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Infectious Molecular Clone of a Recently Transmitted Pediatric Human Immunodeficiency Virus Clade C Isolate from Africa: Evidence of Intraclade Recombination

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Although human immunodeficiency virus type 1 (HIV-1) clade C continues to dominate the pandemic, only two infectious clade C proviral DNA clones have been described (N. Mochizuki, N. Otsuka, K. Matsuo, T. Shiino, A. Kojima, T. Kurata, K. Sakai, N. Yamamoto, S. Isomura, T. N. Dhole, Y. Takebe, M. Matsuda, and M. Tatsumi, AIDS Res. Hum. Retrovir. 15:1321–1324, 1999; T. Ndung’u, B. Renjifo, and M. Essex, J. Virol. 75:4964–4972, 2001). We have generated an infectious molecular clone of a pediatric clade C strain, HIV1084i, which was isolated from a Zambian infant infected either intrapartum or through breastfeeding. HIV1084i is an R5, non-syncytium-inducing isolate that bears all known clade C signatures; gag, pol, and env consistently mapped within clade C. Interestingly, gag resembled Asian isolates, whereas pol and env resembled African isolates, indicating that HIV1084i probably arose from an intraclade recombination. As a recently transmitted clade C strain, HIV1084i will be a useful vaccine development tool. 

Human immunodeficiency virus type 1 (HIV-1) genetic diversity is reflected by three groups (M, N, and O), at least nine group M clades, and 14 circulating recombinant forms (16). Given the high error rate of its reverse transcriptase and the potential for coinfecting clades to recombine, HIV has great potential for diversifying (18). Currently, clade C viruses account for 56% of all global HIV infections (2).

Rapidly expanding within regions with a high prevalence of HIV, such as sub-Saharan Africa, HIV clade C is considered to be a more virulent circulating form than other clades (2, 18). In geographic areas where HIV is predominantly clade C, coinfection of different clades is common (2). The potential for coinfecting clades to recombine, HIV has great diversity is reflected by three groups (M, N, and O), at least nine group M clades, and 14 circulating recombinant forms (16). Given the high error rate of its reverse transcriptase and the potential for coinfecting clades to recombine, HIV has great potential for diversifying (18). Currently, clade C viruses account for 56% of all global HIV infections (2).

To date, numerous HIV isolates have been cloned and sequenced (3, 7–9, 11–15, 17–19, 21, 22). Among these, only Indie-C1 (9) and MJ4 (12) are infectious clade C viruses that resembled clade B LTRs, which contain only two, a characteristic which was postulated to enhance clade C proviral transcriptional activation (10). Indeed, the level of NF-κB translocation induced by the 5′LTR through the vpr open reading frame was amplified by using Expand High Fidelity Taq polymerase (Roche, Alameda, Calif.) and the following Indie-C1-based primers: 5′-LTR-NotI (primer 1 in Fig. 1: 5′-AATGCCGGCCGCTGGA AGGTTAATTTACTCCAAGAAAAGGCAAG-3′) and 5′-reverse-AscI (primer 2 in Fig. 1: 5′-GTCATGAAACACTAT GGGCGGCTTGGACAGGAGTGC-3′) (Invitrogen, Carlsbad, Calif.). Similarly, a 4.3-kb fragment extending from the vpr open reading frame beyond the 5′ LTR was amplified using the following Indie-C1-based primers: 5′-forward-Ascl (primer 3 in Fig. 1: 5′-CGACTCTCTGCAGGCGCCACATATCT TCTATAGAC-3′) and 3′-LTR-NotI (primer 4 in Fig. 1: 5′- CGCGGGCCGACTGACAAAAGGGTGTCAGAGGAT CTCATAGTAC-3′) (Invitrogen). As indicated, NotI restriction sites were added upstream of the 5′ LTR and downstream
of the 3' LTR, while an Ascl restriction site was introduced in the vpr open reading frame using the following nucleotide changes: G5672C, A5674C, T5675G, and A5676C.

The amplicons were individually cloned into pCR 2.1-Topo TA cloning vectors (Invitrogen) and expanded through transformation of chemically competent Top 10 E. coli cells (Invitrogen). Plasmid DNA was extracted with the QIAprep Spin Miniprep kit (QIAGEN, Valencia, Calif.); full-length proviruses were reconstructed from the subgenomic segments. Briefly, all vectors were digested with XhoI and AscI restriction sites, and an AscI restriction site was introduced into primers 2 and 3, which spanned the vpr open reading frame. Subcloning the PCR product into pCR 2.1 Topo cloning vectors, followed by bacterial amplification, restriction endonuclease-mediated linearization, and subsequent ligation yielded the 14.7-kb proviral plasmid, HIV1084i.

The infectious molecular clone of HIV1084i was sequenced by using a primer walking method and more than 50 plndie-C1-derived primers. Individual contiguous stretches of proviral DNA were assembled using the DNASIS program. HIV1084i is 9,665 bp in length, and all reading frames for major and accessory genes are open. Both LTRs are flanked by NotI restriction sites, and vpr contains an AscI restriction site not found in plndie-C1. Although Vpr contained two nonconservative mutations (D52A and T53P), HIV1084i productively infected PBMCs from three independent donors.

To perform phylogenetic analysis, a multiple sequence alignment was carried out on gag, pol, and env and the expected Vpu and Rev sequences with Clustal X (version 1.81) (Fig. 3). Comparison of HIV1084i gag, pol, and env genes with those of other HIV isolates placed HIV1084i within the clade C lineage, despite having origins in Zambia, where the dominant circulating HIV forms include clades C, D, and G; group

Indie-C1 were added and incubated overnight at 37°C; controls included uninfected PBMCs cultured with AZT. The next day, cells were washed three times with medium and resuspended in RPMI medium supplemented with 15% FBS with or without 10 μM AZT. Supernatants were collected at regular intervals. Wells containing AZT did not produce p24. PBMCs from three independent donors supported replication of HIV1084i (Fig. 2 and data not shown), and HIV1084i env-specific primers were used to amplify a 700-bp fragment from genomic DNA of infected PBMCs (data not shown).

To determine coreceptor usage, the following U87.CD4 cells expressing one of the following chemokine coreceptors were used (1, 6): CCR1, CCR2b, CCR3, CXCR4, or CCR5, as well as Ghost.CD4 cells expressing the CCR5, BOB, or BONO coreceptors (National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, Md.). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and infected with HIV1084i viral stock in polybrene (Sigma). Supernatants were collected on days 3, 5, 7, and 10 for p24 Gag titration. HIV1084i replicated only in the U-87.CD4.CCR5 cells (p24 Gag levels, >1 ng/ml; data not shown).
O; and A/C and B/C recombinants (Fig. 3A to C) (4, 23). HIV1084i had no evidence of interclade recombination; however, HIV1084i pol and env clustered closely with AF110963, a Botswana isolate (Fig. 3B and C), while HIV1084i gag clustered with two Indian and two Chinese isolates (AF067254 and AB023804 and AF286229 and AF286230, respectively) (Fig. 3A). It is important to note that this differential clustering could not have resulted from our cloning strategy (Fig. 1), as the entire gag-pol region was contained within the 5′/H11032 half that was initially amplified en bloc using primers located within the 5′/H11032 LTR and in vpr at the AscI restriction site (nucleotides 5670 to 5671). Thus, the recombination breakpoint region within the gag-pol overlap region (nucleotides 2058 to 2253) was left untouched. We conclude that the differential clustering of gag and pol within HIV1084i probably resulted from an intraclade recombination event.

The predicted HIV1084i Rev and Vpu sequences revealed several clade C signature sequences. Vpu contained the ARVDY sequence, a 5-amino-acid (aa) extension upstream of the amino-terminal transmembrane domain (Fig. 3D). This extension was also present in Vpu of MJ4, a hybrid constructed from two distinct African clade C isolates; however, it was absent from Indie-C1 and the non-C isolates examined, as reported previously (19). Furthermore, the clade C-specific LRLL motif appeared upstream of the Vpu C terminus for HIV1084i, MJ4, and Indie-C1 but was absent from all other non-C infectious clones. Phylogenetic analysis placed HIV1084i Vpu into the clade C cluster as a branch off the MJ4 lineage (Fig. 3D).

Compared to the clade B reference, HBX-2R, the Rev aa sequences for HIV1084i, MJ4, and Indie-C1 contained premature stop codons, which shortened HIV1084i and MJ4 by 9 aa and Indie-C1 by 16 aa (Fig. 3E). Phylogenetic analysis of the HIV1084i Rev localized it within the clade C cluster, as a branch of the MJ4 lineage (Fig. 3E).

Next, we surveyed the number of NF-κB binding sites found in 16 distinct clade C LTRs. HIV1084i and eight other LTRs contained three NF-κB binding sites, two of which contained the sequence GGGACTTTCC, while the third site’s sequence was 93IN101 (AB023804).
was GGGGCCGTTC. The remaining seven LTRs, including that of MJ4 (12), displayed two of the three characteristic NF-κB binding sites with the sequence GGGACTTTCC.

In conclusion, we have constructed an infectious molecular clade C clone, HIV1084i, which was replication competent in PBMCs from three different donors, was exclusively R5 tropic, and did not induce syncytia. Isolated from a Zambian infant whose infection was first detected by PCR at 4 months of age, HIV1084i represents a recently transmitted virus. Consistent with recent transmission, many viral isolates recovered from the 1084 mother-infant pair had uncharacteristically close env sequence homology (25). As a recently transmitted virus, HIV1084i will be a useful tool for testing novel passive (24) and active vaccine strategies.

**Nucleotide sequence accession number.** The nucleotide sequence of HIV1084i is available through GenBank (no. AY805330).

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