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Drastic decrease of transcription activity due to hypermutated long terminal repeat (LTR) region in different HIV-1 subtypes and recombinants

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ABSTRACT

Transcriptional activation of HIV-1 gene expression is partially controlled by the interaction between viral and cellular transcription factors acting at HIV-1 long terminal repeat (LTR) sequences. HIV-1 subtyping at LTR region and nucleotide LTR variability from clinical samples in 48 subjects carrying different HIV-1 subtypes (9A, 5C, 3D, 3F, 21G, 2H, 3J and 2 undefined) at the protease (PR) gene, were performed. LTR sequences from each HIV-1 clade were cloned in luciferase-expression vectors to determine basal and Tat-induced transcriptional activities in the presence and absence of PMA stimulation. A high number (37.8%) of recombinants at LTR/PR regions were identified. All HIV-1 promoters presented low basal transcriptional activity that was nevertheless induced by Tat and PMA. LTR activity was similar across the majority of HIV-1 variants in response to Tat and cell activation. Only subtype C and CRF01_AE LTRs presented higher basal and induced-PMA transcription activities than HXB2 clade B promoter. No basal or Tat/PMA induced activity was found in those promoters presenting G to A hypermutation compared to the wild type promoter activities. G to A hypermutation at some important transcription binding-factor sites within LTR compromised the activity of the viral promoter, decreasing the in vitro viral transcription of the luciferase gene.

1. Introduction

Transcription is a crucial step for human immunodeficiency virus 1 (HIV-1) gene expression in infected host cells. After fusion-mediated entry within cells, uncoating, reverse transcription of the RNA genome and nuclear entry of the pre-integration complex, the proviral DNA is integrated into the host cell genome. Transcription of the HIV-1 provirus is then regulated by distinct cellular transcription factors that interact with consensus sequences for regulatory elements in the LTR region and are involved in LTR trans-activation (Pereira et al., 2000). Multiple cell type-specific interplays between cellular and viral factors lead the virus to leave latency and to replicate in a great diversity of cells (Krebs et al., 2001).

Two identical LTR sequences are located in both extremes of HIV-1 provirus. Each LTR is divided in three regions: U3, R and U5. U3 is subdivided into three elements: promoter, enhancer and modulatory regions, which contain important binding sites for cellular proteins such as Sp-1, NF-κB, NF-AT, USF, TCF-1a and RBF-2 that regulate viral transcriptional levels (Jones et al., 1986; Perkins et al., 1993; Estable et al., 1998) (Fig. 1). The R region contains the Tat-responsive element hairpin, TAR, which acts as an RNA enhancer through binding to the viral Tat (trans-activator of transcription) protein, essential for the production of viable viral transcripts. The U5 region contains other binding sites for cellular transcription factors such as AP-1, AP3-like (corresponding to an NF-AT site) and Sp1, which are important for virus infectivity (El Kharroubi and Martin, 1996; Van Lint et al., 1997).

The high genetic diversity of HIV-1 is due to rapid virus turnover, high mutation rate, nucleotide deletions and insertions, and recombination events. Nine subtypes (A, B, C, D, F, G, H, J, K), at least 47 circulating recombinant forms (CRFs) and multiple unique recombinant forms (URFs) are included within the HIV-1 group M. Genetic variability within LTR binding sites in U3 and TAR regions has been described in several HIV-1 subtypes (Montano et al., 1997, 1998; Naghavi et al., 1999; De Baar et al., 2000; Jeeninga et al., 2000; Roof et al., 2002; Ramírez de Arellano et al., 2005; Rodríguez et al., 2007). However, it remains unclear if clade-specific variability at the LTR region could influence viral transcription efficiency and if it could contribute to differences in the virulence of distinct

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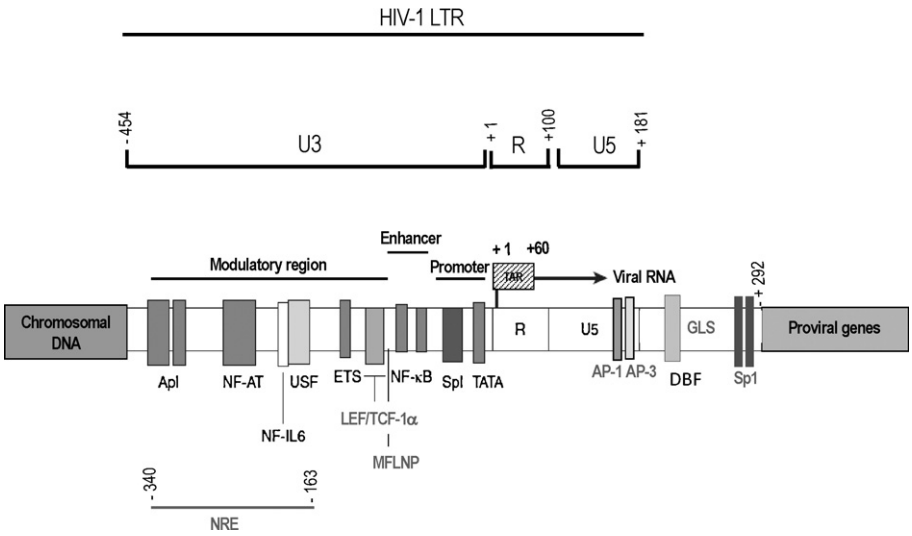

Fig. 1. Schematic representation of HIV-1 LTR and "leader" gag (GLS) sequences. LTR regions and cellular transcription binding-factors are indicated. Numbers are relative to the transcription start site nucleotide +1. Ap-1, 2 or 3: "activator protein 1, 2 or 3"; NF-AT-1: "nuclear factor activator T-cells"; NF-IL6: "nuclear factor-IL6"; USF: "upstream stimulator factor"; NRE: "negative regulatory element"; ETS: "E26 transformation-specific sequence"; LEF/TCF-1: "lymphoid enhancer factor/T-cell factor 1 alpha"; MFNLP: "most frequent naturally length polymorphism"; NF-κB: "nuclear factor kappaB"; Sp1: "specific protein 1"; TAR: "trans-activation region"; DBF: "downstream binding-factor site".

subtypes and recombinant forms (Montano et al., 1997, 1998; Naghavi et al., 1999; Jeeninga et al., 2000; Rodríguez et al., 2007).

2. Materials and methods

2.1. Study population and HIV-1 subtyping at LTR region

A total of 48 HIV-1 infected patients, previously identified as infected by non-B subtypes by phylogenetic analysis of *protease* (*PR*) sequences, were selected (Table 1). All were under clinical follow-up at an AIDS/HIV Reference Centre located in Madrid, Spain. Clinical and epidemiological data, and subtypes at *PR* are summarized in Table 1.

HIV-1 subtyping was performed by phylogenetic analysis of LTR consensus sequences (positions from −454 to +181, 630 bp) from purified amplicons obtained from proviral DNA from peripheral blood mononuclear cells of HIV-1 infected patients, as we previously reported (Ramírez de Arellano et al., 2005). HIV-1 sequences belonging to HIV-1 group M available at the Genbank were used as references. The tree topology was obtained using the Neighbor-Joining program. After alignment of DNA sequences using the Clustal W method, columns containing gaps were eliminated. The distance matrix was estimated using the Kimura two-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap (1000 data sets) of the multiple alignments was done to test the statistical robustness of the tree.

2.2. LTR luciferase constructs and transfection assays

In order to perform transcriptional analysis comparing LTRs from different HIV-1 variants, the LTR region was amplified by nested polymerase chain reaction (PCR) directly from proviral DNA extracted from peripheral blood mononuclear cells (PBMC) of HIV-1 infected patients. The outer PCR amplification was performed using the following primers: LTR-1D (5′-TGGAWGGGYTAATTTACTCCARGAAAAG-3′) and LTR-R (5′-CAAGCCGAGTCCRGCGTCGAGAG-3′). The inner PCR (positions from −454 to +181 in HXB2 isolate, 630 bp) was done with primers LTR-Xho I (5′-GGGCCCC/TCGAGCCCTGATTGGCAGAAYTACACACCAGG-3′) and LTR-Hind III (5′-GGGCCCA/AGCTTCCTGCGTCGAGAGAGCTYCTCTGG-3′) including Xho I and Hind III restriction sites.

Two LTR purified amplicons from each HIV-1 subtype or CRF found were selected according to their similarity to previous LTRs consensus sequences from different HIV-1 subtypes (Montano et al., 1997, 1998; Naghavi et al., 1999; De Baar et al., 2000; Jeeninga et al., 2000; Roof et al., 2002). Then they were digested with Xho I and Hind III to generate LTR amplicons containing the U3 and R region (−454 to +100), and inserted into a pG12-Basic Luciferase reporter vector (Promega, reference E1641), previously treated with Hind III and Xho I restriction enzymes. The generated recombinant plasmids containing the different LTRs inserts were confirmed by sequencing. Two recombinant constructs representative from each HIV-1 subtype or CRF were used to transfect lymphoid Jurkat T-cell line (phenotype CD3+, CD4+, DR) (Fig. 2). Cells were grown in RPMI medium (Biowhittaker) supplemented with 10% of fetal bovine serum, 200 nM glutamine and 5000 U/ml of penicillin/streptomycin. Jurkat T lymphocytes (4.5×10^6 cells) were transfected by electroporation (280 V, 1500 μF). The amount of each plasmid was kept constant at 1 μg of DNA per 10^6 cells. Basal and Tat-induced LTR activity was measured through co-transfection of each different LTR luciferase construct with a pcDNA3.1 vector as control (Invitrogen, reference V79020) or pcDNA3.1 containing the full length HIV-1–Tat sequence from subtype B (Schwartz et al., 1990; Hazan et al., 1990), respectively. Transfections were performed in quadruplicate. Cells were seeded in culture medium in the absence or presence of PMA (phorbol miristate acetate, Sigma; final dilution of 1:10,000).

2.3. Measure of LTR transcriptional activity by luciferase assay

Twenty four hours after transfection, culture medium was removed and Jurkat cells were washed once with phosphate-buffered saline (PBS 1×). Cells were lysed (300 μl of Luciferase Cell Culture Lysis 5× Reagent, Promega). Lysates were kept during 30 min at room temperature. Then, 50 μl of substrate (Luciferase Assay System, Promega) were added to 50 μl of each lysate. Luciferase activity (expressed as relative light units, RLUs) was determined by a Berthold luminometer (Sirius model).

Co-transfection with a pSV-β-galactosidase commercial plasmid (Promega, reference E1081) was performed to normalize

Table 1
Clinical and epidemiological data from 48 subjects infected with different HIV-1 variants.

No.	Origin	CD4+ count (cells/μl)	HIV-1 RNA copies/ml (log)	Sex/age/risk group	PR subtype	LTR subtype
1	Morocco	104	5.6	M/43/3	D	A
2	EG	504	3.7	M/34/3	U	A
3	Spain	378	3.3	M/55/3	C	A
4	Mozambique	132	1.7	M/38/3	A	A
5	EG	440	4.6	M/45/3	A	A
6	EG	192	1.7	F/52/3	G	A
7	EG	777	2.5	F/nd/3	A	A
8	Mozambique	348	1.7	F/32/3	A	A
9	D.R. Congo	nd	1.7	M/44/5	H	A
10	EG	380	1.7	M/50/3	A	A
11	Switzerland	684	1.7	M/56/2	C	B
12	Panama	1040	2.6	M/32/2	A	B
13	France	528	2.8	M/40/1	G	B
14	Ethiopia	120	1.7	F/nd/3	A	C
15	EG	700	1.7	M/52/3	C	C
16	EG	468	3.7	F/46/3	C	C
17	EG	110	5.1	F/51/3	U	C
18	EG	265	1.5	M/27/3	F	C
19	EG	nd	5.8	M/50/1	C	C
20	Angola	168	1.7	F/32/3	D	D
21	Thailand	nd	4.4	M/nd/3	A	CRF01_AE
22	Thailand	nd	3.6	nd	A	CRF01_AE
23	Argentine	nd	3.7	F/nd/3	F	F
24	Argentine	560	2.2	F/nd/3	F	F
25	EG	620	1.8	F/nd/3	G	G
26	Nigeria	320	3.6	M/43/3	G	G
27	EG	567	2.8	F/11/5	G	G
28	Ghana	800	4.2	M/34/3	G	G
29	Liberia	693	1.7	F/nd/3	G	G
30	D.R. Congo	440	2.9	F/nd/3	G	G
31	EG	378	1.7	F/nd/3	G	G
32	Nigeria	126	1.7	M/30/3	G	G
33	EG	841	3.9	F/32/3	G	G
34	EG	50	2.1	M/44/3	G	G
35	Liberia	1100	4.3	M/34/3	G	G
36	Nigeria	1008	2.4	F/23/3	G	G
37	Cameroon	544	1.7	F/34/3	G	G
38	EG	483	5.3	F/49/3	G	G
39	Portugal	140	4.3	M/34/1	G	G
40	EG	nd	1.7	F/32/3	G	G
41	EG	154	1.9	F/40/3	H	H
42	EG	640	1.7	M/57/3	G	H
43	Cameroon	100	5	F/35/3	J	CRF11_cxp
44	EG	357	1.7	M/34/3	J	CRF11_cxp
45	Cameroon	342	4.7	M/40/3	J	CRF11_cxp
46	Cameroon	28	1.8	M/29/3	G	U
47	Spain	360	3.9	F/nd/3	G	U
48	EG	448	3.6	M/37/3	D	D

EG, Equatorial Guinea; D.R. Congo; Democratic Republic of Congo; F, female; M, male; risk group: 1, intravenous drug user; 2, homosexual; 3, heterosexual; 4, transfusion; 5, vertical; nd, not determined.

transfection efficiency. β-Galactosidase activity was measured in transfected cell lysates using the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer according to Manufacturer's instructions (Promega). We applied to each absolute RLUs value a correction factor being the ratio among the lowest value of each β-galactosidase assay and each β-galactosidase lysate value.

3. Results

3.1. High rate of HIV-1 recombinants at LTR/PR

The LTR subtypes from the viruses infecting 48 HIV-1 patients were: 10A, 3B, 6C, 2D, 2E, 2F, 16G, 3J, 2H, 3CRF11_cpx and 2U (undefined variants, not ascribed to any known subtype or CRF by phylogenetic analysis) (Fig. 1). We found a high rate of inter-subtype recombinant HIV-1 variants (37.5%), carrying different subtypes at both LTR/PR regions: 1A/D, 1A/U, 1A/C, 1A/H, 1B/C, 1B/A, 1B/G, 1C/A, 1C/U, 1C/F, 1H/G, 2CRF01_AE/A, 3 CRF11_cpx/J and 2U/G (Table 1). The remaining non-LTR/PR recombinants were: 6A/A, 3C/C, 2D/D, 2F/F, 16G/G, and 1H/H. LTR sequences were deposited at the GenBank (Accession numbers: from AY610959 to AY611005 and EF380393).

3.2. Clade-specific LTR markers at different HIV-1 subtypes and CRF

Several subtype-specific mutations were found in regulatory regions within LTR across different non-B subtypes and CRF (Fig. 2). Three NF-κB binding sequences (positions −84 to −109 in HXB2 subtype B isolate) were present in LTR subtype C sequences, two in subtypes A, B, D, F, G, H and CRF11_cpx, and only one in CRF01_AE (former clade E) variants, in agreement with prior reports (Montano et al., 1997; Naghavi et al., 1999; Jeeninga et al., 2000; Roof et al., 2002; Ramírez de Arellano et al., 2005; Rodríguez et al., 2007).

The Core-NRE region within U3 (position −163 to −174) presented subtype-specific changes, as previously reported (Ramírez de Arellano et al., 2005). The conserved consensus sequences found were: CGCAGACACAT (clade C), TTTGAACAT/CAAG/A (subtype D), CGAACACACAAA (recombinants CRF01_AE), and CGAAGACACA/GTA (CRF11_cpx).

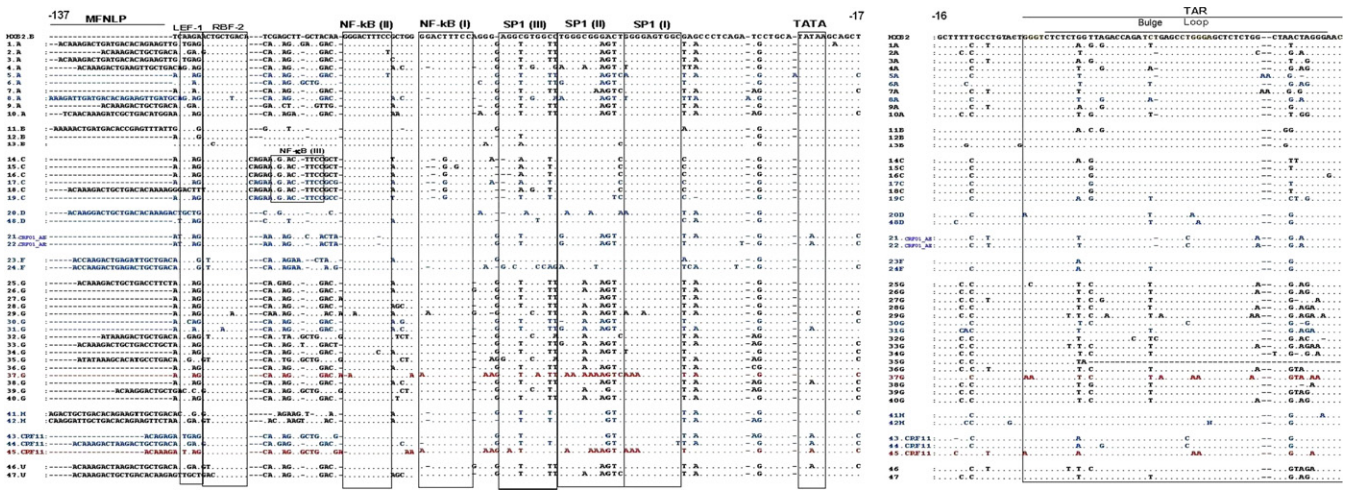

Fig. 2. Partial LTR consensus sequences (−137 to +60) from PCR amplicons of 48 HIV-1 subtypes and circulating recombinant forms (CRFs). In boxes, the most important LTR transcription factor-binding sites. At the top, LTR sequence from HXB2 subtype B reference virus. Dots indicate nucleotides identical to HXB2. Dashes indicate nucleotide deletions. In red, hypermutated sequences. In blue, LTR sequences that were cloned. Clones from patient 20 (subtype D) were obtained from another sequential sample showing hypermutated LTR sequence (see Fig. 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The C24T mutation and T25 nucleotide deletion in the "bulge" region (nucleotides T23C24T25) within TAR were previously described at different HIV-1 variants as subtype-specific markers (Montano et al., 1997, 1998; Ramírez de Arellano et al., 2005). We observed C24T in several sequences: subtype A (3/10), C (1/6), D (2/2), F (1/2) and in most clade G (14/16). C24A mutation appeared in 2 subtype A specimens. T25 deletion was present in both CRF01_AE and in 5 subtype A sequences (Fig. 2).

3.3. Guanine (G) to Adenine (A) hypermutation in transcription factor-binding sites within LTR

Viruses infecting patient 37 (subtype G) and patient 45 (recombinant CRF11_cpx) showed G to A hypermutation at LTR consensus sequence derived from the purified nested amplification (positions −137 to +60 in HXB2 isolate). Hypermutation affected to important regulatory LTR domains of the LTR, such as SP1 and TAR (Fig. 2). In patient 37 the G to A hypermutation was also present in all analyzed molecular clones derived from the same amplicon product (clones 37.1, 37.2, and 37.3), although the number of G-to-A mutations was lower in a molecular clone (37.G partial) derived from another amplicon using a different PBMC specimen (Fig. 3). When hypermutated viruses were obtained, patient 45 was *naïve* for any antiretroviral therapy and patient 37 was under highly active antiretroviral therapy (HAART), and both presented undetectable viral load (<50 HIV-1 RNA copies per ml). Interestingly, hypermutation was not present in the LTR consensus sequence (37.G WT) from the virus infecting the first available specimen col-

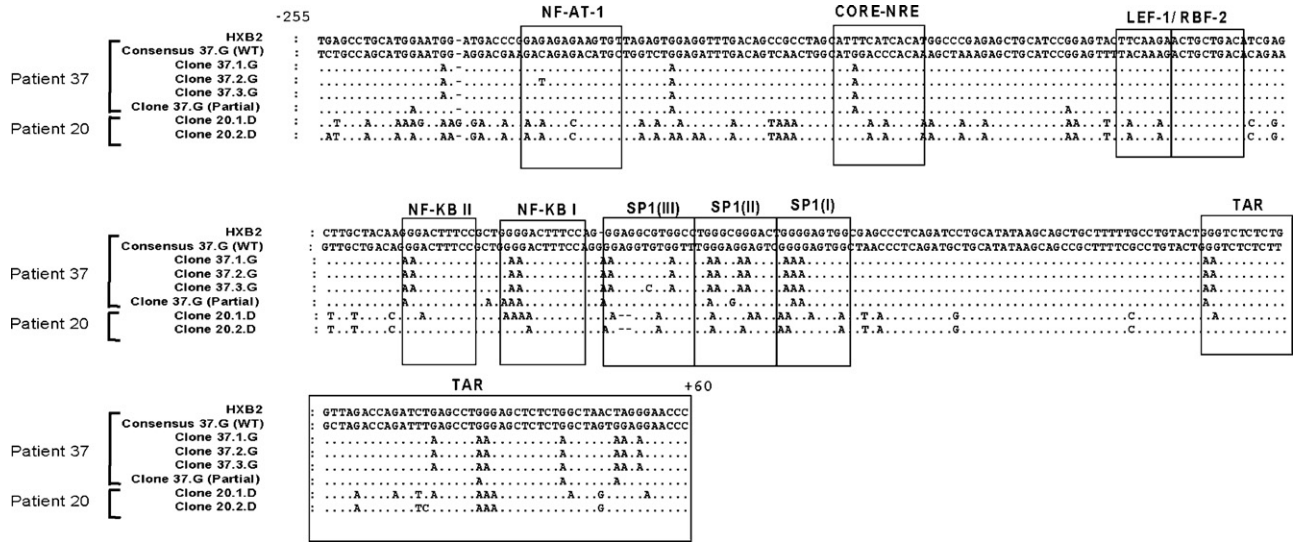

Fig. 3. Hypermutated LTR sequences in molecular clones. Alignment of the hypermutated sequences at important LTR transcription factor-binding sites (NF-κB, SP1, and TAR regions) in different molecular clones (37.1 G, 37.2 G, 37.3 G, 37 G partial, 20.1 D, 20.2 D) from two patients infected with G and D subtypes or consensus sequences (37.G WT) from PCR amplicons of patient with subtype D. In boxes, the most important transcription factor-binding sites within LTR. At the top, LTR HXB2 reference sequence. LTR subtype G sequences (patient 37): hypermutated (37.1, 37.2 and 37.3), and partially hypermutated (37.G partial) molecular clones compared to HXB2 and consensus LTR wild type sequence (37.G WT). LTR subtype D sequences (patient 20): molecular clones 20.1 y 20.2 compared to HXB2 sequence. Dots indicate nucleotides identical to the corresponding reference virus. Functional analysis of these LTR was performed (except for clones 37.2 y 37.3) in the presence and absence of PMA and the viral protein Tat (see Fig. 4c).

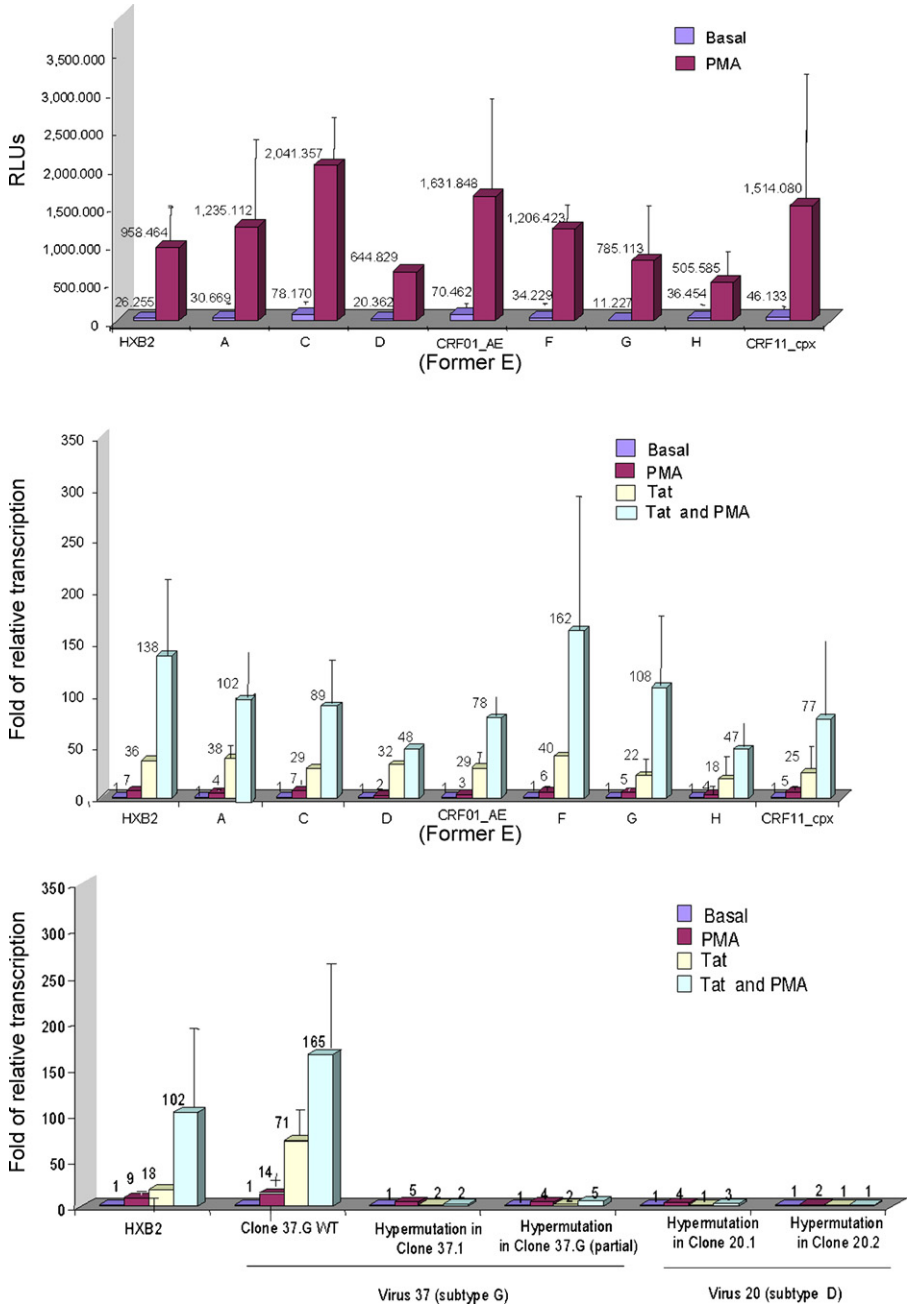

Fig. 4. LTR transcription activity at different HIV-1 variants in Jurkat cells. The mean standard deviation from two representative LTRs clones containing the R and U3 regions of each variant is shown. The RLU final value was corrected according to β-galactosidase activity (see Section 2). (A) Effect of PMA on basal LTR transcription. The mean values for the basal transcription activity (−Tat) and for induced transcription (+PMA) in samples with the same subtype or CRF is expressed in relative luciferase units (RLUs). (B) Folds of transcription activity induced (+Tat and/or +PMA) in samples with the same subtype relative to each basal activity (considered value 1). (C) Folds of transcription activity induced (+Tat and/or +PMA) in hypermutated LTR clones compared to HXB2 clade B and wild type subtype G (37.G WT) promoters relative to each basal activity (value 1).

lected when patient 37 was naive for any antiretroviral treatment and presented a high viral load (Fig. 3). Neither hypermutation was observed in several derived molecular clones analyzed (data not shown).

Hypermutated LTRs sequences were also found in two molecular clones analyzed (20.1 and 20.2) derived from a sample of subtype D in patient 20 under HAART (Fig. 3), with undetectable viral load. Again, hypermutation was also absent in the consensus sequence of another amplicon derived from a previous specimen collected during antiretroviral therapy (Fig. 2).

3.4. Similar transcriptional activity across HIV-1 subtypes and recombinants

Two LTR cloning sequences from each HIV-1 available variant were selected according to their similarity with the corresponding PCR amplicon (Fig. 2). Then, transcriptional activity of different molecular clones including LTR sequences from each HIV-1 subtype or CRF were performed. The luciferase gene activity led by the corresponding LTR promoter was measured in Jurkat cells, in the absence or presence of HXB2 subtype B Tat (trans-activator) protein and cellular activation with PMA (described in methods). All

LTRs were functional promoters regardless of the HIV-1 subtype, and most HIV-1 subtypes showed similar transcriptional activities after Tat or/and PMA stimulation. All LTR subtypes presented low basal transcriptional activity that was increased after Tat expression or PMA activation (Fig. 4). As expected, a synergistic effect between the Tat and PMA was observed (Fig. 4b), as previously described (Alcamí et al., 1995). Nevertheless, LTR subtype C and LTR CRF01_AE presented higher basal and induced-PMA transcription activities related to the HXB2 promoter (Fig. 4a). Results indicated that subtype B Tat protein was functional with all analyzed HIV-1 subtypes and recombinants, as previously suggested (Naghavi et al., 1999; Jeeninga et al., 2000).

3.5. Drastic decrease in transcription activity of hypermutated LTR promoters

Transcriptional activity of LTRs carrying G to A hypermutation at important transcription initiation (SP1 consensus) and elongation (TAR domain) sites was examined in two subtype G LTR clones (37.1 and 37.G partial, patient 37) and two subtype LTR D clones (20.1 and 20.2, patient 20). A drastic decrease in transcription activity of hypermutated LTR promoters was observed. No basal or induced activity with Tat or PMA was observed compared to HXB2 subtype B or wild type G (clone 37.G WT) promoters (Fig. 4c).

4. Discussion

This study provides several results. Firstly, the phylogenetic analysis of a series of HIV proviral clinical isolates revealed the presence of LTR sequences ascribed to pure subtypes or CRF recombinants. Secondly, the viral LTR promoter is highly trans-activated by Tat (HIV subtype B) or PMA in all tested subtypes and CRFs.

This study analyzes LTR transcriptional activities in a wide spectrum of HIV-1 subtypes and recombinants CRF defined in LTR sequences by phylogenetic analysis. A high rate of recombinants, analyzing *pol* and *env* (Lospitao et al., 2005; Holguín et al., 2005a) or *gag* and *pol* (Holguín et al., 2005b) genes, for viral clinical isolates in Spain has been previously reported. In our study, we observed that a third of the 48 studied specimens were LTR/PR recombinants when comparing subtypes provided by phylogenetic analysis of both regions from the same virus. HIV-1 subtyping using phylogenetic analysis of LTR sequences is recommended before transcriptional activity assays from different HIV-1 subtypes and recombinant variants are performed.

It was also observed that some genetic changes at different LTR regulatory regions represented subtype-specific sequence patterns, as has been extensively reported in other genetic regions such as in *pol* (Kantor and Katzenstein, 2003). Although some LTR mutations could reflect structural tolerance or could be fixed during evolution without influence on the functional activity of cellular factors binding the HIV-LTR is still unknown. Additional experiments testing the functional effect of each subtype-specific marker are necessary to determine if they could establish distinct interactions with viral and cellular transcription factors leading to different transcriptional levels among distinct HIV-1 variants (Montano et al., 1997, 1998; Naghavi et al., 1999; De Baar et al., 2000; Jeeninga et al., 2000; Roof et al., 2002).

Accordingly, since replication rate depends on the expression of a set of transcription factors by diverse stimuli (Van Opijnen et al., 2004), we cannot exclude the possibility that the expression of other transcriptional factors in different replicate conditions could affect the LTR activity of different HIV-1 subtypes. In this sense, the GALT (gut-associated lymphoid tissue) cytokine network and an IL-7 rich microenvironment favored HIV-1 subtype C replication during primary infection in rhesus macaques compared to its counterparts carrying clade B and CRF01_AE LTR sequences (Centlivre et al., 2006). A predominance of HIV-1 chimera carrying LTR clade C vs. those carrying LTR clade B or CRF01_AE during primary infection was also confirmed after simultaneous infection of rhesus macaques with the 3 chimeras (Centlivre et al., 2005). These results would suggest that clade C promoter could be particularly adapted to sustain viral replication in primary infection. Whether or not the high rates (50%) of HIV-1 subtype C worldwide could result in enhanced transmission needs to be clarified. In our study, LTR subtype C presented the higher basal and induced-PMA transcription activities in relation to HXB2 promoter, although a similar response to Tat expression was found among all subtypes. However, previous studies reported differences in the trans-activator activity of Tat proteins belonging to distinct HIV-1 subtypes (B and C) and recombinant CRF01_AE (Roof et al., 2002; Kurosu et al., 2002; Desfosses et al., 2005). Thus, additional studies for testing LTR activity induced by Tat protein belonging to other HIV-1 subtypes and CRF are required to confirm our results.

Despite displaying a high genetic variability, LTRs from different subtypes and CRF were functional in response to clade B Tat protein and cell activation. As previously described, a synergistic effect between the Tat viral protein and cellular activation was found in all HIV-1 clades. However, a strong decrease in transcriptional activity was found in hypermutated LTRs.

The high degree of G-to-A mutations in these hypermutated LTR sequences are expected to disrupt the NF-κB and Sp1 consensus binding sites as well as to destabilize the secondary structure of the TAR element, compromising the activity of the viral promoter. In this sense, the Sp1 transcription factor contains a zinc finger protein motif, by which it binds directly to a GC box element and enhances gene transcription. Additionaly, NF-κB factor specifically recognizes kappaB DNA elements with a consensus sequence of 5′-GGGRNYYYCC-3′ (R is an unspecified purine; Y is an unspecified pyrimidine; and N is any nucleotide) (Chen et al., 1998). In our hypermutated LTRs sequences, most G to A changes occurred at 5′ N-terminal of kappaB DNA elements and within the GC box of the three SP1 sites. This could cause the destruction of the consensus sequences for the binding of NF-κB and Sp1 transcription factors, decreasing the *in vitro* luciferase gene transcription at hypermutated LTR clones.

A decrease in HIV-1 transcription level was described in one HIV-1 long term non progressor (LTNP) subject infected by HIV-1 variants carrying multiple changes of G to A at proviral LTR binding sites for NF-κB, SP1, and TAR region (Zhang et al., 1997). Patients 45, 20 and 37 with hypermutated viruses at LTR were normal progressors according to clinical data. The first patient displayed high HIV-1 viraemia, and the other two were under antiretroviral therapy at the moment of the study and their samples presented an undetectable viral load. This finding suggests that defective non-replicative variants due to hypermutation in the HIV-1 LTR, can be detected in *naïve* subjects and with higher frequency in patients under efficient HAART, in which proviral DNA corresponds to long-lasting integrated copies, and is not the result of ongoing replication. According to our results, one of the mechanisms of generation of integrated but replication-defective genomes could be hypermutation in critical sequences such as these located in the HIV-1 LTR region.

In addition, It has been previously reported that impaired activity in the Tat–TAR axis may contribute to the establishment of latent infection in CD4+ T-cells (Yukl et al., 2009). Since HIV-1 infection is still productive in our analyzed patients, it is probably that a high fraction of the total HIV-1 sequences present in the PBMCs of the patients would show non-hypermutated LTRs, with an efficient Tat function and Tat–TAR interaction in the majority of the cells, with replicative-competent proviruses able to restart viraemia. Sequencing more clones as well as reverse-transcribed circulating

viral RNA would probably show non-hypermutated LTRs, since is unlikely that defective hypermutated proviruses lead to competent circulating viruses.

Several publications hypothesized the idea that increased expression of APOBEC3G host factor, which carry out deamination of cytidine/deoxycytidine, may inhibit HIV-1 infectivity by mediating dC-to-dU mutation on minus-strand DNA during reverse transcription and corresponding creation of templates for dG-to-dA transition during plus-strand synthesis. Although the HIV-1 vif-protein counteracts the lethal hypermutation effect of the cellular antiviral factor APOBEC3G/F in HIV-1, an increased expression of APOBEC3G/3F could lead to higher hypermutation in the presence of functional vif. Thus, enhanced APOBEC3G gene expression in humans may provide protective effects against HIV disease progression by reducing viral burden and increasing CD4 T-cell count over time (Jin et al., 2005). Additional studies testing the expression of APOBEC3G/3F in our LTR hypermutated sequences are required to confirm this hypothesis.

However, vif mutations would give rise to hypermutated defective viral genomes *in vivo* that could be potentially integrated without producing competent progeny viruses. In contrast with circulating plasma viruses, the presence of G to A hypermutated protease and retrotranscriptase coding regions at resting CD4+ T lymphocytes has been described (Kieffer et al., 2005), suggesting that an inefficient vif function could be responsible for an unusual high rate of G to A hypermutation. Since patient 37 before therapy presented high viral load, high level of replication-competent viruses with vif integrity would be expected. Moreover, it is unlikely that integrated defective proviruses carrying multiple G to A changes at LTR recovered from PBMCs of the patient 37 after therapy, led to competent circulating viruses. Circulating viruses in this patient could be the result of replicating viruses without LTR hypermutation in infected cells with an efficient vif function.

Recently, it has been demonstrated that HIV-1 inter-subtype vif variability affected APOBEC-3G-mediated host restriction and the authors observed differences in viral replication according to vif subtype (Lisovsky et al., 2010). Others described the impact of K22H vif mutation in both vif function and APOBEC-3 activity, suggesting its possible influence in G to A hypermutation *in vivo*, mainly in the context of specific polymorphisms associated to drug resistance including M36I (Fourati et al., 2010), highly prevalent in non-B subtypes (Yebra et al., 2010).

More functional experiments are essential to clarify if clade-specific variability at different HIV-1 regions could have consequences on the virulence of the different HIV-1 subtypes and recombinants, particularly in virus transmission and disease progression.

5. Conclusions

This is one of the largest studies analyzing LTR transcriptional activities in a wide spectrum of HIV-1 variants. Some of the genetic changes found at different LTR regulatory regions in the distinct HIV-1 variants represent subtype-specific sequence patterns presented in all analyzed samples.

Despite showing a high LTR genetic variability, the functional LTR activity in response to Tat protein and cell activation was similar across all HIV-1 analyzed subtypes and CRF. Furthermore, we observed a synergistic effect between the Tat viral protein and cellular activation.

The response to subtype B Tat *trans-activator* protein was similar in all HIV-1 tested subtypes and CRF, reflecting that Tat subtype B is functional with all analyzed LTR HIV-1 variants.

Changes of nucleotide G to A at some important transcription binding-factor sites in the LTR compromise the activity of the viral promoter, and could decrease the transcription of HIV-1 genes.

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