 14 CpG's in the viral *env*, *tat*, *rev*, and *nef* genes were analyzed. (B) Methylation profiles of the HIV-1 proviral DNA from PBMC's of chronically infected patient 901271. (C) Methylation profiles of the HIV-1 proviral DNA from PBMC's of chronically infected patient 901251. (D) Methylation profiles of the HIV-1 proviral DNA from PBMC's of long-term non-progressor (LTNP) TR19 03/1997. (E) Methylation profiles of the HIV-1 proviral DNA from PBMC's of elite controller W-350 05/2002. (F) DNA methylation patterns exclusively in the 5′-region of HIV-1 proviruses in PBMC's from an additional three HIV-1 infected individuals, 135945, 138399, and 139847. Each square denotes either unmethylated □ or methylated ■ CpG's. Each row of squares depicts the data from one proviral molecule, each column the data of one CpG position in the proviral DNA. Detailed information on the HIV-1 infected individuals was summarized in Table 1.

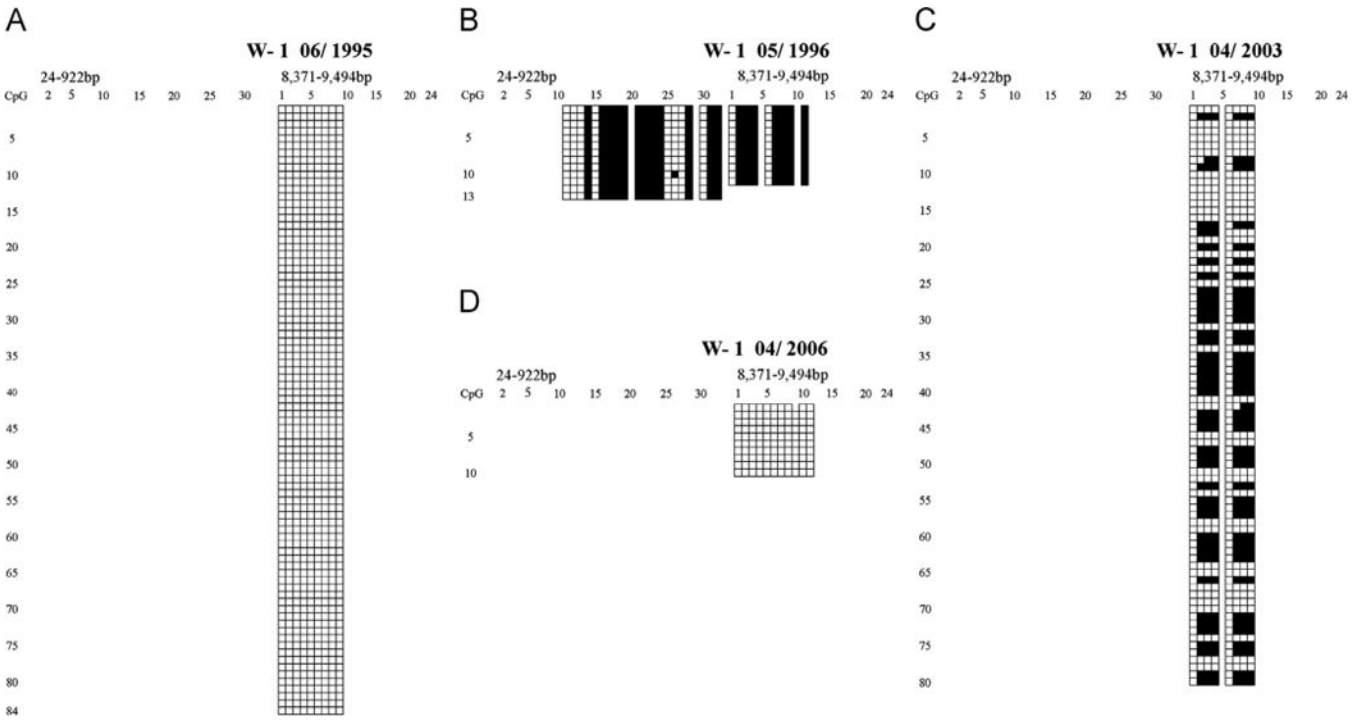

Fig. 2.

(A)–(D) Longitudinal study of DNA methylation profiles in the HIV-1 progenomes in PBMC's from long term non-progressing individual W-1. Blood samples were drawn at times as indicated on top of each graph. Individual W-1 was infected with HIV-1 probably in the late 1980s. All other symbols are as listed in the legend to Fig. 1.

Table 1

Characteristics of HIV-1-infected individuals whose PBMC derived proviral genomes were analyzed. AA, African American; H, Hispanic; C, Caucasian, NA, information not available.

Subject	Sex	Ethnicity	Date	Plasma HIV-1 RNA Load (copies/ml)	CD4 Count (cells/mm^3)	ART	Clinical status
W-1	F	AA	06/1995	464	ND	None	LTNP
			05/1996	431	800	None	LTNP
			04/2003	280	1074	None	LTNP
			04/2006	250	623	None	LTNP
W-21	F	AA	01/1997	240	1849	None	LTNP
			03/2001	530	1774	None	LTNP
			06/2006	2,000	1278	None	LTNP
W-360	F	C	07/1996	21,130	972	None	LTNP
TR19	M	C	03/1997	5579	560	None	LTNP
W-20	F	H	09/1997	<48	1174	None	Elite Controller
			04/2006	<80	1826	None	Elite Controller
			07/2006	230	1143	None	Elite Controller
W-350	F	AA	05/2002	<80	917	None	Elite Controller
			10/2002	<80	784	None	Elite Controller
W-351	F	AA	03/2002	110	886	None	Elite Controller
W-352	F	AA	03/2002	<80	ND	None	Elite Controller
			01/2006	<80	601	None	Elite Controller
			07/2006	<80	1032	None	Elite Controller
TR15	M	C	03/2007	<50	650	None	Elite Controller
TR16	M	C	12/2006	110	610	None	Elite Controller
W-4	F	AA	08/1999	110,000	135	None	AIDS
W-17	F	H	06/1999	480,000	0	D4T, EFV, NVF	AIDS
901148	M	NA	02/2009	12,000	NA	None	Chronic infection
901196	M	C	02/2009	38,000	NA	None	Chronic infection
901251	F	NA	02/2009	63,000	NA	None	Chronic infection
901271	M	C	02/2009	130,000	NA	None	Chronic infection
901330	M	C	02/2009	470,000	NA	None	Chronic infection

Subject	Sex	Ethnicity	Date	Plasma HIV-1 RNA Load (copies/ml)	CD4 Count (cells/mm^3)	ART	Clinical status
901448	M	C	02/2009	36,000	NA	None	Chronic infection
901496	M	NA	02/2009	670,000	NA	None	Chronic infection
901498	M	NA	02/2009	180,000	NA	None	Chronic infection
135945	M	C	05/2013	1400,000	NA	None	Chronic infection
138399	M	C	07/2013	37,000	NA	None	Chronic infection
139847	M	C	08/2013	32,000	NA	None	Chronic infection

F=female, M=male; AA=African American; H=Hispanic; C=Caucasian; NA=information not available; D4T=Stavudine; EFV=Efavirenz, NVF=Nelfinavir.

Table 2

Search for 2-LTR HIV-1 DNA circles. PCR analyses were performed with DNA samples from individuals as indicated. Primers were selected inside the two LTR's. In samples W-1, W-21, W-20, W-350, and W-351, two or three time points after HIV-1 infection were screened. To document the presence of proviral genomes, one primer was placed inside the LTR, the second one in a cellular Alu I sequence. In all instances, signals characteristic for proviral genomes were detected. The successful extraction of small circular DNA in the size range between 5.65 and 14.8 kbp i.e. that of 2-LTR circles, was verified by the control experiment described under Methods.

Long term nonprogressors	2-LTR circles	Proviral DNA
W-1	Negative	Positive
W-21	Negative	Positive
W-360	Negative	Positive
TR19	Negative	Positive
Elite controllers		
W-20	Negative	Positive
W-350	Negative	Positive
W-351	Negative	Positive
W-352	Not determined	Not determined
TR15	Positive	Positive
TR16	Negative	Positive
Progressive disease		
W-4	Negative	Positive
W-17	Negative	Positive

Table 3

Extent of CpG methylation in the 5′-region and 3′-region of HIV-1 proviruses. CpG methylation was determined by the bisulfite sequencing method (Frommer et al., 1992; Clark et al., 1994). The percentage of unmethylated CpG's in genome regions as indicated was recorded. ND—not determined.

Subject	Date	24–922 bp Unmethylated CpGs (%)	8371–9494 bp Unmethylated CpGs (%)
W-1	06/1995	ND	100
	05/1996	35	28
	04/2003	ND	68
	04/2006	ND	100
W-21	01/1997	ND	100
	03/2001	ND	100
	06/2006	ND	100
W-360	07/1996	100	100
TR19	03/1997	100	98.9
W-20	09/1997	ND	100
	04/2006	ND	100
	07/2006	ND	100
W-350	05/2002	100	100
	10/2002	ND	98.8
W-351	03/2002	ND	100
W-352	03/2002	ND	100
	01/2006	ND	100
	07/2006	ND	100
TR15	03/2007	ND	100
TR16	12/2006	ND	100
W-4	08/1999	98.8	98.6
W-17	06/1999	ND	99
901148	02/2009	97.5	98.5
901196	02/2009	99.6	100
901251	02/2009	98.9	100
901271	02/2009	99.8	99.7
901330	02/2009	100	100
901448	02/2009	98.4	100
901496	02/2009	97.5	100
901498	02/2009	100	98.5
135945[a]	05/2013	100	ND
138399[a]	07/2013	100	ND
139847[a]	08/2013	100	ND

ND=not determined.

[a]Only CpG pairs 2 to 11 were analyzed.

Table 4

All primers used in the experiments described here are listed and assigned to individual PCR experiments. Primers were those used by HYPERLINK \l "MEP_L_tbl4fna" \o "[a]Blazkova et al. (2009). "[a]Blazkova et al. (2009), [b] (2012); [c] primers used by Friedrich et al. (2010).

Primer bisulfite sequencing	Sequence 5′→3
MB[a]	TGGTAGAATTATATATTAGGGTTAGGGATT
MH[a]	CACCCATCTCTCTCCTTCTAACCTC
MC[a]	AGAGAAGGTAGAAGAAGTTAATGAAGGAGA
MF[a]	AAATCTAAAAAATCTCTAATTACCAAAATC
F1[b]	TAGATATTTATTGATTTTTGGATGGTG
R1a[b]	CACCCATCTCTCTCCTTCTAACCTC
F2[b]	AGTGTTAGTGTGGAGGTTTGATA
R1b[b]	AAAAAACTCCTCTAATTTYHCTTTC
HIV1 P1 f	TTTTTTTGGTTAGATTAGATTTGAGTTT
HIV1 P2 r	ATAATAATCTAAATTCTTCTAATCCTATCT
HIV1 P3 f n	TTGTTTTGAGTGTTTTAAGTAGTGTGTGTT
HIV1 P4 r n	ATCCCAATATTTATCTACAACCTTCTAATA
HIV P1n f	TTTGGTTAGATTAGATTTGAGTTTG
HIV P3n r	AATCTAAATTCTTCTAATCCTATCT
HIV P2n f n	TTTGAGTGTTTTAAGTAGTGTGTGTT
HIV P4n r n	TCCCAATATTTATCTACAACCTTCT
HIV1 P5	TTTAAGAATAGTTTTTGTTGTATTTTTTAT
HIV1 P6a r	ACATTAAACAAACTAACAACACTATTCTTT
HIV1 P7 f n	AGTGAATAGAGTTAGGTAGGGATATTTATT
HIV1 P8a r n	AATTCCTAACTCCAATACTATAAAAAATTC
HIV1 P5a f	AATATTGGTGGAATTTTTTATAGTATT
HIV1 P6 r	ACACAAACAACATTAATAACTACTATATTA
HIV1 P7a f n	AGTTAGGAATTAAAGAATAGTGTTGTTAGT
HIV1 P8 r n	CTACTTATAATTACTCCATATTTTTCCAAA
HIV1 P5.2a f	TAGTTTTTGTTGTATTTTTTATAGTGAA
HIV1 P6.2a r	TTCTTTAATTCCTAACTCCAATACT
HIV1 P5i f n	GAGTTAGGTAGGGATATTTATTATT
HIV1 P6i r n	TTACAATCAAAAATAAATCTCTCAA
HIV1 P9 f	AGAAGAGGTTAATAAAGGAGAGAATATTAG
HIV1 P10 r	CAAATCTAATCTAACCAAAAAAACCCAATA
HIV1 P11 f n	TTTGTTATATTTTGTGAGTTTGTATGGGAT
HIV1 P12 r n	CAAACAAAAAACAACTACTTATATACAAAA
HIV-1 Integration	
*Alu*1 forward f[b]	GCCTCCCAAAGTGCTGGGATTACAG
HIV-1 gag reverse r [b]	GCTCTCGCACCCATCTCTCTCC

Primer bisulfite sequencing	Sequence 5′→3
LTR forward f n [b]	GCCTCAATAAAGCTTGCCTTGA
LTR reverse r n [b]	TCCACACTGACTAAAAGGGTCTGA
2-LTR circles	
MH535 f[b]	AACTAGGGAACCCACTGCTTAAG
MH536 r[b]	TCCACAGATCAAGGATATCTTGTC
Detection of small circular DNA pEGFP-FMR1	
Kan.pEGFP 5′ f	ATGATTGAACAAGATGGATT
Kan.pEGFP 3′ r	TCAGAAGAACTCGTCAACAA
pNL4-3-dE-EGFP	
HXB2o P1 f	TGGAAGGGCTAATTCACTCC
HXB2o P2 r	CACACACTACTTGAAGCACTCAAG
HXB2o P3 f n	AAGATATCCTTGATCTGTGG
HXB2o P4 r n	CTTAAGCAGTGGGTTC

f=forward, r=reverse, n=nested.

[a] Blazkova et al. (2009).

[b] Blazkova et al. (2012).

[c] Friedrich et al. (2010).