ORIGINAL

Growth ability in simian cells of monkey cell-tropic HIV-1 is greatly affected by downstream region of the *vif* gene

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Abstract : To obtain monkey-tropic (mt) HIV-1 derivatives with distinct biological characteristics and to improve the viral growth property, we have generated several variants from a prototype mt HIV-1 designated NL-DT5R (X4-tropic). The prototype HIV-1 contains a portion of *gag* and entire *vif* genes from SIVmac in its genome. The two derivatives carrying 3' half-genomic region of the SF162 (R5-tropic) or 89.6 (dual-tropic) isolate displayed very retarded or no viral growth, respectively, in a simian cell line HSC-F. In contrast, the three clones containing a part of *env* gene (encoding the V1-V4 region) from SF162, YU-2 (R5-tropic) or 89.6 showed different growth kinetics in HSC-F cells, although they grew somewhat more poorly than the NL-DT5R. Comparison of various viral proteins potentially involved in the different biological properties has revealed that, while amino acid sequences of Tat, Rev, Vpr, Vpu and Nef are quite conserved among the clones, those in the surface (SU) region of Env are relatively heterologous. Our data described here have shown that the 3' half of viral genome other than *gag* and *vif* genes greatly affects the growth property of mt HIV-1 in simian cells. J. Med. Invest. 55 : 236-240, August, 2008

INTRODUCTION

The narrow host range of human immunodeficiency virus type 1 (HIV-1) has been a major impediment for establishing animal models for studies of viral replication and pathogenesis *in vivo* (1). To overcome this difficulty, we have recently generated an mt HIV-1 designated NL-DT5R (1-3). The parental clone of NL-DT5R contains a 21-nucleotide simian immunodeficiency virus from rhesus monkeys (SIVmac) Gag capsid (CA) element, cor-

responding to the HIV-1 cyclophilin A-binding loop, and the entire *vif* gene (2). Long-term passage of the virus in simian HSC-F cells has resulted in the acquisition of two non-synonymous changes in the *env* gene, which did confer an improved replication potential on the virus. A molecular clone thus obtained from the infected HSC-F cells was named NL-DT5R (2) and used for subsequent monkey infection experiments as a prototype mt HIV-1 (3). From the results of these experiments, we have learned that NL-DT5R virus grew more poorly both *in vitro* and *in vivo* than a standard SIV designated SIVmac239, which induces the AIDS in monkeys and is widely used for model studies of HIV-1/ AIDS. As an apparent result of its biological property, NL-DT5R was unable to induce AIDS in the animals (3). Moreover, NL-DT5R is tropic for cells

expressing the CXCR4 (X4) molecule but not for CCR5 (R5). It is well known that R5 viruses are clinically more important than X4 viruses (1). Taken together, new mt HIV-1s with R5 tropism and pathogenic potential are absolutely required to develop tractable animal models for AIDS research.

As a first step towards this purpose, we have generated five new mt HIV-1s by recombinant DNA techniques in this study. The proviral clones used here included R5-tropic NF462 (4), R5-tropic YU-2 (5) and dual-tropic 89.6 (6). The 3' half of NL-DT5R genome or a sequence within the *env* gene was replaced with the corresponding region of the other viral clones to obtain biologically distinct viruses. We demonstrate here that the regions other than Gag-CA and Vif certainly contain the determinants on accelerated viral growth and severe cytopathic effects in simian cells.

MATERIALS AND METHODS

Cells

A human monolayer cell line 293T (7) was maintained in Eagles's minimal essential medium containing 10% heat-inactivated fetal bovine serum. A simian lymphocytic cell line HSC-F (8) was maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum.

Transfection

Sub-confluent 293T cells in 90 mm dishes were transfected with 20 μg of proviral clones in Fig. 1 by the calcium-phosphate co-precipitation method as previously reported (9). On day 2 post-transfection, cell-free culture fluids were prepared for virus samples for infection experiments (9).

Infection

HSC-F cells (3×10^6) were infected with an equal amount of viruses (1 to 2×10^7 reverse transcriptase (RT) units) prepared from transfected 293T cells, and monitored for RT production at intervals as previously described (9). Infected HSC-F cells were cultured in the presence of recombinant human IL-2 (50 units/ml) during the observation period.

RT assay

Viral growth property was determined by monitoring RT activity of culture supernatants prepared from infected HSC-F cells. RT assay using ^{32}P-dTTP has been previously described (10).

DNA constructs

An mt infectious DNA clone of HIV-1 designated NL-DT5R has been previously described (2). Infectious DNA clones of HIV-1 designated NF462 (4), YU-2 (5) and 89.6 (6) have been previously described. Construction of proviral clones in Fig. 1 were carried out by routinely used recombinant DNA methods. Appropriate DNA fragments from NF462, YU-2 and 89.6, generated by digestion with the restriction enzymes in Fig. 1, were inserted into NL-DT5R to make new full-length clones.

Amino acid alignments

Amino acid sequences of various HIV-1 proteins were aligned by the GENETYX system (Version 7). GenBank accession nos. for NL4-3, NL-DT5R, SF 162, 89.6 and YU-2 are AF324493, AB266485, M
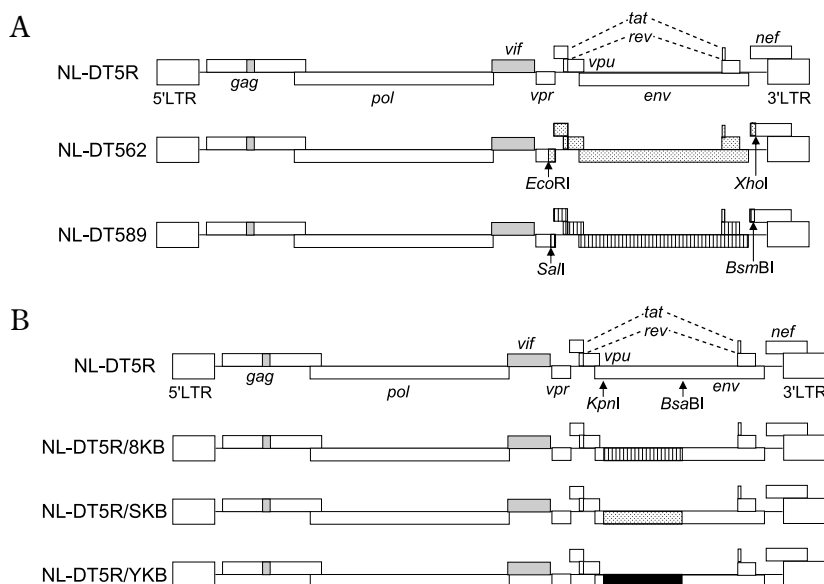

Fig. 1 Genome structure of various proviral clones used in this study. (A) Genomes of R5- and dual-tropic clones derived from NL-DT5R. The 3' half of the NL-DT5R genome was replaced with those of R5-tropic NF462 (4) and dual-tropic 89.6 (6) genomes at the sites indicated. White, grey, dotted and striped areas represent sequences from NL-DT5R (2), MA239 (SIVmac239) (13), NF462 and 89.6, respectively. LTR, long terminal repeat. (B) Genomes of *env* variants of NL-DT5R. Sequence within the *env* gene encompassing the V1-V4 region of NL-DT5R was substituted with those of 89.6, NF 462 and YU-2 (5) at the sites indicated. White, grey, striped, dotted and black areas represent sequences from NL-DT5R, MA239, 89.6, NF462 and YU-2, respectively. LTR, long terminal repeat.

65024, U39362 and M93258, respectively. NF462 clone carries the SF162 sequence in the backbone of NL4-3 genome (4).

RESULTS AND DISCUSSION

Our previous results have indicated that biologically significant mutations in the viral genome readily occur after long-term culture of infected cells (1, 2). Many of them were mapped to the *env* gene (2 ; our unpublished data). We, therefore, exchanged the 3' half of X4 tropic NL-DT5R genome with the corresponding regions of the other viral genomes to obtain biologically distinct and better-growing proviral clones (Fig. 1A). The exchanged sequences encompassing the *env* gene came from infectious molecular clones designated NF462 (4) and 89.6 (6), which are R5-tropic and dual-tropic, respectively. The replaced regions also contained *tat*, *rev*, *vpu*, and a part of *vpr* and *nef* genes (Fig. 1A).

To examine growth potentials in simian cells of new viral clones (NL-DT562 and NL-DT589 in Fig. 1A), they were transfected into 293T cells, and cell-free virus samples were prepared on day 2 post-transfection. Viruses obtained were then inoculated into HSC-F cells, and viral growth was monitored by RT assay. As shown in Fig. 2, the parental virus NL-DT5R readily established a spreading infection, which peaked on day 12 or 15 post-infection. In contrast, NL-DT562 grew much more poorly than NL-DT5R, and no virus growth was detected for NL-DT589. The infection experiments were repeated with similar results. Thus, it was clear that the 3' genomic region of the viruses harbors determinants on regulation of viral growth rate. We noticed that the slow-growing NL-DT562 is able to induce profound and severe cytopathic effects (mostly fusion-type) in HSC-F cells. Although NL-DT562 grew poorly, this property is quite evident and unique between the two growth-competent viruses.

In order to examine whether the growth property as described above is ascribed to the *env* gene, we next constructed three *env*-substitution variants as shown in Fig. 1B. In addition to the NF462 and 89.6 clones, another proviral clone YU-2, which is R5-tropic, was used to generate a variant. The substituted *env* sequence contained the regions of V1, V2, V3 and V4, which are important for X4/R5 tropism (V3 in particular). Cell-free virus samples derived from these proviral clones were prepared as described above, and inoculated into HSC-F cells. As
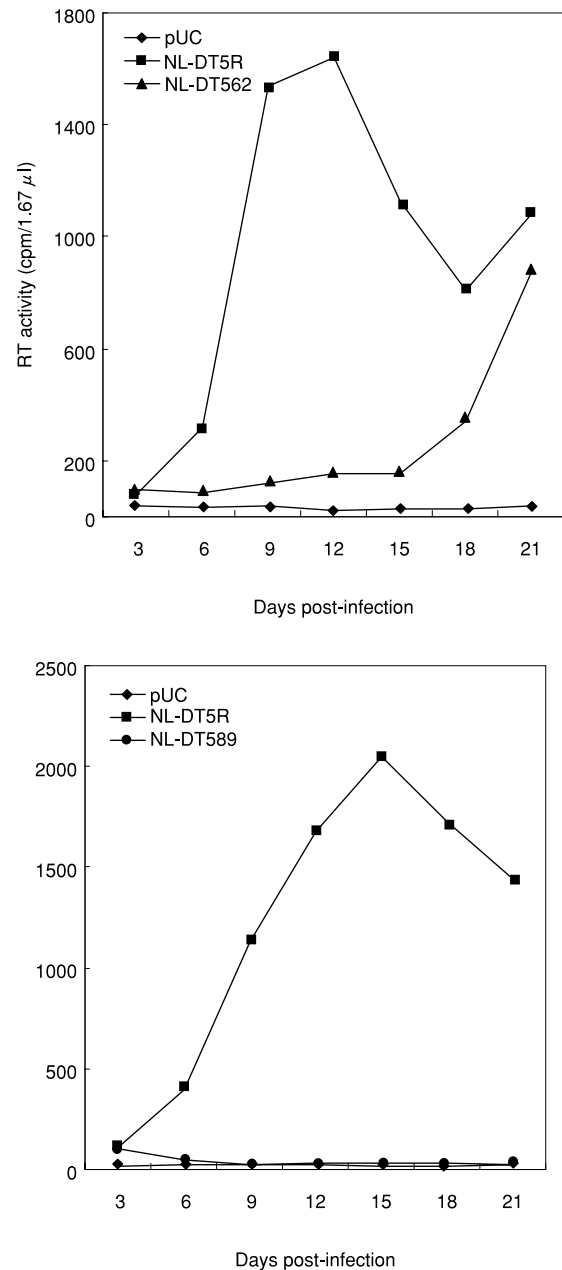

Fig. 2   Growth kinetics of various clones in simian HSC-F cells. Input cell-free viral samples were prepared from 293T cells transfected with the clones indicated, and an equivalent RT units were inoculated into HSC-F cells. Viral growth was monitored at intervals by RT activity in the culture supernatants. As a negative control, pUC19 was used.

shown in Fig. 3, while NL-DT5R grew best in the cells as above, all the other viruses did propagate. NL-DT5R/SKB virus consistently grew better than NL-DT5R/8KB and NL-DT5R/YKB in HSC-F cells. The data in Fig. 3 showed that the V1-V4 region (Fig. 1B) of *env* gene has in fact some sequences(s) controlling viral growth rate. However, when all the results in Figs. 2 and 3 were taken into consideration, it was concluded that region(s) other than V1-V4 affect very much the growth prop-
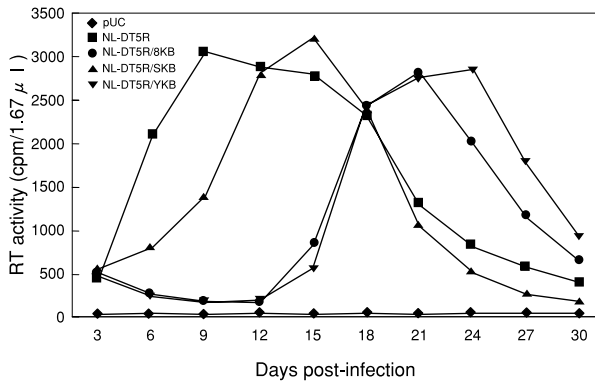
Fig. 3   Growth kinetics of various *env*-variants in simian HSC-F cells. Input cell-free viral samples were prepared from 293T cells transfected with the clones indicated, and an equivalent RT units were inoculated into HSC-F cells. Viral growth was monitored at intervals by RT activity in the culture supernatants. As a negative control, pUC19 was used.

Fig. 5   Amino acid alignments of accessory proteins Vpr, Vpu and Nef from NL-DT5R, -DT562 and -DT589. Identical amino acid residues are boxed. The sites used to construct NL-DT562 and and NL-DT589 are indicated (see Fig.1).

erty of mt HIV-1.

We were interested in evaluating how different the amino acid sequences of various viral proteins are among the clones used. Amino acid alignments were made, and sequences were compared for Tat and Rev proteins (Fig. 4), for Vpr, Vpu and Nef proteins (Fig. 5) and for Env protein (Fig. 6). As is clear in the figures, all the proteins compared are different, to various degrees, with respect to the primary amino acid sequence. However, sequences of Tat, Rev, Vpr, Vpu, and Nef are quite conserved, especially those of functionally important domains of Tat and Rev (11). In addition, the accessory proteins except for Vif are known to affect relatively slightly the viral replication *in vitro* (11 ; our unpublished results). Furthermore, a *vpu*-minus mutant of SIV/HIV-1 chimeric virus, in fact, grew similarly well with wild-type virus in the HSC-F cells (12). As for Env, sequences are quite different, the N-terminal region in particular. More importantly, mutations in the *env* gene occurred quite readily
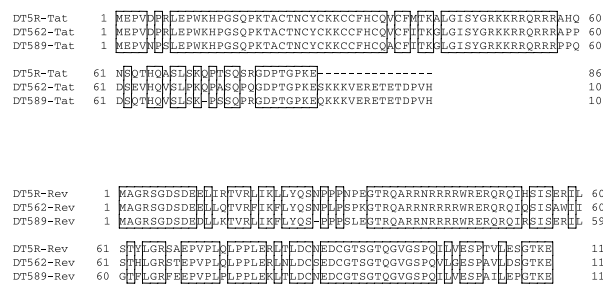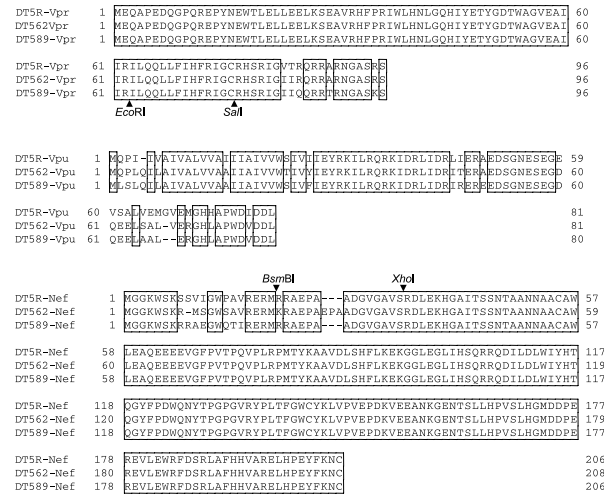
Fig. 4   Amino acid alignments of regulatory proteins Tat and Rev from NL-DT5R, -DT562 and -DT589. Identical amino acid residues are boxed.
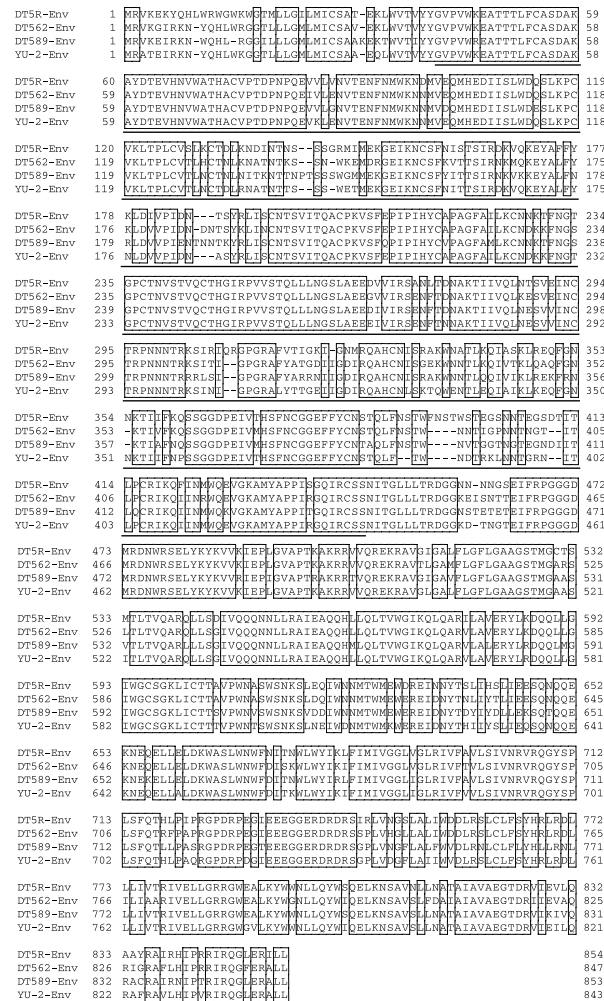
Fig. 6   Amino acid alignments of a structural protein Env from NL-DT5R, -DT562, -DT589 and YU-2. Identical amino acid residues are boxed. The substituted region is underlined (see Fig.1).

within cells, conferring augmented replicative properties on the virus (2, 10).

Taken all together, although it can not be excluded that some *cis*-acting elements in the viral genome may contribute to the growth potential of viruses, it is not unreasonable to assume that certain amino acid residues in Env are primarily important for the growth property of mt HIV-1 in simian cells. Consistent with this, we have readily found mutations in the *env* gene of cell-adapted mt HIV-1s (our unpublished observations). The identification of sequences in the *env* gene (and/or the other genes) responsible for altered virus growth phenotype, and the elucidation of the underlying mechanism need to be carried out.

ACKNOWLEDGMENTS

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