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TRANSCRIPTION OF HIV1 IS INHIBITED BY DNA METHYLATION

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A possible role of DNA methylation as a factor in HIV latency was studied by methylating a HIV1-LTR-CAT plasmid in vitro and measuring its expression after transfection on Vero cells. Methylation with a eukaryotic DNA methylase resulted in a 70 % inhibition of chloramphenicol acetyltransferase expression, in the absence as well as in the presence of the HIV1 trans-activator protein TAT in the cell. A similar degree of transcription inhibition was obtained by methylation of the only Hpa II site at position -143 in the HIV1-LTR with the bacterial Hpa II methylase. In contrast to the effect by eukaryotic methylation, the inhibition by Hpa II methylation could be partially reversed by cotransfection of the TAT gene. The reason may lie in an about 40 % demethylation at the Hpa II site which was concomitantly observed.

The retrovirus HIV is generally accepted as the etiologic agent of AIDS (1, 2). Characteristic of the disease is a latency period of 5-8 years, during which only few provirus DNA are transcribed (3). Factors playing a role in the maintenance of latency are under investigation in several laboratories, but are not yet well understood. We are, at present, studying a possible contribution of DNA methylation to viral latency.

DNA methylation has been shown to have a function in the shut off of tissue specific genes in tissues in which they are not expressed (reviewed in 4, 5, 6). In general, methylation of the promoter region of a gene leads to inhibition of transcription, whereas undermethylation of the same region allows transcription (7). Viral DNA is normally not methylated in lytic infections and in the viral particle (8), but can become methylated in latent infection or in viral transformation of cells (9, 10). In such cases the same correlation of promoter methylation and gene inactivation as with cellular genes is observed. A first indication that DNA methylation could be a regulatory factor in HIV1 transcription came from Bednarik et al. (11). They obtained reactivation of a HIV1-LTR-CAT plasmid, stably integrated in Vero cells, by 5-azacytidine, an inhibitor of DNA methylation. They could demonstrate a change of methylation after 5-azacytidine treatment only in a Pvu II site containing a methylatable CA dinucleotide.

Abbreviations: CAT: chloramphenicol acetyltransferase, HIV: human immunodeficiency virus, LTR: long terminal repeat, NRE: negative regulating element, TAT: HIV1 trans-activator protein.

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The methylation of cytosine in other sequences than the main methylation sequence CG is, however, a rare event (12) and it is not known whether CA methylation can inhibit transcription.

In order to understand the influence of methylation on HIV1 transcription and to explore a possible role in viral latency, we performed transient expression assays with a HIV1-LTR-CAT plasmid after prokaryotic or eukaryotic in vitro methylation. The HIV1-LTR contains all the regulatory sites for the initiation of transcription on the HIV1 genome. One of them, the TAR site, is the target for the strong viral trans-activator protein TAT (13). We therefore included the effect of the TAT protein on transcription inhibition by methylation in our study.

MATERIALS AND METHODS

Plasmids: pHIV1-LTR-CAT (donated by L. Montagnier) contains the 3'-LTR of the HIV1-BRU isolate (14). pS2 HIV1-LTR-CAT was derived from pHIV1-LTR-CAT by deleting the Ava I site in pBR322. pCV-1 (15) (donated by R.C. Gallo) was used as TAT expression vector.

Enzymes: DNA methylase (E.C. 2.1.1.37) was extracted from regenerating rat liver (16) and purified as described (17). 1 unit incorporates 1 pmol CH_3 into denatured M.luteus DNA in 1 hr at 37°C. Hpa II methylase was obtained from Boehringer Mannheim, restriction enzymes were from BRL.

Methylation: The whole LTR or parts of the LTR were cut out with restriction enzymes and separated by agarose gel electrophoresis. The DNA was methylated at 30°C for 40 hours in 20 mM Tris, pH 7.9, 40 mM KCl, 0.5 mM dithiothreitol, 70 μM S-adenosyl-L-methionine, 10% glycerol, 0.05% Triton X-100, 200 μg/ml bovine serum albumin with 1.5 ng DNA/μl and 600 U DNA methylase/μg DNA. To control the efficiency of methylation, an aliquot was incubated with S-adenosyl-L-methyl-[^{3}H]-methionine (15 Ci/mmol) and the [^{3}H]-incorporation determined by scintillation counting. After the incubation, the mixture was treated with 1% sodium lauroyl sarcosinate and 250 μg proteinase K per ml for 40 min at 37°C followed by two phenol and three ether extractions and a dialysis. Then the LTR was religated into the plasmid and transfected.

The M•Hpa II incubation contained: 50 mM Tris, pH 7.6, 5 mM EDTA, 1 mM dithiotreitol, 5% glycerol, 70 μl S-adenosyl-L-methionine, 200 μg/ml bovine serum albumin, 50 μg/ml DNA and 0.5 U methylase/μg DNA. Subsequently, the DNA was digested with Hpa II to destroy unmethylated DNA.

Transfection: DNA transfection was carried out by the calcium phosphate precipitation method of Graham and van der Eb (18) with the modifications of Wigler et al. (19) and an additional treatment with 15% glycerol in DMEM 4 hours after transfection. 24 hours prior to transfection, Vero cells were seeded at 1×10^6 cells per dish. Cell harvesting 48 hours later was done as described by O'Hare (20).

CAT assay: CAT enzyme activity assays were performed with 15 μl cell extract, 0.7 mM acetyl-CoA, 50 μg/ml chloramphenicol and 40 μl 250 mM Tris, pH 7.8, in a total volume of 70 μl. The incubation time was varied between 0.5 and 3 hours at 37°C to avoid greater conversion than 30%. With longer incubation times, more acetyl-CoA was added and the linearity was controlled. The reaction mixture was extracted with 0.5 ml ethylacetate, the organic layer was evaporated to dryness and taken up in methanol. The CAT assay products were analyzed by reversed-phase HPLC. In 45% methanol as eluent the retention times are 4.4 min for unreacted chloramphenicol, 7.1 min for 3-acetate chloramphenicol and 10.9 min for 1,3-diacetate chloramphenicol.

Test for demethylation: Vero cells were transfected with 3 μg/dish of pHIV1-LTR-CAT DNA which had been extensively methylated with Hpa II methylase together with 15 μg unmethylated pCV-1 DNA containing the TAT gene. To test for demethylation at the Hpa II site, the

plasmid DNA was extracted from the cells 36 or 48 hours after transfection by the method of Hirt (21). After digestion with Hpa II, Hind III and Bgl II, the DNA was separated by agarose gel electrophoresis and blotted on a nylon membrane. Hybridisation was performed under stringent conditions with the nick-translated Bgl II-Hind III fragment as a probe (22).

RESULTS

Fig. 1 shows some characteristic features of the HIV1-LTR fragment from the Bgl II to the Hind III site. The CGs, the target sites for the known regulatory factors (23) and some restriction sites are indicated. In the first experiment, the whole Bgl II-Hind III fragment was excised, methylated with eukaryotic methylase and religated into the CAT plasmid for transfection.

To control the efficiency of methylation, the percentage of 5-methylcytosine was determined for each reaction. Typically, about 3% of all bases were found methylated. As complete methylation of the 10 CGs in the fragment amounts to 2%, the additional 1% methylation must be contained in the dinucleotides CA and CT (24).

Methylation of the Hpa II and the Ava I sites was determined as a further criterion for complete methylation of CG. The fragment was methylated in the presence of S-adenosyl-L-methyl-[^{3}H] -methionine and digested with Ava I, Hpa II or Msp I.

Ava I and Hpa II do not cut the DNA when the CG in their recognition sequence is methylated. Msp I, an isoschizomer of Hpa II, cuts irrespective of CG methylation. The fluorogram in Fig. 2A shows the same 566 bp band for the Hpa II- or Ava I-digested as for the unrestricted methylated fragment, arguing for complete methylation. After Msp I digestion, bands of 343 and 223 bp were obtained as expected. Even on a highly overexposed fluorogram (14 days instead of overnight) no indication for an undermethylation was found.

When pHIV1-LTR-CAT with the LTR methylated was transfected on Vero cells, 70 % inhibition of CAT expression was observed in comparison with the mock-methylated control (Table1). Cotransfection of a 5fold excess of the TAT gene in an expression vector did not influence the degree of inhibition by methylation. To determine which CGs are responsible for the transcription inhibition by methylation, the LTR was further subdivided and smaller frag-

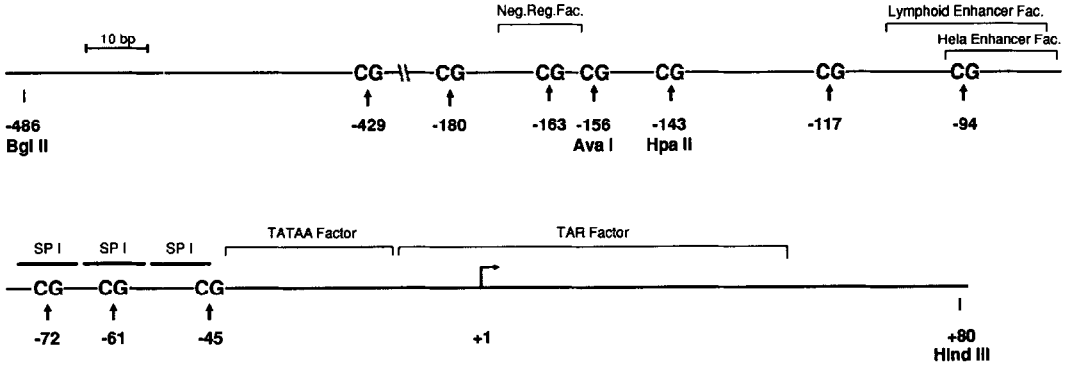

Figure 1: Regulatory region in the 3'-LTR of the HIV1-BRU isolate. Selected restriction sites, CGs (methylation sites) and protein protected regions are displayed. The transcription start is marked by an arrow.

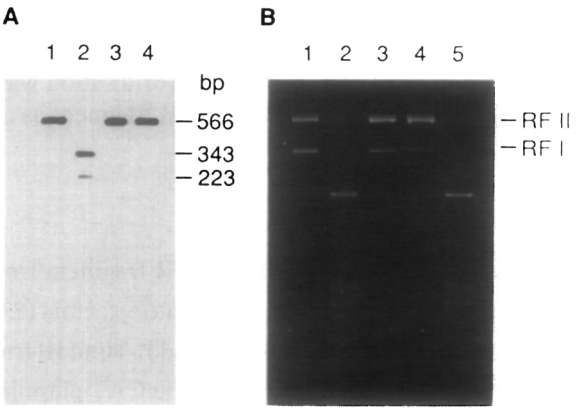

Figure 2: In vitro methylation of pHIV1-LTR-CAT by eukaryotic or prokaryotic DNA methylases. A): Fluorography of the Bgl II-Hind III fragment after methylation with the rat methylase and digestion with Hpa II (lane 1), Msp I (lane 2), Ava I (lane 4) or undigested (lane 3). B): Ethidiumbromide-stained agarose gel of pHIV1-LTR-CAT after Hpa II methylation. lanes 1, 2: mock-methylated; lanes 3-5: methylated with M•Hpa II. The plasmid was digested with Hpa II (lanes 2, 4), Msp I (lane 5) or was left undigested (lanes 1, 3).

ments were methylated. For that purpose the plasmid pS2 HIV1-LTR-CAT was constructed in which the second Ava I site in the vector was deleted by partial Ava I digestion and fill-in with the Klenow enzyme. This allowed us the separate methylation of the Ava I-Bgl II fragment including the NRE site and of the Ava I-Hind III fragment containing the majority of the CG sites and the promoter/ enhancer region. While methylation of the more upstream fragment from Ava I to Bgl II resulted in no inhibition in Vero cells, methylation of the promoter/

Table 1: Influence of DNA methylation on HIV1-LTR-CAT expression in Vero cells

DNA fragment methylated	TAT	acetylated chloramphenicol(%)		inhibition(%)
		mock-methylated	methylated	
A) rat methylase				
Bgl II-Hind III	+	30.6	9.9	67.6
Bgl II-Hind III	-	6.0	1.8	70.0
Ava I-Hind III	+	33.0	12.1	63.3
Bgl II-Ava I	+	24.4	25.6	0.0
B) Hpa II methylase				
pHIV1-LTR-CAT	-	8.8	2.2	75.0
pHIV1-LTR-CAT	+	25.0	15.5	38.0

pHIV1-LTR-CAT was methylated in vitro with the rat liver DNA methylase or the bacterial Hpa II methylase and transfected on Vero cells. 48 hrs later the conversion to acetylated chloramphenicol was measured in a CAT assay. In the case of eukaryotic methylation only the LTR or fragments of the LTR were methylated and religated into the plasmid; in the case of prokaryotic methylation the whole plasmid was methylated.

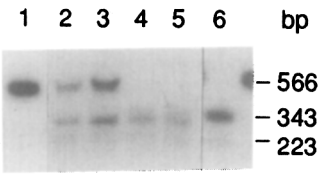

Figure 3: Demethylation of the HIV1-LTR in Vero cells.
The pHIV1-LTR-CAT plasmid was methylated with the Hpa II methylase and transfected on Vero cells. 36 or 48 hrs later the plasmid DNA was reextracted from the cells by the method of Hirt. The 566-bp fragment containing the only Hpa II site of the HIV1-LTR was cut out with Hind III and Bgl II, probed for methylation by digestion with Hpa II and blotted on a nylon membrane.
lanes 1, 6: methylated and mock-methylated DNA, respectively, before transfection; lanes 2, 3: methylated DNA 36 and 48 hrs after transfection; lanes 4, 5: mock-methylated DNA 36 and 48 hrs after transfection.

enhancer fragment from Ava I to Hind III showed the same inhibition as methylation of the entire LTR (Table 1).

The presence of a Hpa II site (CCGG) in the Ava I-Hind III fragment 30 bp upstream of the enhancer, opened the possibility to study the influence of a single methylgroup on transcription. Complete methylation of this site with Hpa II methylase was shown by Hpa II digestion (Fig. 2B). Hpa II cleaved the unmethylated (lane 2) but not the methylated plasmid (lane 4). Msp I cleaved also the methylated DNA (lane 5). The inhibition of CAT expression in Vero cells was 75% in the absence and 38% in the presence of TAT (Table 1). TAT protein therefore seems to be able to partly neutralize the Hpa II methylation effect.

The reason for that is evidently a partial demethylation of the LTR-CAT plasmid (Fig. 3). When the methylation state of the M•Hpa II-methylated plasmid was analyzed 36 or 48 hrs after transfection an about 40% demethylation of the Hpa II site in the LTR was observed.

DISCUSSION

Our results show a 70 % inhibition of transcription of the HIV1-LTR-CAT plasmid by eukaryotic methylation of the LTR, irrespective of whether TAT protein is present in the cell or not. Separate methylation of the three CGs in the region upstream of the Ava I site including the NRE site did not effect transcription. Methylation of the CGs downstream of the Ava I site inhibited as effectively as methylation of the whole LTR. Moreover, a similar degree of transcription inhibition could be achieved by methylation of only the Hpa II site at position -143 with the Hpa II methylase. In contrast to eukaryotic methylation, prokaryotic Hpa II methylation was partially overcome by TAT cotransfection. The inhibition of transcription was diminished from 75 to 38% in the presence of TAT. Bednarik et al. (25) recently reported the complete reversion of the Hpa II methylation effect by TAT in a T-cell line with a different plasmid. Accompanying the partial reactivation of the HIV1-LTR promoter, we found an about 40% demethylation of the methylated Hpa II site in the HIV1-LTR. As replication of the plasmid in the transfected cell should not occur, an active demethylation process is probably operating. Active demethylation has recently been described in several cases (26, 27). Further experi-

ments will have to show whether the HIV-LTR methylated with the eukaryotic enzyme is not demethylated or why else it is not reactivated by TAT.

With the above results pointing to a possible role of DNA methylation in HIV1 latency, it will now be necessary to look for Hpa II methylation of the HIV1-LTR in latently infected persons. Because of the low percentage of infected T-cells during latency (about 1 in 1000), methylation of the few HIV1 genomes cannot be analyzed by Southern blotting after digestion of the DNA with methylation sensitive restriction enzymes (e.g. Hpa II). More refined techniques including methylation sensitive restriction enzymes and PCR (polymerase chain reaction) will have to be developed.

That provirus genomes of retroviruses after integration into the cellular genome can also be inactivated by de novo methylation in the cell has been amply documented (10, 28, 29). There have even been speculations that DNA methylation in higher eukaryotes could serve the main purpose to permanently inactivate retroviral information by mutation of 5-methylcytosine to thymine (10, 28).

In general DNA methylation has been found in transforming retroviruses which are inherited via the germe line (10, 26, 27). But, as the example to HTLV-I (human T-cell lymphotropic virus type I) shows (31), DNA methylation is also observed in infectious retroviruses.

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