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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 general, clade C long terminal repeats (LTRs) contain three NF-κB sites compared to clade B LTRs, which contain only two, a characteristic which was postulated to enhance clade C proviral transcriptional activation (10). Indeed, the level of tumor necrosis factor alpha stimulation correlated with the number of NF-κB sites, indicating some difference among HIV LTRs (5, 20).

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 use CCR5 as coreceptor. Indie-C1 is a primary Indian isolate (9), and MJ4 is a chimeric infectious clone, containing the 96MOLE1 envelope and the replication-incompetent backbone of 96BW06. 96MOLE1 and 96BW06 were originally isolated from anonymous infected donors in Botswana (12). The HIV disease stages of the source persons for both Indie-C1 and MJ4 are unknown (9, 12).

We constructed a replication-competent, infectious proviral DNA clone of a pediatric HIV clade C isolate, HIV1084i. This virus was recovered by cocultivation from a 4-month-old, HIV-positive Zambian infant whose umbilical cord blood had been HIV negative by PCR. HIV-negative donor peripheral blood mononuclear cells (PBMCs) were purified by using Lymphoprep (Life Technologies, Grand Island, N.Y.) and propagated in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 5 μg of phytohemagglutinin (PHA) (Sigma, St. Louis, Mo.)/ml for 40 h. Then, the infant's PBMCs were cocultured with an equal number of PHA-stimulated PBMCs from the seronegative donor to a combined final concentration of 2×10^6 cells/ml. Equal numbers of fresh uninfected PHA-stimulated PBMCs were added to the culture weekly. Virus production was monitored by measuring HIV-1 p24 antigen levels with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Beckman Coulter, Somerset, N.J.).

Genomic DNA from the cocultivated PBMCs served as template for the following PCRs. A 5.4-kb fragment extending from 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′-AATGCGGCCGCCTGGAAGGGTTAATTTACTCCAAGAAAAGGCAAG-3′) and 5′-reverse-AscI (primer 2 in Fig. 1: 5′-GTCTATGAAACATATGGCGCGCCTTGGACAGGAGTCG-3′) (Invitrogen, Carlsbad, Calif.). Similarly, a 4.3-kb fragment extending from the *vpr* open reading frame beyond the 3′ LTR was amplified using the following Indie-C1-based primers: 3′-forward-AscI (primer 3 in Fig. 1: 5′-CGACTCCTGTCCAAGGCGCGCCATATGTTTCATAGAC-3′) and 3′-LTR-NotI (primer 4 in Fig. 1: 5′-CGCGCGGCCGCACTGACTAAAAGGGTCTGAGGGATCTCTAGTTAC-3′) (Invitrogen). As indicated, NotI restriction sites were added upstream of the 5′ LTR and downstream

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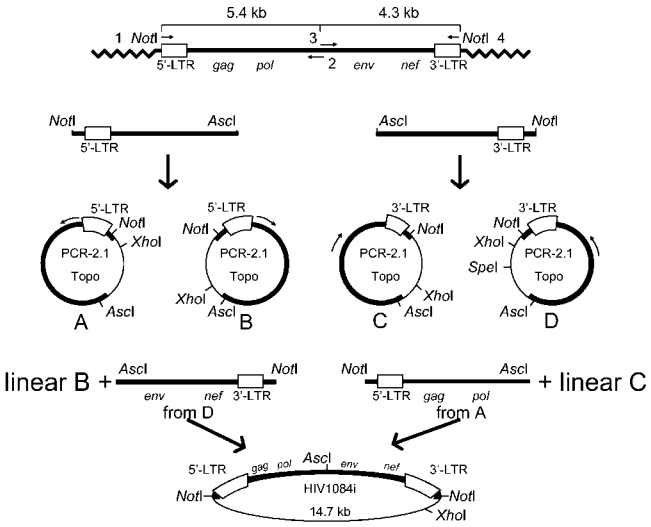

FIG. 1. Strategy for the cloning of HIV1084i. Full-length HIV1084i was constructed from two subgenomic amplicons containing NotI and AscI restriction sites at alternate ends of the molecule. NotI restriction sites were added to the LTR primers (1 and 4), while AscI restriction sites were introduced into 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.

of the 3′ LTR, while an AscI 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 *Escherichia 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 (New England Biolabs, Beverly, Mass.), and the 5′ and 3′ vectors (vectors B and C in Fig. 1) were subsequently treated with alkaline phosphatase. The 3′ insert (vector D in Fig. 1) was treated with SpeI, and the 5′ insert (vector A in Fig. 1) was left unmodified. Overnight ligation of gel-purified vectors A and C (or vectors D and B) (Fig. 1) with T4-DNA ligase was followed by transformation of chemically competent Top 10F′ *E. coli* cells (Invitrogen).

Next, 293T cells grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Sigma) were transfected with 6 μg of HIV1084i DNA by calcium phosphate precipitation (Promega, Madison, Wis.).

pIndie-C1 served as a positive control, and pIRES-hrGFP (Life Technologies) served as a negative control. Cell-free supernatants that were positive for p24 Gag ELISA (Beckman Coulter) 72 h posttransfection were used to infect human PBMCs. Supernatants were monitored every 3 days until day 15 for p24 Gag production; HIV1084i replication peaked on day 9 (data not shown).

Next, we assessed the sensitivity of HIV1084i to zidovudine (AZT). Half of the wells containing PBMCs were pretreated with 10 μM AZT (Sigma) for 30 min at 37°C. HIV1084i or 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 BONZO 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).

The infectious molecular clone of HIV1084i was sequenced by using a primer walking method and more than 50 pIndie-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 pIndie-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

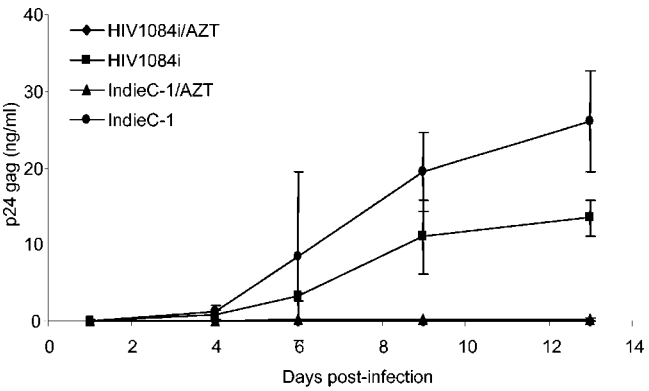

FIG. 2. Kinetics of replication of HIV1084i and Indie-C1 in PBMCs with or without AZT. PBMCs with or without 10 μM AZT were infected with excess HIV1084i or HIV Indie-C1. Supernatants were collected at various days postinfection and analyzed by p24 Gag ELISA. The figure depicts the average of results from two independent experiments.

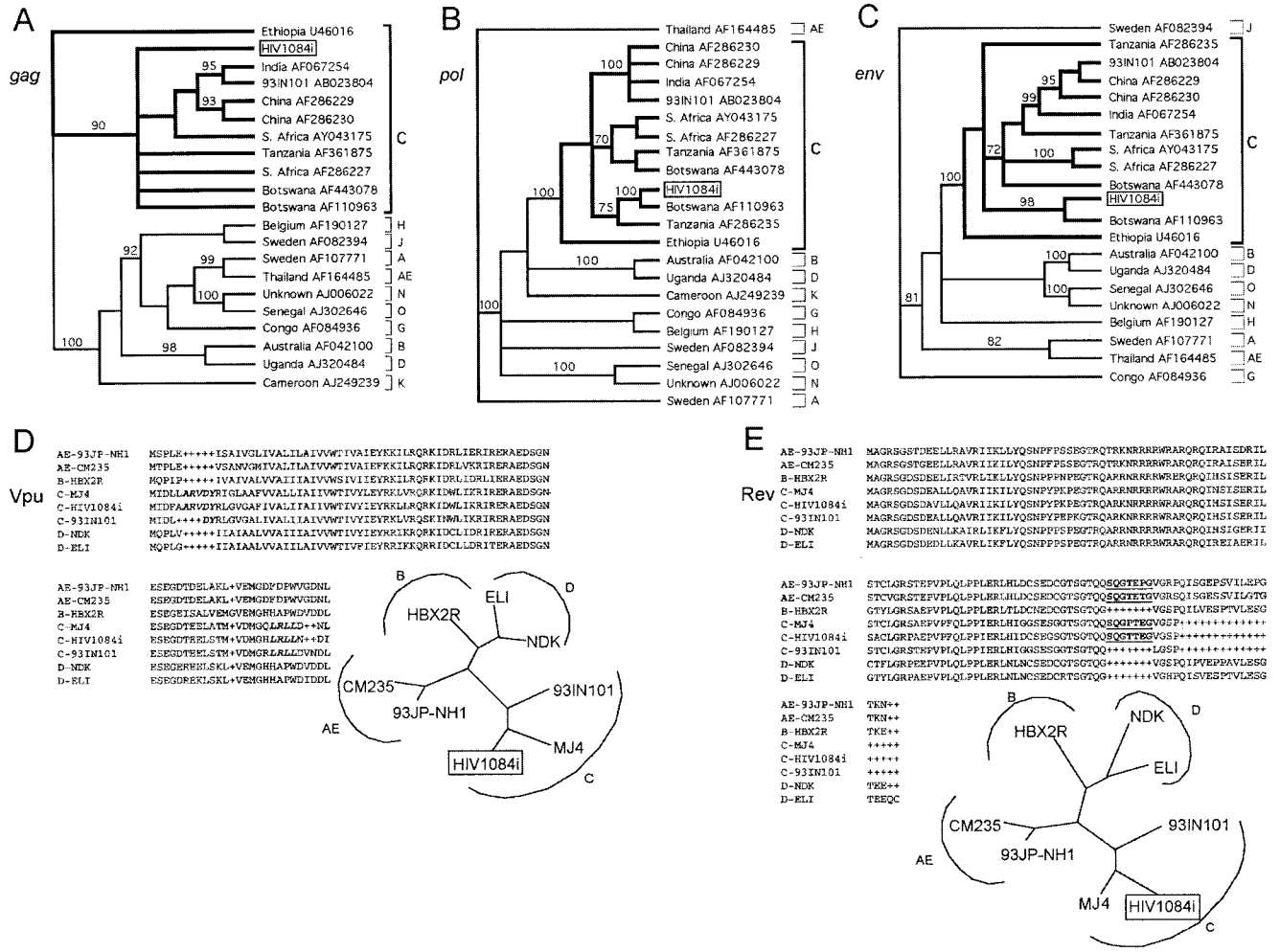

FIG. 3. Phylogenetic analysis of *gag*, *pol*, *env*, Vpu, and Rev. Using Clustal X (version 1.81) followed by PAUP (version 4.0), unrooted bootstrapped phylogenetic trees were generated for *gag* (A), *pol* (B), *env* (C), Vpu (D), and Rev (E) of HIV1084i. One thousand bootstrap replicates, a gap opening penalty of 50 (or 10), a gap extension penalty of 5 (or 0.1), and the International Union of Biochemistry DNA (or Gonnet 250 protein) weight matrix were used to generate the trees. Only bootstrap values greater than 70 are indicated. All reference DNA sequences were obtained from the Los Alamos National Laboratory HIV database (http://hiv-web.lanl.gov/). 93IN101 (AB023804) is referred to herein as Indie-C1 (9).

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′ half that was initially amplified en bloc using primers located within the 5′ 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 GGGGCGTTCC. 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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