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# Alterations in HIV-1 LTR promoter activity during AIDS progression

Kirsten Hiebenthal-Millow,[a] Thomas C. Greenough,[b] Doreen B. Bretttler,[c] Michael Schindler,[d] Steffen Wildum,[d] John L. Sullivan,[b] and Frank Kirchhoff[d,*]

[a] *Institute for Clinical and Molecular Virology, University of Erlangen-Nürnberg, Schlossgarten 4, 91054 Erlangen, Germany*
[b] *Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA*
[c] *New England Area Comprehensive Hemophilia Center, University of Massachusetts Memorial Health Care, Worcester, MA 01605, USA*
[d] *Department of Virology–Universitätsklinikum, Albert-Einstein-Allee 11, 89081 Ulm, Germany*

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**Abstract**

HIV-1 variants evolving in AIDS patients frequently show increased replicative capacity compared to those present during early asymptomatic infection. It is known that late stage HIV-1 variants often show an expanded coreceptor tropism and altered Nef function. In the present study we investigated whether enhanced HIV-1 LTR promoter activity might also evolve during disease progression. Our results demonstrate increased LTR promoter activity after AIDS progression in 3 of 12 HIV-1-infected individuals studied. Further analysis revealed that multiple alterations in the U3 core-enhancer and in the transactivation-response (TAR) region seem to be responsible for the enhanced functional activity. Our findings show that in a subset of HIV-1-infected individuals enhanced LTR transcription contributes to the increased replicative potential of late stage virus isolates and might accelerate disease progression.


## Introduction

Variants of HIV-1 and SIV evolving during or after progression to AIDS show genotypic and phenotypic differences from early stage virus isolates. These changes seem to be associated with increased virulence. It has been reported that HIV-1 variants with enhanced replicative capacity emerge before disease progression (Connor and Ho, 1994). AIDS develops faster in infants born to mothers with low $CD4^+$ T-cell counts at the time of delivery (Tovo et al., 1994). Experiments in the SIV-macaque model confirmed that late virus variants show increased pathogenicity (Kimata et al., 1999). It is known that an expanded coreceptor tropism (Van't Wout et al., 1998; Xiao et al., 1998) and functional changes in the *nef* gene contribute to the pathogenic properties of late stage HIV and SIV variants (Carl et al., 2001; Patel et al., 2002). However, it is currently unclear whether alterations in the transcriptional activity of the HIV-1 promoter, located in the 5′ end of the long terminal repeats (LTR), are also selected during disease progression.

The HIV-1 LTR is approximately 640 bp in length and segmented into the U3, R, and U5 regions. The U3 region is further subdivided into the modulatory region, the enhancer element comprising two NF-κB-binding sites and the core region with the TATAA box and three GC-rich binding sites for Spl transcriptions factors (Gaynor, 1992). The R region contains the 59 nucleotide *trans*-activation-responsive (TAR) element forming a highly stable stem-loop RNA structure (Feng and Holland, 1988). The TAR element is important for Tat activation, efficient reverse transcription, and efficient packaging of viral genomic RNA (Feng and Holland, 1988; Harrich et al., 2000; Helga-Maria et al., 1999).

The transcriptional activity of naturally occuring promoter/TAR variants varies considerably (Delassus et al., 1992; Michael et al., 1994; Estable et al., 1996; Kirchhoff et al., 1997). A study of one single patient did not give indications for LTR alleles that were transcriptionally more active during disease progression (Delassus et al., 1991). Nevertheless, it remained unclear whether changes in tran-

* Corresponding author. Fax: +49-731-50023337.
*E-mail address:* frank.kirchhoff@medizin.uni-ulm.de (F. Kirchhoff).

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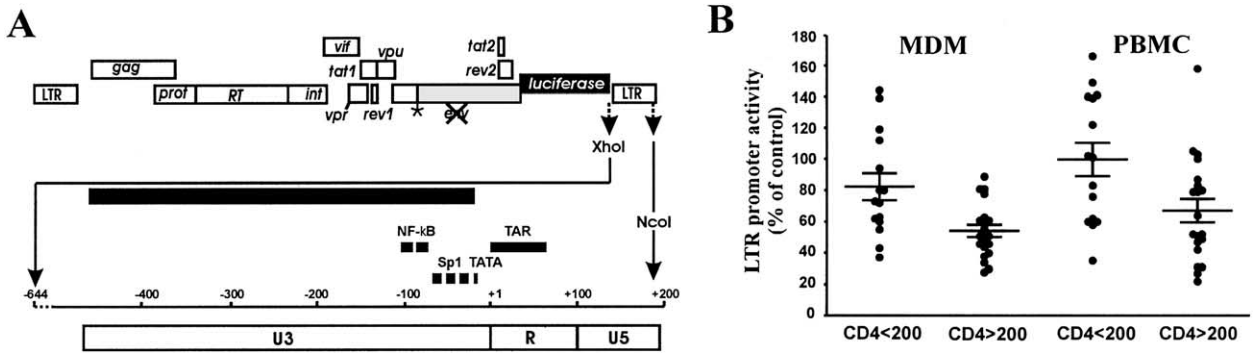



Fig. 1. Schematic presentation and transcriptional activity of proviral HIV-1 NL4-3 LTR variants. (A) The proviral NL4-3 reporter construct carries a frame-shift mutation in the *env* open-reading frame as indicated by the asterisk and the *nef* gene is replaced by the luciferase gene (upper). Mutated LTRs were inserted at the 3′ end of the proviral genome using the *Xho*I and *Nco*I restriction sites indicated. The deletion in NL43(e-n-L+)Δ3LTR is marked by a black bar. The NF-κB and Sp1-binding sites, the TATA box, and the TAR region are shown. (B) The average promoter activities relative to the NL4-3 wt LTR (100%) were grouped according to the $CD4^+$ T-cell count at the time of PBMC sampling (Table 1) and analyzed as described in the methods section. Indicated are mean values and the standard error of the mean.

scriptional activity are selected during AIDS progression, because either small numbers of patients were analyzed or no sequential samples were available. Furthermore, most data were obtained in immortalized cell lines transfected with LTR reporter constructs and might not accurately reflect the situation in HIV-1-infected cells.

We analyzed the promoter activity of LTRs amplified directly from peripheral blood mononuclear cells (PBMC) derived from HIV-1-infected individuals prior to, during, and after AIDS progression. We demonstrate that in some patients transcriptional HIV-1 LTR activity is increased after AIDS progression. Accordingly, emerging LTR variants with enhanced transcriptional and replicative capacity might drive disease progression in a subset of HIV-1 infected individuals.

## Results

Alterations in LTR promoter activity during the course of HIV-1 infection were investigated in the context of the integrated provirus. We tried to ensure that the LTRs analyzed were representative for each patient and time point:(1) DNA was isolated directly from patient PBMC samples without any cell culture passage; (2) a relatively high quantity of template DNA containing an average number of 130 proviral copies was used in a nested PCR approach to ensure that multiple proviral LTR sequences are amplified, and (3) mixtures of primary LTRs were inserted as a pool at the 3′ end of the pNL4-3.Luc.$R^-E^-$ clone (Fig. 1A). Control experiments demonstrated that ≥95% of the proviral constructs contained the correct inserts and that each plasmid preparation represented at least ≥100 independent transformants (not shown).

Jurkat and U937 cells as well as primary human PBMC and MDM were transduced with the pseudotyped HIV-1 particles (Table 1). During reverse transcription the patient-derived 3′ promoter sequences are copied to both the 5′- and 3′-LTRs of the provirus allowing a readily quantitation of the activity of the primary LTRs by Luciferase assay. The Mann-Whitney test revealed a significant inverse relationship between $CD4^+$ T-cell counts and LTR activities in both PBMC ($P = 0.022$) and MDM ($P = 0.005$). To further elucidate whether enhanced LTR activity is associated with AIDS progression we categorized all patient samples into two groups defined by $CD4^+$ T-cell counts greater ($n = 20$) or smaller ($n = 15$) than 200/μl at the time of PBMC sampling. The transcriptional activity of late stage LTRs was significantly higher compared to those obtained earlier in infection in both PBMC (99.6 ± 10.6% vs 67.1 ± 7.5%; $P = 0.0147$; given are mean values ± standard error) and MDM (82.2 ± 8.5% vs 53.4 ± 3.9%; $P = 0.0021$) (Fig. 1B). This relationship was also observed in Jurkat and U937 cell lines, but differences were less pronounced (60.5 ± 8.4% vs 44.0 ± 3.7%; $P = 0.04$ and 48.3 ± 6.5% vs 36.1 ± 6.4%, $P = 0.118$, respectively). Notably, the activity of the primary LTR alleles was higher in primary cells than in the transformed cell lines (PBMC, 81.0 ± 39.9% compared to 51.1 ± 25.6% in Jurkat T cells; $P = 0.0005$; and MDM, 65.7 ± 28.8 compared to 41.3 ± 22.7 in U937 cells; $P = 0.0001$; $n = 35$; given are mean values ± standard deviation).

Thus, in primary cells the activity of LTRs obtained after progression to AIDS was significantly higher than of those obtained earlier in infection. However, this difference was mainly due to strongly increased activity of LTR alleles derived from individuals SP13, P7, and P10 (Table 1). LTR alleles obtained from SP13 showed a slight increase in activity from the early to the intermediate stage of infection and a strong increase after AIDS progression. These differences were stronger in PBMC (5.5-fold) and Jurkat cells (4-fold) than in MDM (3-fold) or U937 cells (2-fold). In

Table 1
Promoter activities of LTR alleles obtained at different stages of HIV-1 infection

| Patient | Sampling[a] | Age[b] | $CD4^+$ counts | | LTR promoter activity[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | | | No./$mm^3$ | % | Jurkat | U937 | PBMC | MDM |
| SP8 | 1984 | 12 | 616 | 27 | 81 ± 15 | 73 ± 2 | 105 ± 14 | 77 ± 9 |
| | 1987 | 14 | 474 | 29 | 50 ± 8 | 50 ± 7 | 79 ± 6 | 50 ± 11 |
| | 1996 | 24 | 39 | 4 | 70 ± 12 | 80 ± 3 | 101 ± 12 | 43 ± 9 |
| SP13 | 1984 | 6 | 1622 | 53 | 39 ± 4 | 46 ± 7 | 22 ± 8 | 43 ± 6 |
| | 1990 | 11 | 722 | 29 | 58 ± 5 | 73 ± 4 | 42 ± 3 | 33 ± 3 |
| | 1993 | 14 | 66 | 6 | 155 ± 18 | 103 ± 1 | 122 ± 15 | 139 ± 21 |
| P5 | 1983 | 11 | 616 | 25 | 61 ± 10 | 28 ± 6 | 100 ± 34 | 60 ± 5 |
| | 1991 | 18 | 121 | 8 | 82 ± 6 | 38 ± 1 | 149 ± 21 | 119 ± 13 |
| | 1995 | 22 | 11 | 1 | 75 ± 9 | 26 ± 3 | 141 ± 19 | 94 ± 22 |
| P7 | 1982 | 8 | 1000 | 48 | 16 ± 2 | 15 ± 1 | 47 ± 4 | 27 ± 3 |
| | 1985 | 11 | 822 | 33 | 43 ± 4 | 33 ± 3 | 87 ± 7 | 55 ± 14 |
| | 1993 | 18 | 7 | 1 | 56 ± 1 | 48 ± 2 | 140 ± 15 | 73 ± 24 |
| P8 | 1983 | 43 | 526 | 26 | 26 ± 3 | 21 ± 2 | 103 ± 7 | 62 ± 4 |
| | 1987 | 47 | 126 | 6 | 50 ± 2 | 71 ± 3 | 102 ± 15 | 80 ± 12 |
| | 1989 | 49 | 12 | 1 | 35 ± 2 | 45 ± 5 | 139 ± 5 | 80 ± 3 |
| P9 | 1984 | 45 | 636 | 37 | 64 ± 8 | 69 ± 8 | 158 ± 13 | 80 ± 12 |
| | 1989 | 50 | 118 | 12 | 69 ± 16 | 80 ± 5 | 166 ± 29 | 112 ± 7 |
| | 1998 | 58 | 95 | 9 | 59 ± 4 | 58 ± 5 | 83 ± 7 | 144 ± 7 |
| P10 | 1984 | 3 | 1021 | 40 | 36 ± 4 | 17 ± 3 | 31 ± 7 | 45 ± 4 |
| | 1986 | 4 | 1357 | 34 | 26 ± 4 | 14 ± 3 | 27 ± 3 | 29 ± 3 |
| | 1996 | 14 | 9 | 1 | 91 ± 9 | 45 ± 8 | 76 ± 8 | 62 ± 4 |
| P13 | 1985 | 9 | 598 | 16 | 44 ± 6 | 21 ± 2 | 79 ± 16 | 45 ± 6 |
| | 1991 | 15 | 222 | 6 | 49 ± 3 | 34 ± 2 | 64 ± 5 | 39 ± 2 |
| | 1995 | 20 | 11 | 2 | 35 ± 2 | 27 ± 2 | 58 ± 3 | 37 ± 6 |
| P15 | 1984 | 27 | 657 | 36 | 19 ± 3 | 18 ± 2 | 49 ± 4 | 50 ± 4 |
| | 1986 | 29 | 591 | 37 | 22 ± 1 | 63 ± 12 | 52 ± 4 | 60 ± 7 |
| | 1990 | 33 | 59 | 3 | 20 ± 1 | 38 ± 2 | 60 ± 6 | 63 ± 5 |
| P16 | 1985 | 22 | 771 | 27 | 51 ± 3 | 29 ± 5 | 52 ± 4 | 80 ± 15 |
| | 1987 | 24 | 384 | 28 | 41 ± 1 | 29 ± 4 | 31 ± 5 | 88 ± 12 |
| | 1990 | 27 | 89 | 2 | 45 ± 3 | 22 ± 4 | 35 ± 1 | 72 ± 15 |
| P17 | 1984 | 18 | 666 | 28 | 55 ± 5 | 37 ± 4 | 51 ± 6 | 37 ± 3 |
| | 1989 | 23 | 15 | 4 | 51 ± 5 | 20 ± 2 | 60 ± 7 | 55 ± 21 |
| P18 | 1984 | 16 | 1017 | 23 | 46 ± 9 | 29 ± 2 | 80 ± 12 | 48 ± 6 |
| | 1986 | 17 | 456 | 10 | 52 ± 8 | 23 ± 4 | 83 ± 15 | 59 ± 4 |
| | 1988 | 19 | 15 | 1 | 35 ± 4 | 23 ± 1 | 62 ± 2 | 60 ± 8 |

[a] Year of PBMC sampling for PCR analysis.
[b] Age of the HIV-1-infected individuals at the date of PBMC sampling.
[c] Average values and standard deviations for transcriptional activity. Values are given relative to the NL4-3 activity and were determined as described under Materials and Methods. Results were obtained from triplicate transductions and were confirmed in two to four independent experiments. The activity of the NL43(e-n-L+)Δ3LTR control control construct was consistently < 1% of the wild type NL4-3 activity.

comparison, LTRs derived from subject P7 in 1993 were generally about 3-fold more active than those derived from the 1982 PBMC sample. The first two PBMC samples analyzed from P10 were obtained when the $CD4^+$ T-cell counts were high and the LTR alleles showed low activity. In contrast, LTR sequences derived from a sample drawn in 1996 after AIDS progression were about 3-fold more active. These results demonstrate strongly enhanced transcriptional LTR activity at late stages in these three HIV-1-infected individuals.

Next, we generated NL4-3 LTR variants containing an intact *env* gene to analyze whether the increased LTR activity is associated with enhanced replication. NL4-3 recombinants containing late stage LTRs replicated with enhanced efficiency in human PBMC (Figs. 2A–C). These differences in replication efficiency were consistently observed in four independent experiments performed in PBMC derived from different blood donors. Notably, we did not observe significant differences in the efficiency of viral replication with NL4-3 variants containing sequential LTR alleles derived from individuals P13, P15, and P18, which did not show increased transcriptional activity after AIDS progression (Figs. 2D–E). Thus, the increased transcriptional LTR activity observed in some HIV-1-infected individuals after progression to AIDS is associated with enhanced replicative capacity.

We also investigated which sequence alterations might be responsible for the increase in functional LTR activity. In all three individuals substantial differences were observed between the early and late stage LTR sequences (Fig. 3).

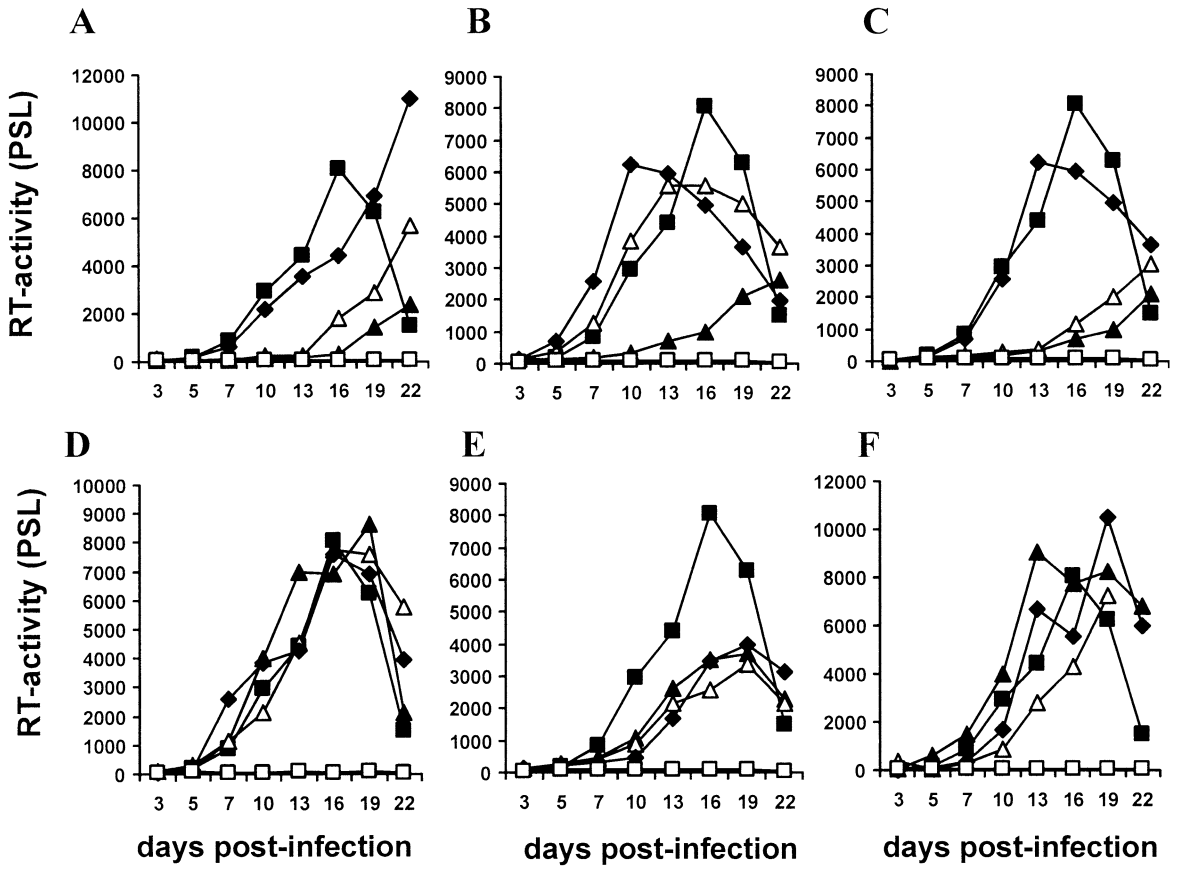
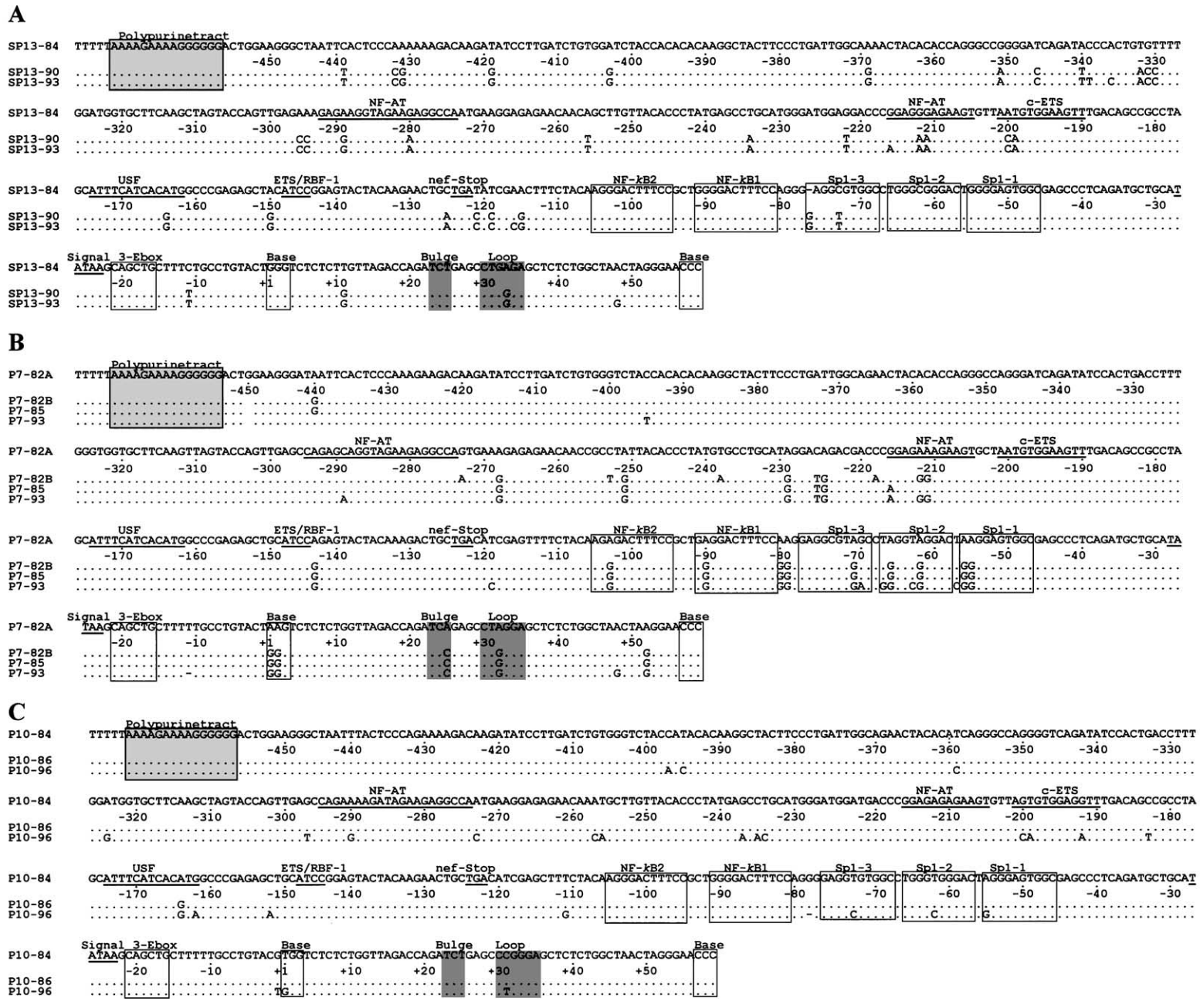
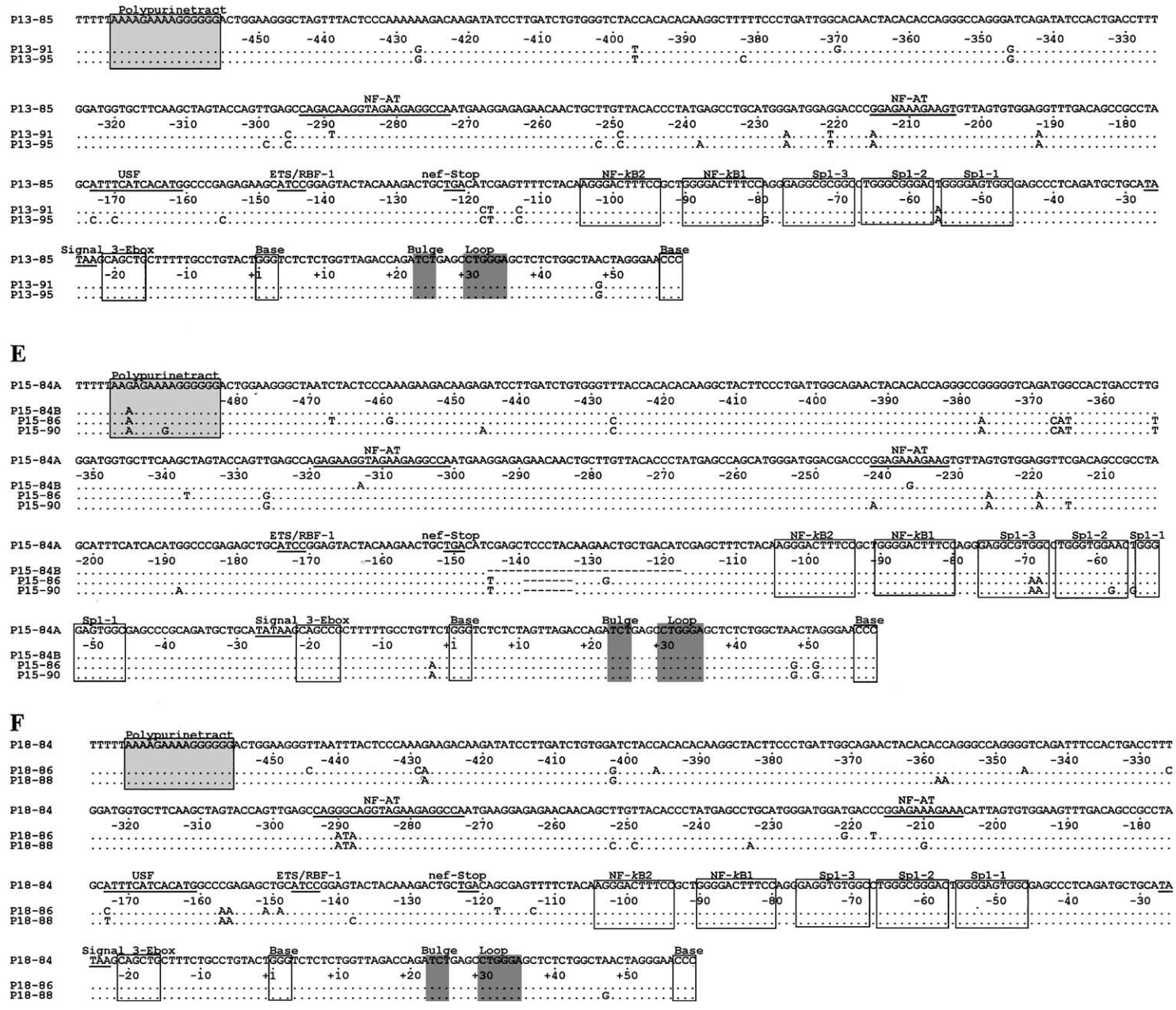



Fig. 2. Replication of sequential HIV-1 LTR variants in PBMC. Cells were infected with HIV-1 NL4-3 variants containing primary LTRs derived from subject SP13 (A), P7 (B), P10 (C), P13 (D), P15 (E), and P18 (F). PBMC were infected with virus stocks containing 100 ng of p24 antigen immediately after isolation and stimulated 3 days later. Viral replication was determined by reverse-transcriptase assay. Shown are the results of one representative experiment out of four performed. Abbreviations: RT, reverse transcription; PSL, photon-stimulated luminescence. Symbols: ■, NL4-3 wt; △, early; ▽, intermediate; ▲, late LTR alleles; and □, uninfected cells.

The consensus 84 and 93 SP13 LTR alleles differed by a total of 39 nucleotide substitutions (Fig. 3A). Most alterations were located in the upstream modulatory U3 region and affected the potential NF-AT, C-ETS, and USF-binding sites. Furthermore, late SP13 LTRs contained changes in the Sp1-3 site as well as in the TAR element. Two groups of sequences were obtained from the P7-82 PBMC samples. Three of eight LTR alleles (consensus designated as P7–82B) showed high sequence homology to the later stage LTRs. The remaining five clones (P7–82A) differed by at least 32 nucleotide substitutions from the P7–93 consensus sequence and contained two G→A nucleotide substitutions at the 5′ base of the TAR region, a single mutation in the TAR hexanucleotide loop, and numerous substitutions in the NF-kB and SpI-binding sites resulting in a substantially higher GC content (Fig. 3B). Little sequence variation was observed between LTRs derived from P10 during asymptomatic infection in 84 and 86 (Fig. 3C). However, the P10–96 LTR sequences differed by approximately 26 nucleotide substitutions from the early sequences and contained several alterations increasing the GC content in the SpI sites. Thus, multiple changes in the U3 and R regions of the LTR, particularly in the core enhancer-promoter and TAR elements, were selected during the course of infection in subjects SP13, P7, and P10. For comparison, we also investigated LTR sequences derived from P13, P15, and P18, which did not show increased LTR activity after progression to AIDS. Notably, late stage LTR sequences de-

Fig. 3. Alignment of sequential LTR promoter/TAR consensus sequences. For clarity only consensus LTR sequences obtained from the sequential SP13 (A), P7 (B), P10 (C), P13 (D), P15 (E), and P18 (F) PBMC samples are shown. Each consensus sequence was derived from five to eight individual clones analyzed. Sequences are aligned with the LTR sequence derived from the early stage PBMC sample. Two different groups of sequences were obtained from the P7–82 PBMC samples; five represented P7–82A and three P7–82B LTR alleles. Dots indicate sequence identity and dashes nucleotide deletions. The NF-κB-binding motifs, the Sp1 sites, and the TATAA sequence are underlined and shaded. The positions of the start of transcription, the TAR core region, and the TAR bulge and loop sequences are also indicated.


A
Polypurinetract
SP13-84
SP13-90
SP13-93
NF-AT
NF-AT
c-ETS
USF
ETS/RBF-1
nef-Stop
NF-kB2
NF-kB1
Sp1-3
Sp1-2
Sp1-1
Signal 3-Ebox
Base
Bulge
Loop
Base
B
Polypurinetract
P7-82A
P7-82B
P7-85
P7-93
NF-AT
NF-AT
c-ETS
USF
ETS/RBF-1
nef-Stop
NF-kB2
NF-kB1
Sp1-3
Sp1-2
Sp1-1
Signal 3-Ebox
Base
Bulge
Loop
Base
C
Polypurinetract
P10-84
P10-86
P10-96
NF-AT
NF-AT
c-ETS
USF
ETS/RBF-1
nef-Stop
NF-kB2
NF-kB1
Sp1-3
Sp1-2
Sp1-1
Signal 3-Ebox
Base
Bulge
Loop
Base


D
Polypurinetract
NF-AT
NF-AT
USF
ETS/RBF-1
nef-Stop
NF-kB2
NF-kB1
Sp1-3
Sp1-2
Sp1-1
Signal 3-Ebox
Base
Bulge
Loop
Base
E
Polypurinetract
NF-AT
NF-AT
ETS/RBF-1
nef-Stop
NF-kB2
NF-kB1
Sp1-3
Sp1-2
Sp1-1
Sp1-1
Signal 3-Ebox
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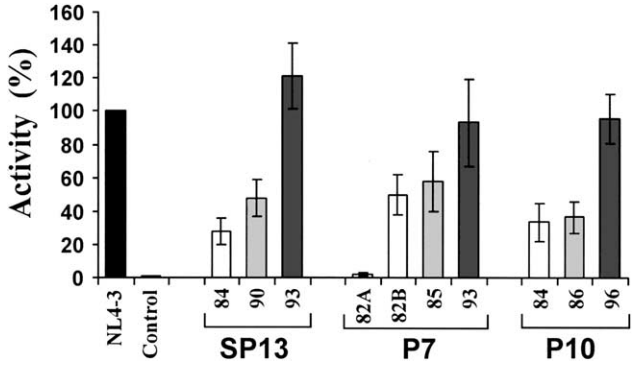


Fig. 4. Promoter activity of individual time-point representative HIV-1 LTRs. PBMC were transduced with the pseudotyped luciferase reporter viruses. Promoter activities together with standard deviation are shown relative to the NL4-3 wt LTR activity (100%). Values were derived from triplicate transductions with each individual LTR allele. The sequences of the LTR variants tested are shown in Fig. 3. Similar results were obtained in one independent experiment and in Jurkat or U937 cells. Control, cells were transduced with the NL43(e-n-L+)Δ3LTR construct.

rived from these individuals did not contain changes increasing the GC content of the Sp1-binding sites or alterations in the base, bulge, and the hexanucleotide loop sequence of the TAR region compared to the early LTR alleles (Figs. 3D–F). Finally, we analyzed the activity of time-point representative individual LTR alleles. Consistent with the previous results (Table 1), early stage LTRs alleles showed lower functional activity than the late stage LTRs (Fig. 4). Concordant with the sequencing data the P7–82A LTRs containing mutations at the base of the TAR element were severely impaired in transcriptional activity, whereas the P7–82B LTRs were functionally active (Fig. 4). Thus, the low average activity of the early P7–82 LTR alleles is due to a high proportion of clones containing attenuating mutations.

## Discussion

A variety of viral factors, e.g., escape from neutralizing Ab and CTL responses, antiviral drug resistance, an expanded cell, and coreceptor tropism or altered Nef function, might mediate a selective advantage allowing a certain HIV-1 quasispecies to become predominant in vivo. Obviously, these variations in biological viral properties can be selected individually or in various combinations. Suboptimal LTRs might become predominant if the viral genome contains mutations elsewhere resulting in a stronger growth advantage. Therefore, it is expected that LTR activities were only increased at late stages of infection in a minority of HIV-1 infected individuals. Many different viral and host factors need to be analyzed to better understand the complex interplay between HIV-1 and its host. This study demonstrates that in some HIV-1-infected individuals alterations in the LTR contribute to the increased replicative capacity of late stage virus isolates. One possible reason for their selection is that with a loss of immune surveillance, viral antigens expressed in cells would no longer label a cell for lysis. Therefore, unrestrained transcription may no longer carry a selective disadvantage.

We analyzed proviral DNA sequences rather than genomic RNA sequences because of the availability of samples. A possible disadvantage is that archival LTR sequences from both intact and defective proviruses are amplified. However, all samples were obtained from progressing individuals with ongoing viral replication and the half-life time of infected PBMC in the periphery should be only about 2 days (Perelson et al., 1996). One important question is whether the proviral LTR sequences analyzed are representative for each patient and time point. Clearly, the possibility that relevant sequence variants are missed cannot be excluded in this type of analysis. However, we feel that most likely the LTRs analyzed in our study were representative because PCR amplification was performed using high quantities of DNA derived directly from patient PBMC samples and complex pools of primary LTRs were inserted into the proviral constructs. Quantitative analysis of eight template DNA samples revealed an average number of 259 proviral copies per microgram DNA. Furthermore, independent PCR amplifications from the same DNA samples yielded LTR sequences that were both patient and time-point specific (data not shown). Taken together, our findings indicate that the LTRs analyzed are representative for the majority of integrated HIV-1 promoters present in the PBMC samples investigated.

Several patients were also analyzed for changes in Nef and Env function (summarized in Table 2). Late stage HIV-1 variants detected in individuals SP13 and P10, in whom LTR activity was strongly increased after AIDS progression, also showed an expanded coreceptor tropism. In comparison, X4- or dual-tropic variants were only detected at late stages in one of six HIV-1-infected individuals tested, who did not show an increase in transcriptional LTR activity. Simm et al. (1996) found that LTRs from X4- and R5-tropic HIV-1 isolates do not differ in transcriptional activity. However, only one AIDS patient and two asymptomatic individuals were analyzed. Thus, it needs further investigation to clarify whether the emergence of rapidly replicating cytopathic X4- or dual-tropic HIV-1 isolates frequently coincides with increased transcriptional LTR activity. Perhaps an altered tropism may necessitate modulation of LTR activity as different cell types are infected. In subjects P7 and P10 late stage *nef* alleles showed a decrease in class I MHC-I down-regulation and an increased ability to down-modulate CD4 and to stimulate viral replication (Carl et al., 2001). Thus, in some HIV-1-infected individuals, e.g., P10, changes in Env, Nef, and LTR function contribute simultaneously to the virulence of late stage HIV-1 variants.

Our data suggest that in subjects SP13, P7, and P10 at least three different mechanisms might contribute to the increased activity of LTRs obtained after progression to

Table 2
Alterations in Env, Nef, and LTR function during the course of HIV-1 infection

| Sample | LTR activity[a] | Coreceptor tropism[b] | Nef function[c] | | | |
|---|---|---|---|---|---|---|
| | | | CD4 | Class I MHC | Infectivity | Replication |
| SP13–84 | 1.00 | R5 | n.d. | n.d. | n.d. | n.d. |
| SP13–90 | 1.91 | R5 | n.d. | n.d. | n.d. | n.d. |
| SP13–93 | 5.55 | R5X4 | n.d. | n.d. | n.d. | n.d. |
| P7–82 | 1.00 | R5 | 1.00 | 1.00 | 1.00 | 1.00 |
| P7–85 | 1.85 | R5 | 1.37 | 0.62 | 2.20 | 1.07 |
| P7–93 | 2.98 | R5 | 1.12 | 0.23 | 2.94 | 1.60 |
| P10–84 | 1.00 | n.d. | 1.00 | 1.00 | 1.00 | 1.00 |
| P10–86 | 0.89 | R5 | 1.03 | 2.68 | 1.22 | 3.65 |
| P10–96 | 2.45 | R5X4 | 4.74 | 0.16 | 1.41 | 6.95 |

[a] Values give promoter activity in PBMC relative to the activity of pooled LTR alleles derived from the early patient sample.
[b] Full-length patient-derived *env* genes were cloned into into the NL4-3 backbone and the coreceptor tropism was determined with X4.15 and R5.3 cells coexpressing CD4 and R5 or X4 as described elsewhere (Münch et al., 2002).
[c] The in vitro activities of these *nef* alleles have been described previously (Carl et al., 2001). The functional activity is shown relative to the early stage *nef* alleles.

AIDS. First, late stage LTRs frequently contained substitutions in the Sp1-binding sites increasing the GC content in the basal promoter region and presumably enhancing the affinity for the cellular transcription factor Sp1 (Jones et al., 1986). Second, early stage TAR sequences frequently contained mutations at the 5′ base of the TAR region impairing perfect pairing with the 3′ base. In contrast, all late LTR alleles contained perfectly matching 5′- and 3′-base TAR regions (Fig. 4). Thus, alterations at the base of the TAR element might attenuate LTR activation by Tat and the efficiency of reverse transcription. Third, several mutations in the hexanucleotide loop region of TAR, shown to decrease the level of cooperative Cyclin T1 and Tat binding (Dingwall et al., 1990; Richter et al., 2002), were exclusively observed in early stage LTR alleles. Further studies with larger numbers of samples are required to clarify whether some individuals are initially infected with HIV-1 variants containing suboptimal promoter/TAR regions or whether forms containing highly active LTR emerge during or after AIDS progression.

Relative to the wild-type NL4-3 LTR, the patient-derived LTRs showed significantly higher activity in human PBMC and MDM, compared to Jurkat and U937 cells, respectively. Furthermore, while a trend toward higher activity of late stage LTR alleles was also observed in immortalized cell lines, this was much more significant in primary cells. Thus, some properties of the patient-derived LTRs seem to be more relevant for efficient transcription in primary human cells than in immortalized cell lines.

Dissecting the relative importance of the various phenotypic changes occurring during the course of infection for HIV-1 virulence and hence disease progression will be a challenging task. In each case, enhanced transcriptional LTR activity would directly lead to increased virus production and should result in higher viral load and accelerate AIDS progression. The phenotypic changes observed in HIV-1 infection (Van't Wout et al., 1998; Xiao et al., 1998; Carl et al., 2001) and experimental studies in the SIV/macaque model (Kimata et al., 1999; Patel et al., 2002) strongly suggest that viruses frequently become more virulent during progression to AIDS. Nevertheless, there is no evidence for an overall increase in virulence in the HIV-1-infected population. It will be interesting to elucidate the different selective forces "counterselecting" the properties that make late stage isolates highly aggressive. A better knowledge of the complex interplay between the virus and the host might lead to new approaches allowing a delay or even prevention of disease progression.

## Materials and methods

### *Study population*

Samples were obtained from 12 individuals in the cohort monitored since 1983 at the New England Comprehensive Hemophilia Center at the UMass/Memorial Health Center, Worcester, Massachusetts (Table 1). These individuals were selected by a $CD4^+$ T-cell profile that showed an early rapid decline or a clear time point of inflection. Samples generally predated the use of highly active antiretroviral therapy. Plasma viral RNA levels were not available for early time points and are not included in the analysis. References to "early" and "late" disease pertain to sample time points when $CD4^+$ T-cell counts were higher than or less than 200/$\mu$l, respectively. All participants have given informed consent to these studies with the approval of the institutional review board on the conduct of research on human subjects at the UMass Medical School.

### *Viral load*

Proviral DNA copy numbers in patients followed by the Worcester cohort were estimated using a modification of the

Amplicor HIV-1 test system (Roche Diagnostics Systems, Inc, Branchburg, NJ) as described (Greenough et al., 1999). Quantitative analysis of viral RNA loads was performed using the Amplicor HIV-1 Monitor assay (Roche Diagnostics Systems) following the manufacturers instructions.

*HIV-1 LTR mutant construction*

PBMCs were isolated from patient blood samples by centrifugation through Ficoll-Histopaque density gradients. Genomic DNA was extracted by standard methods from freshly purified PBMC, or from samples continuously frozen at −70°C since collection. A nested PCR method was employed to amplify the LTR region using the outer primer set pF1 (5′-GCAGTAGCTGAGGGGACAGATAGG-3′) and K114 (5′-CATGCCATGGACTGCTAGAGATTTTC-CACACTG-3′) and the inner primer pair K116-nefXho (5′-GGTGGGAGCAGTATCTCGAGACC-3′) and K112 (5′-CATGCCATGGTCTGAGGGATCTCTAGTTACCA-GAGT-3′). The *XhoI* and *NcoI* sites (underlined) were used to replace the HIV-1 NL4-3 3-LTR with the patient-derived sequences (Fig. 1A). For each sample 0.5 μg of template DNA was used for amplification. The PCR products were inserted as a pool into the pNL4-3.Luc.$R^-E^-$ clone containing the luciferase reporter gene and a disrupted *env* gene (Connor et al., 1995). Cloning efficiencies were determined as described previously (Carl et al., 2001). Briefly, aliquots of the transformed supercompetent *E. coli* XL-2 (Stratagene) were plated on LB-Amp dishes to assess the transformation efficiency, and the remaining 90% of the transformed bacteria were used for direct inoculation of medium-scale plasmid preparations. The percentage of the plasmid population containing an HIV-1 *nef* insert was estimated by restriction and PCR analysis. NL43(e-n-L+)△3LTR missing the U3 sequences upstream of the TATA box served as negative control (Fig. 1A). Sequencing was performed with the PRISM kit (Perkin-Elmer, Foster City, CA) on an Applied Biosystem 373 DNA sequencer. Proviral constructs containing an intact *env* gene were generated by standard cloning techniques.

*Virus stocks*

Generation of virus stocks was performed as described (Hiebenthal-Millow and Kirchhoff, 2002). Briefly, for the generation of pseudotyped viral particles, 293T cells were cotransfected with the proviral constructs and a plasmid expressing the Env protein of the vesicular stomatitis virus (VSV-G). For the production of replication-competent virus, 293T cells were transfected with 10 μg of the full-length NL4-3 proviral constructs. Viral stocks were stored in aliquots at −80°C. The p24 antigen concentrations were quantitated by using an HIV-ELISA provided by the NIH AIDS Research and Reference Reagent Program (Rockville, MD).

*Cell culture*

293T cells were maintained in DMEM supplemented with 10% FCS. Jurkat and U937 cells were kept in RPMI 1640 with 10% FCS. PBMC were isolated using lymphocyte separation medium (Biochrom KG, Germany), stimulated 3 days with 4 μg/ml PHA and cultured in RPMI 1640 medium with 20% FCS and 50 U interleukin-2 (IL-2)/ml. For MDM isolation, cells were isolated from fresh blood as described above. Cells ($7.5 \times 10^5$) were seeded in 48-well dishes and cultured for 1 week in RPMI 1640 supplemented with 10% human serum (GibcoBRL, Carlsbad, CA), 50 U/ml GM-CSF (Boehringer Mannheim, Germany), and antibiotics to allow monocyte differentiation and adherence to the plates. Subsequently, nonadherent cells were removed by extensive washing with PBS.

*Reporter assays*

Jurkat and U937 cells were seeded at $1 \times 10^5$ in 24 well dishes, PBMC at $1 \times 10^6$, and MDM at $2 \times 10^5$ in 48 well dishes and transduced in triplicates with aliquots of virus stocks containing 600 ng p24 antigen in a total volume of 250 or 150 μl medium, respectively. At 4 h after transduction medium was added to a final volume of 1 ml or 500 μl, respectively. At 3 or 4 days after infection the cells were pelleted, washed once with phosphate-buffered saline, and resuspended in 50 μl lysis buffer (Promega, Madison, WI). Cell debris was removed by centrifugation and 5 μl of the supernatant was used to determine the total protein concentration with a commercial reagent (Bio-Rad Laboratories, CA). Luciferase assays were performed with the Promega assay system as recommended by the manufacturer.

*Viral replication*

PBMC were infected immediately after isolation with aliquots of viral stocks containing 100 ng p24 antigen. Three days postinfection cells were stimulated with PHA (4 μg/ml, Sigma) for 3 days, washed, and maintained in RPMI 1640 supplemented with 20% FCS and 50 U/ml IL-2. Cell culture supernatants were sampled at regular intervals and stored at −20°C. Virus production was measured by reverse-transcriptase assay as described elsewhere (Hiebenthal-Millow and Kirchhoff, 2002). At the end of culture PCR amplification and sequence analysis of the 5′-LTRs were performed to verify that the patient-derived LTR sequences were still present and that no recombination with wild-type NL4-3 LTR sequences occurred.

*Statistical methods*

The mean and median LTR activities for subgroups defined by $CD4^+$ T-cell numbers at the time of PBMC sampling were compared by using Student's *t* test with Welch's

correction. Similar results were obtained with the Mann-Whitney test. The software package StatView version 4.0 (Abacus Concepts, Berkeley, CA) was used for all calculations.

*GenBank accession numbers*

The LTR sequences analyzed in this study have been assigned Genbank Accession Nos. AY376247 to AY366266.

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