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

Received 2 July 2003; returned to author for revision 24 July 2003; accepted 26 August 2003

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.

© 2003 Elsevier Inc. All rights reserved.

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 transcription 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 occurring 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-
transcriptional 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'U6 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).
The 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).

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Samplinga</th>
<th>Ageb</th>
<th>CD4+ counts</th>
<th>LTR promoter activityc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No./mm³</td>
<td>%</td>
</tr>
<tr>
<td>SP8</td>
<td>1984</td>
<td>12</td>
<td>616</td>
<td>27</td>
</tr>
<tr>
<td>1987</td>
<td>14</td>
<td>474</td>
<td>29</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>1996</td>
<td>24</td>
<td>39</td>
<td>4</td>
<td>70 ± 12</td>
</tr>
<tr>
<td>SP13</td>
<td>1984</td>
<td>6</td>
<td>1622</td>
<td>53</td>
</tr>
<tr>
<td>1990</td>
<td>11</td>
<td>722</td>
<td>29</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>1993</td>
<td>14</td>
<td>66</td>
<td>6</td>
<td>155 ± 18</td>
</tr>
<tr>
<td>P5</td>
<td>1983</td>
<td>11</td>
<td>616</td>
<td>25</td>
</tr>
<tr>
<td>1991</td>
<td>18</td>
<td>121</td>
<td>8</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>1995</td>
<td>22</td>
<td>11</td>
<td>1</td>
<td>75 ± 9</td>
</tr>
<tr>
<td>P7</td>
<td>1982</td>
<td>8</td>
<td>1000</td>
<td>48</td>
</tr>
<tr>
<td>1985</td>
<td>11</td>
<td>822</td>
<td>33</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>1993</td>
<td>18</td>
<td>7</td>
<td>1</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>P8</td>
<td>1983</td>
<td>43</td>
<td>526</td>
<td>26</td>
</tr>
<tr>
<td>1997</td>
<td>47</td>
<td>126</td>
<td>6</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>1989</td>
<td>49</td>
<td>12</td>
<td>1</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>P9</td>
<td>1984</td>
<td>45</td>
<td>636</td>
<td>37</td>
</tr>
<tr>
<td>1989</td>
<td>50</td>
<td>118</td>
<td>12</td>
<td>69 ± 16</td>
</tr>
<tr>
<td>1998</td>
<td>58</td>
<td>95</td>
<td>9</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>P10</td>
<td>1984</td>
<td>3</td>
<td>1021</td>
<td>40</td>
</tr>
<tr>
<td>1986</td>
<td>4</td>
<td>1357</td>
<td>34</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>1996</td>
<td>14</td>
<td>9</td>
<td>1</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>P13</td>
<td>1985</td>
<td>9</td>
<td>598</td>
<td>16</td>
</tr>
<tr>
<td>1991</td>
<td>15</td>
<td>222</td>
<td>6</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>1995</td>
<td>20</td>
<td>11</td>
<td>2</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>P15</td>
<td>1984</td>
<td>27</td>
<td>657</td>
<td>36</td>
</tr>
<tr>
<td>1986</td>
<td>29</td>
<td>591</td>
<td>37</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>1990</td>
<td>33</td>
<td>59</td>
<td>3</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>P16</td>
<td>1985</td>
<td>22</td>
<td>771</td>
<td>27</td>
</tr>
<tr>
<td>1987</td>
<td>24</td>
<td>384</td>
<td>28</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>1990</td>
<td>27</td>
<td>89</td>
<td>2</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>P17</td>
<td>1984</td>
<td>18</td>
<td>666</td>
<td>28</td>
</tr>
<tr>
<td>1989</td>
<td>23</td>
<td>15</td>
<td>4</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>P18</td>
<td>1984</td>
<td>16</td>
<td>1017</td>
<td>23</td>
</tr>
<tr>
<td>1986</td>
<td>17</td>
<td>456</td>
<td>10</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>1988</td>
<td>19</td>
<td>15</td>
<td>1</td>
<td>35 ± 4</td>
</tr>
</tbody>
</table>

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.
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 Sp1-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 Sp1 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. 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.

derived 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, unrestricted 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
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 infection. 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/μl, 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′-GCAGTAGCTAGGGGCAGATAGG-3′) and K114 (5′-CATGCCATGGACTGCTAGAGATTTC-CACACTG-3′) and the inner primer pair K116-nefXho (5′-GGTGGGAGCAGTATCTCGAGACC-3′) and K112 (5′-CATGCGATGTCTGAGGATCTCTAGTTACCA-GAGT-3′). The XhoI and NcoI sites (underlined) were used to replace the HIV-1 NL4-3 3-LTR with the patient-derived GAGT-3

**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 10% FCS 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.

Acknowledgments
We thank Thomas Mertens for support and Ingrid Bennett for critical reading of the manuscript. We are also indebted to all the individuals who participated in the study. We thank Ann Forsberg and Pat Forand for their efforts in the clinic and the excellent technical support of laboratory members in the Division of Pediatric Immunology. This work was supported in part by NIH Grants HL42257 and AI39400, by the UMass CFAR Grant AI42845, and by grants from the Deutsche Forschungsgemeinschaft (DFG) and the Wilhelm-Sander-Stiftung. T.C.G. was also supported by NIH K08 Grant AI01382 and is currently supported by the Campbell Foundation.

References

Connor, R.I., Ho, D.D., 1994. Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. J. Virol. 68, 4400–4408.


Patel, P.G., Yu, M.T., Kimata, J., Biggins, J.E., Wilson, J.M., Kimita, J.T., 2002. Highly pathogenic simian immunodeficiency virus mne variants that emerge during the course of infection evolve enhanced infectivity and the ability to downregulate CD4 but not class I major histocompatibility complex antigens. J. Virol. 76, 6425–6434.


