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Sequence Note

Isolation and Characterization of a Full-Length Molecular DNA Clone of Ghanaian HIV Type 1 Intersubtype A/G Recombinant CRF02_AG, Which Is Replication Competent in a Restricted Host Range

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ABSTRACT

We have isolated a replication-competent, full-length molecular clone of HIV-1 CRF02_AG, designated p97GH-AG1, by reconstituting two separately amplified genomic regions of an HIV-1 provirus of a 1997 Ghanaian isolate. The phylogenetic and recombination breakpoint analyses revealed that 97GH-AG1 had an A/G recombinant structure similar to that of prototype Nigerian isolate IbNG. The 17-nucleotide insertion downstream of the primer-binding site appeared to be a common sequence signature specific to most CRF02_AG strains, including 97GH-AG1. 97GH-AG1 showed an R5 phenotype and exerted productive infection in both HOS and NP2 cell infectivity assays, whereas it failed to show a detectable level of progeny production in peripheral blood mononuclear cells (PBMCs). The data may suggest the presence of unknown determinant(s) that dictate efficient replication in PBMCs, but that are not required for replication in immortalized cell lines.

Globally circulating HIV-1 strains are classified into three groups, designated M, N, and O, which are defined as distinct clusters on phylogenetic trees. Group M comprises the great majority of HIV-1 isolates and is further divided into at least nine nonrecombinant subtypes, designated A to D, F to H, and J and K.[1] Analyses of subgenomic and full-length HIV-1 sequences identified some numbers of intersubtype recombinants that clustered with different subtypes in different parts of their genome. Some recombinants showed a widespread geographic dissemination, referred to as circulating recombinant forms (CRFs).[2] Four CRFs, CRF01 through CRF04, are currently recognized: CRF01_AE from Southeast Asia, CRF02_AG from Africa, CRF03_AB found in the epidemic among injecting drug users in Kaliningrad in Russia, and CRF04_cpx from Cyprus and Greece.[1]

Our understanding of the pathogenesis and the molecular biology of HIV-1 has been mainly based on the analysis of a few strains of subtype B, a subtype that is not often found in the major epicenter of the HIV epidemic, including Africa and south and Southeast Asia. Accordingly, the molecular reagents for nonsubtype B viruses are limited. In particular, replication-competent HIV-1 molecular clones are critically needed for the studies requiring functional gene products with the defined and uniform genetic and immunological properties of respective subtypes, or CRFs. Only seven replication-competent nonsubtype B molecular clones are so far available, including one sub-

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type C (pIndieCI[3]), four subtype D (NDK,[4] Z2Z6, ELI,[5] and 94UG114.1), one African strain of CRF01_AE (90CF402.1[6]), and MAL (HIV-1 A/D/K/? recombinant).[5] The availability of replication-competent molecular clones would facilitate the studies particularly aimed at determining the biological consequence of HIV-1 genetic diversity and its impact on cellular and humoral immune responses. Here we describe the first full-length molecular DNA clone of HIV-1 intersubtype A/G recombinant (CRF02_AG) from Ghana and discuss on its structural and virological properties.

An HIV-1 strain (NJ97-42) was isolated in 1997 from a symptomatic HIV-seropositive 60-year-old consenting Ghanaian woman (NJ97-42) by cocultivation of the peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin (PHA)-stimulated donor PBMCs. She was a sexually transmitted disease clinic patient seropositive for *Treponema pallidum* antigen. At the time of blood sampling, her CD4$^+$ cell count was 440 cells/μl and her CD4$^+$:CD8$^+$ cell ratio was 0.85. The HIV-1 strain, NJ97-42, was originally classified as HIV-1 subtype A by phylogenetic tree analysis based on the *env* (C2/V3) sequence in a previous study (data not shown). The virus was plaque purified in the MAGIC5A indicator cell line, a derivative of HeLa-CD4-LTR-β-Gal (MAGI), which expressed high level of CD4 and CCR5 as well as CXCR4.[3] MAGIC5A cells were infected with a serially diluted virus stock of NJ97-42, overlaid with medium containing 0.7% agarose, and cultured for 3 days. After staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside, viruses were recovered from plaques and propagated in HeLa 4.5 cells, which express both CXCR4 and CCR5 with CD4, to avoid the possible complication of recombination events with the HIV-1 long terminal repeat (LTR) sequence in MAGIC5A cells.

High molecular weight DNAs were extracted from HeLa 4.5 cells infected with plaque-purified NJ97-42 and were used as templates for polymerase chain reaction (PCR) amplification of HIV-1 proviral sequences by the Expand Long Template PCR system (Boehringer Mannheim, Indianapolis, IN). The replication-competent provirus clones were reconstituted from two separately amplified genomic regions[3,7] (Fig. 1). Briefly, the 658-bp fragment spanning the viral LTR, and the untranslated leader sequence preceding the *gag* gene, was amplified by using the primer pair of HIV-LTR1(+)/*Eco*RI (5′-CGGAATTCT^{1}GGATGGGCTAATTTACTCCAA^{22}-3′, sense; the *Eco*RI site is underlined. The positions of the nucleotides in HIV relative to HXB2CG, which were determined by the HXB2 Numbering Engine available at http://hiv-web.lanl.gov/NUM-HXB2/HXB2.Nuc.html, are hereinafter shown as superscripts in the first and last nucleotides of the corresponding HIV-1 se-

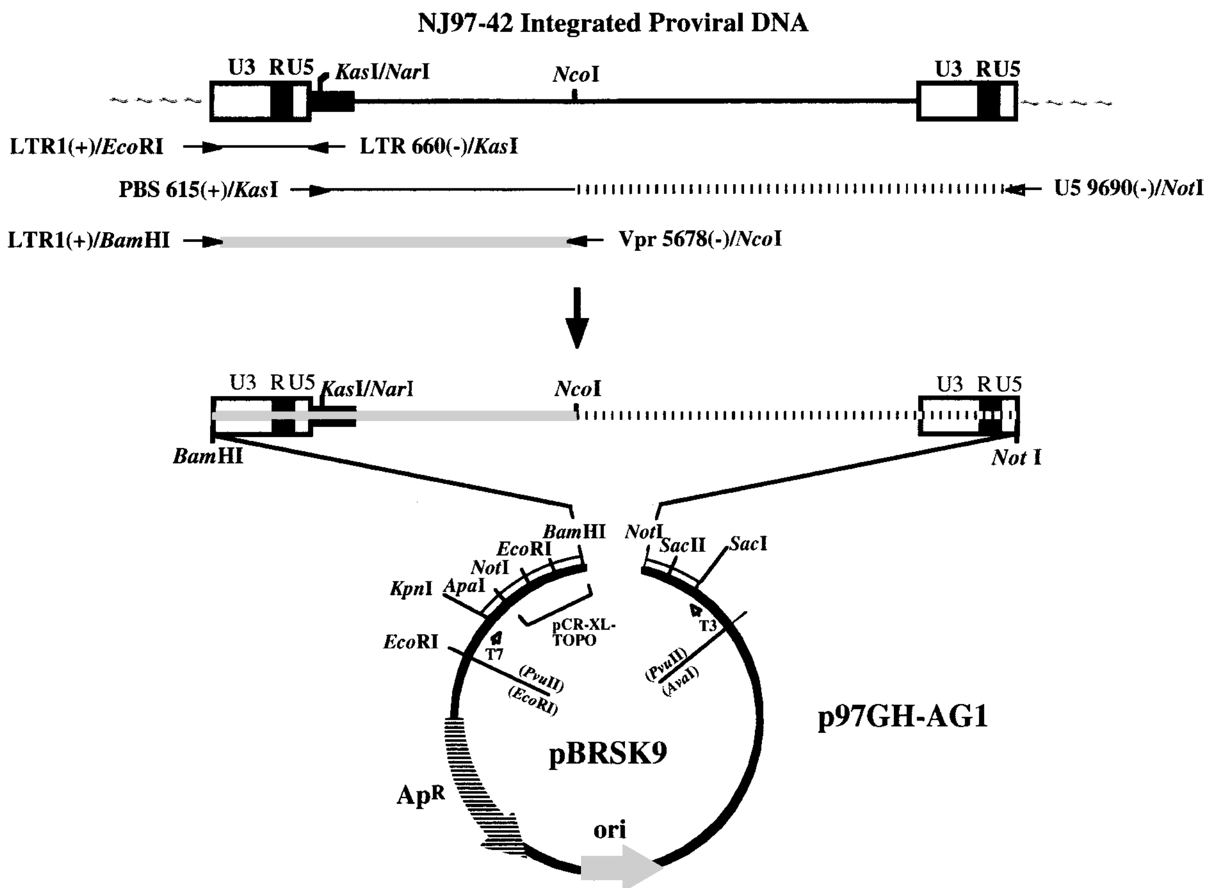

FIG. 1. The scheme of construction and structure of the full-length HIV-1 CRF02_AG molecular clone, p97GH-AG1. The positions of PCR primers that were used to reconstitute the full-length clone are shown at the top. Details of the construction are described in text.

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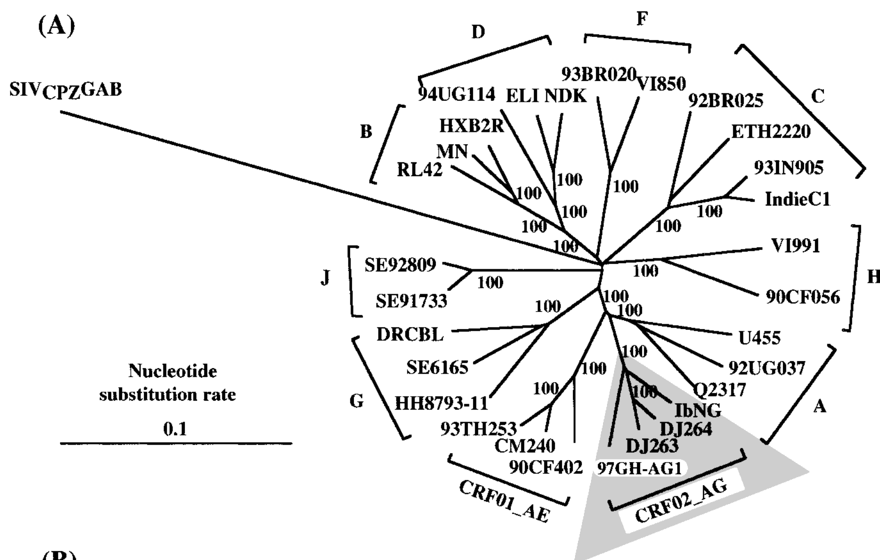

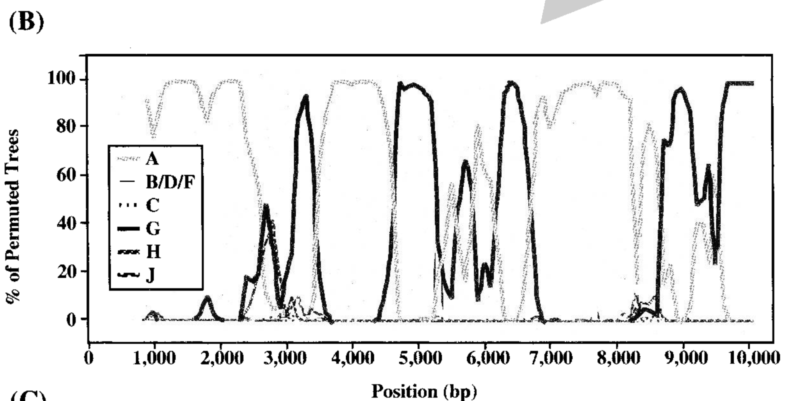

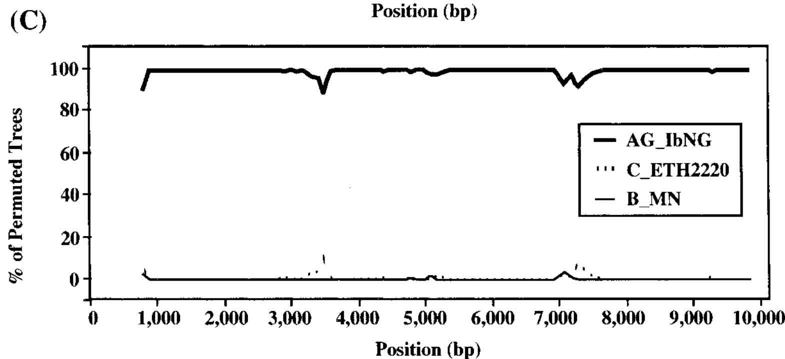

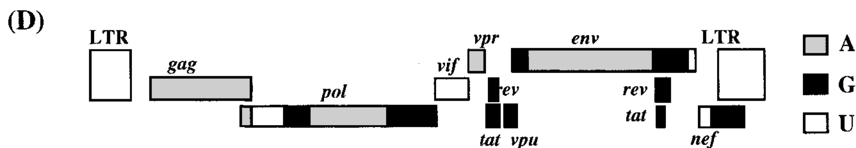

FIG. 2. Structural profiles of 97GH-AG1. (**A**) Phylogenetic tree based on full-length nucleotide sequence. Full-length nucleotide sequence of 97GH-AG1 was aligned with those of the newly proposed HIV-1 group M reference strains (http://hiv-web.lanl.gov/ALIGN_CURRENT/subtype_alignments.html) in the Los Alamos HIV sequence database. Sites where there was a gap in any of the sequences were excluded. The tree was generated by the neighbor-joining method based on the Kimura two-parameter distance matrix, using PHYLIP. $SIV_{CPZ}GAB$ was used as outgroup. Subtype or CRF clades are indicated outside the tree. The bootstrap values are shown at corresponding nodes. (**B**) Bootstrap plots depicting the relationship to the indicated reference strain of respective subtype. Since the bootstrap values against subtype B, D, E, and G references were negligibly low, only plots for subtypes A, C, G, H, and J are shown. Trees were constructed from the multiple genome alignment, and the percentage of bootstrap replicates (*y* axis) that support the clustering of 97GH-AG1 with the reference strains was plotted for a window of 500 bp moving in increments of 100 bp along the alignment. Regions of subtype A or G origin are identified by high bootstrap values (>90%). Points of cross-over of the two curves indicate recombination breakpoints. (**C**) Bootscan plot of 97GH-AG1 with the prototype CRF02_AG strain IbNG, in comparison with the subtype C and B reference strains. (**D**) The deduced recombinant structure of p97GH-AG1. The regions in white could not be assigned to any known subtype.

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quence in respective primers) and HIV-LTR 660(–) (5′-C[660]TTCTAGAACCCTGTTCGGGCGCCACTGCT[631]-3′, antisense; *Kas*I/*Nar*I site is underlined). For the amplification of the approximately 9.1-kb 3′ HIV segment containing most of the HIV genome, a primer set of HIV PBS 615(+) (5′-A[615] GTCTAGAAAATCTCTAGCAGT GGCGCCCGAACAG[649]-3′, sense; *Kas*I/*Nar*I site is underlined) and HIV U5 9690(–) (5′-AGACGCGGCCGCG[9690]GTCTGAGGGATCTCTA GT-TACCAGAGT[9663]-3′, antisense; *Not*I site is underlined) was used. The fragment containing the 5′ LTR that was cloned into the pBRSK9 vector (Fig. 1), a derivative of pBR322, containing the multiple cloning site derived from pBluescript-SK(–) (Stratagene, La Jolla, CA), was subsequently cleaved with *Kas*I/*Nar*I in the primer-binding site and by *Not*I in the polylinker to allow the insertion of the 9.1-kb PCR products cleaved with the same restriction enzymes. The full-length HIV-1 DNA sequence was determined on both strands, using the fluorescent dye terminator method in an Applied BioSystems model 373A DNA sequencer (Applied Biosystems, Foster City, CA). The resultant plasmid was found to contain a small deletion immediately upstream of the primer-binding site and to have weak replication capability in the indicator cells. To repair this defect, a DNA segment containing the 5′ half of the HIV genome, encompassing from the 5′ LTR to the *Nco*I site in the *vpr* gene, was amplified with the primer pair HIV-LTR1(+)/*Bam*HI (5′-CGGGATCCT[1]GGATGGGCTAA-TTTACTCCAA[22]-3′, sense; *Bam*HI site is underlined) and HIV Vpr 5678(–) (5′-A[5678]TGGAGCCATGGTCTAGGAAAGT-GTCTG[5651]-3′, antisense; *Nco*I site is underlined) and was cloned by the TA cloning method into the *Xcm*I site in pCR-XL-TOPO (InVitrogen, San Diego, CA). The insert containing the 5′ half of the HIV-1 genome was cleaved with *Apa*I and *Nco*I and then cloned into the *Apa*I–*Nco*I sites in vector DNA.

The infectivity of HIV-1 DNA clones was tested with HeLa4.5-nEGFP, a highly sensitive indicator cell line that expresses a high level of CD4 and both major HIV-1 coreceptors, CCR5 and CXCR4, and carries the HIV-1 LTR-driven enhanced green fluorescent protein (EGFP) gene with nuclear localizing signal (nEGFP). HeLa4.5-nEGFP cells were transfected with each candidate DNA clone by the method using FuGENE 6 (Boehringer Mannheim). The replication-competent clones were identified by the following three criteria: (1) detection of fluorescence in the nuclei of transfected HeLa4.5-nEGFP cells, assuring the intactness of *tat* gene function and that of its *cis*-acting TAR sequence in the candidate clone; (2) formation of syncytia, reflecting the functional intactness of *env* gene and the related genetic elements required for *env* gene expression (*tat* and *rev* and their *cis*-acting elements) to trigger membrane fusion; and (3) production of progeny virion, which is assessed by reverse transcriptase assay of the supernatants of the GFP-positive cultures. One replication-competent full-length molecular DNA clone, designated p97GH-AG1 (Fig. 1), was identified among 10 candidate plasmids.

The size of the full-length HIV-1 genome in p97GH-AG1 was 9748 bp (since 29 bp of the 3′-terminal part in the 3′ LTR sequence was missing because of the 3′ LTR primer that we used, the total length of this clone after reverse transcription should be 9777 bp), with intact open reading frames for all nine HIV-1 genes, including *gag*, *pol*, *env*, *vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*. p97GH-AG1 had two NF-κB sites, three SP-1 sites, a normal TATA box (TATAAA), and a typical 3-nucleotide bulge (UCU) in the TAR stem region (data not shown). Neighbor-joining analysis based on full-length HIV-1 sequences revealed that p97GH-AG1 is clustered with the reference strains for HIV-1 intersubtype A/G recombinants (CRF02_AG), including IbNG[8] and DJ263 and DJ264[2] (Fig. 2A). The bootstrap and diversity plot analyses showed that p97GH-AG1 shared an almost identical structural profile with IbNG[8,9] (Fig. 2B–D).

The alignment of the nucleotide sequences near the LTR and the *gag* leader regions, in comparison with that of HIV-1 subtype B strain HXB2, revealed that 97GH-AG1 had a 17-nucleotide insertion similar to that of two CRF02_AG reference strains, including IbNG and DJ263 (DJ264 has one extra nucleotide) (Fig. 3A). The other form of HIV-1 intersubtype A/G recombinants (92NG003 and 92NG083), as well as CRF01_AE, subtype G, and some of subtype A strains (92UG03 and SE8131), have 24-nucleotide insertions[10,11] (Fig. 3A). The 17-nucleotide insertion has not been detected so far in any other HIV-1 subtypes, CRF clades, or other forms of recombinants that contained subtype A segments (Fig. 3A), and thereby appeared to be specific to most of the CRF02_AG strains, including 97GH-AG1. As shown in Fig. 3A, the 24-nucleotide insertion[10] is generated by a duplication of the 3′ part of the PBS stem–loop structure, which is composed of duplication units i and ii.[11] These two duplication units, with an intervening AT-rich 5-nucleotide stretch,[11] were composed of three purine-rich segments of 6, 7, and 6 bp (Fig. 3A). The 24-bp insertion comprises units of 7, 6, 5, and 6 nucleotides (Fig. 3A). In contrast, the 17-nucleotide insertions uniquely found in most of the CRF02_AG strains are apparently generated by the deletion of a 7-nucleotide segment of duplication i, and thereby were composed of two 6-bp units with an intervening 5-bp AT-rich sequence (Fig. 3A).

It is noted that the insertion of 9 amino acids was observed in the proximal part of the amino-terminus coding region of the *nef* gene[12] in p97GH-AG1. This insertion appears to be unique in this clone, since it is not found in any other CRF02_AG so far reported in the database (Fig. 3B). However, it is uncertain whether this has any consequences for the viological properties of the virus.

FIG. 3. Sequence features unique to 97GH-AG1. (**A**) Alignment of nucleotide sequence near the primer-binding sites (PBS). Locations of the PBS, PBS insertion,[10,11] and U5–PBS hairpin structure[15] with the positions of nucleotides numbered relative to HXB2CG (determined by HXB@ numbering engine, available at http://hiv-web.lanl.gov/NUM-HXB2/HXB2.Nuc.html), are shown at the top. The shaded areas indicate the locations of duplicative sequences. Duplications i and ii[11] are composed of units of duplicative sequences 6, 7, and 6 bp long, with an intervening 5-bp AT-rich stretch. The 17-bp insertions unique to most of CRF02_AG, including 97GH-AG1, consisting of two 6-bp units with a 5-bp intervening sequence, are shown at the bottom. (**B**) Alignment of deduced amino acid sequences of the amino-terminus regions of Nef proteins. Dots indicate identity with the HXB2 sequence, shown at the top; dashes indicate a gap in the alignment.

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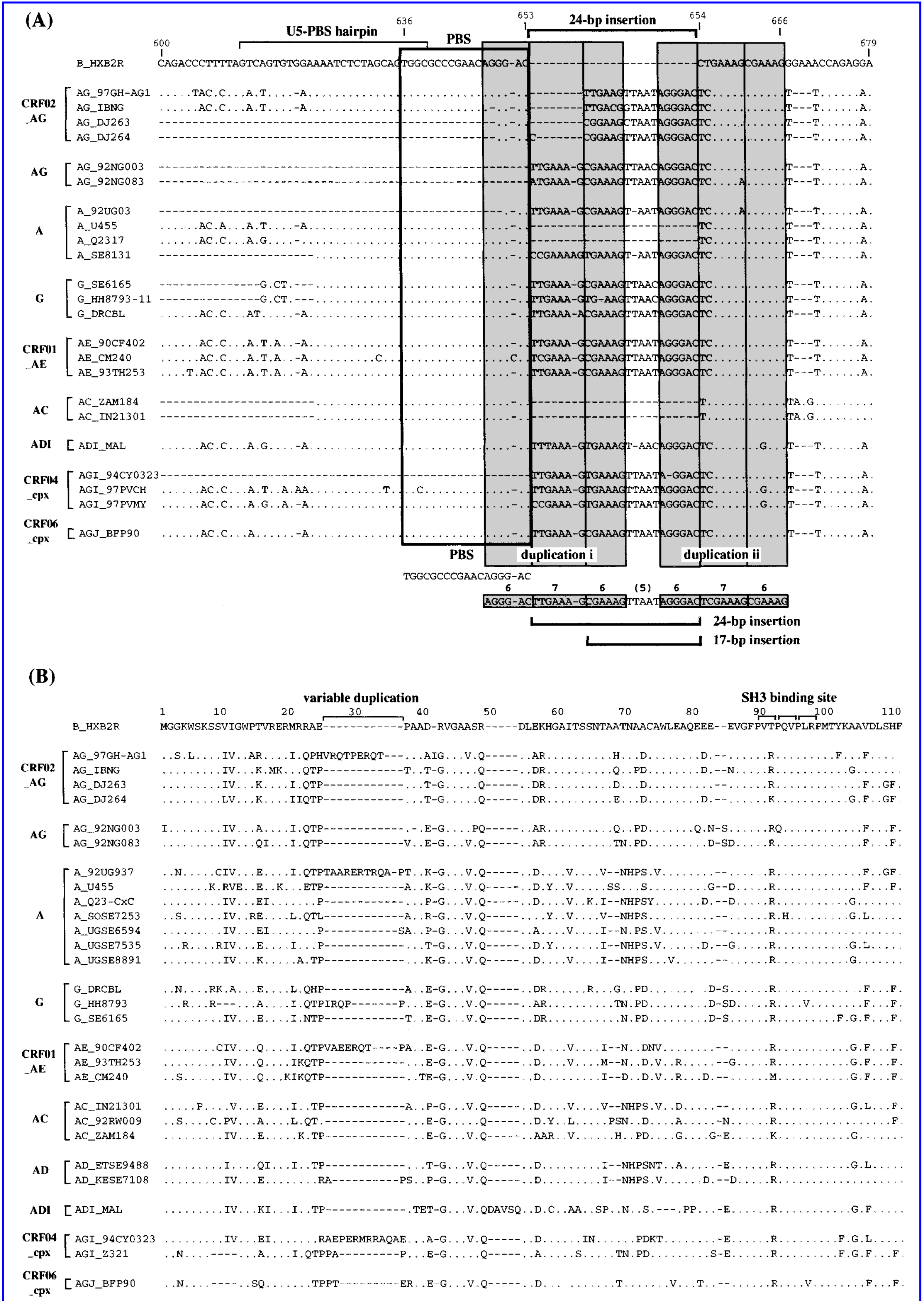

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The NP2-CD4[13] and HOS-CD4 cell[14]-based infectivity assays indicated that 97GH-AG1 used CCR5, but not CXCR4, as a primary coreceptor (Fig. 4). 97GH-AG1 showed syncytium formation in NP2-CD4 cells expressing CCR5 (NP2-CD4-CCR5 cells), but not in those expressing CXCR4 (NP2-CD4-CXCR4 cells) (Fig. 4A). The virion-associated reverse transcriptase assay of culture supernatants demonstrated that p97GH-AG1 exerted productive infection in NP2-CD4-CCR5 cells, but not in NP2-CD4-CXCR4 cells (Fig. 4B). Essentially similar results were obtained in the HOS cell-based infectivity assay (data not shown). However, p97GH-AG1 did not show any detectable level of progeny production in phytohemagglutinin (PHA)-stimulated PBMCs even after $CD8^+$ T lymphocytes were depleted (data not shown). It is known that pro-

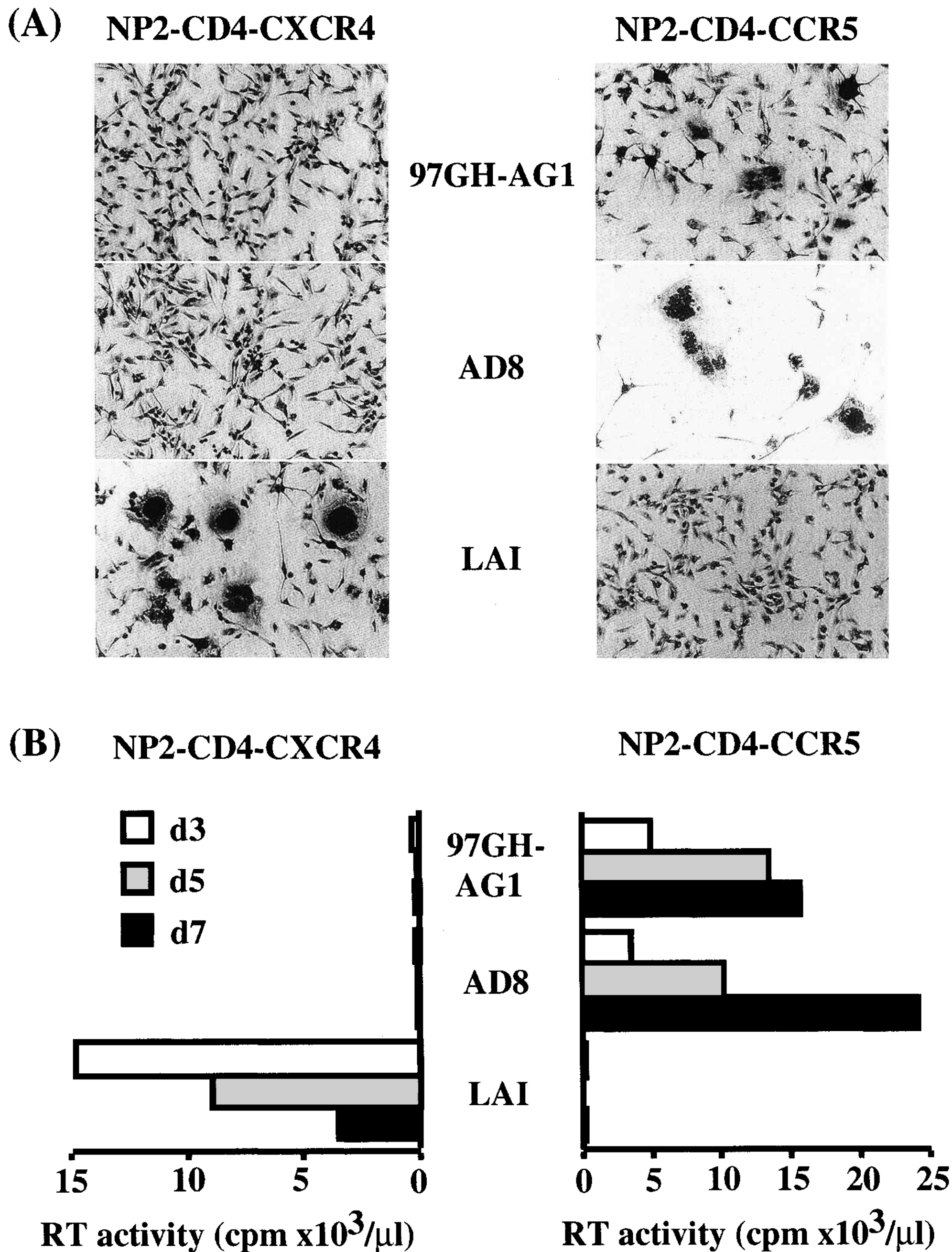

FIG. 4. Virological properties of HIV-1 CRF02_AG molecular clone p97GH-AG1. (**A**) Syncytium formation in NP2-CD4 indicator cell lines. *Left*: NP2-CD4-CXCR4 cells. *Right*: NP2-CD4-CCR5 cells. Syncytium formation was monitored by Giemsa staining 3, 5, and 7 day postinfection. (**B**) Coreceptor usage of p97GH-AG1 in NP2-CD4 cell infectivity assay. The virion-associated RT activities in culture supernatants collected 3, 5, and 7 days after infection with respective strains are shown as a histogram. Each plot was an average of triplicated assays.

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longed activation with interleukin 2 (IL-2) in addition to PHA is required for the induction of sufficient surface CCR5 expression in PBMC cultures. Indeed, the previously isolated HIV-1 subtype C infectious molecular clone, pIndieC1, which uses CCR5 as a coreceptor,[3] was not able to replicate in the standard PBMC cultures stimulated only with PHA, while it could replicate in PBMCs stimulated with both PHA and IL-2 prior to the infection. Even under the same experimental conditions in which pIndieCl resulted in productive infection, p97GH-AG1 was not able to show any detectable progeny production in PBMCs.

There are several possible explanations for the replication competency of p97GH-AG1 in the restricted host range. The indicator cell lines that were used for screening the infectious molecular clones in the present study overexpressed CD4 and the coreceptors, and thereby under these extreme conditions it is possible to select HIV-1 DNA clones that are dependent on high levels of CCR5 and/or CD4 to propagate efficiently in target cells. p97GH-AG1 might require higher CCR5 and CD4 expression levels for efficient propagation in PBMCs. Alternatively, p97GH-AG1 may represent a viral species that can replicate slowly in certain cell populations *in vivo*. This phenomenon may also suggest the presence of unknown determinant(s) that are required for efficient propagation in PBMCs but not for replication in immortalized cell lines. Further analyses are currently in progress to understand the mechanism underlying the difference in permissiveness of 97GH-AG1 propagation in different target cells.

In conclusion, our screening system, which facilitates the identification of replication-competent HIV-1 clones, enabled us to isolate several infectious molecular clones of nonsubtype B and CRF clades, including subtype C,[3] CRF02_AG (in the present study), and CRF01_AE recombinants.[16,17] Replication-competent HIV-1 molecular clones of nonsubtype B/CRF clades would facilitate the investigation of differences in virological and immunological properties (if any), and the development of clade-specific molecular and immunological reagents.

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SEQUENCE DATA

The full-length sequences of HIV-1 97GH-AG1 were submitted to GenBank and are available under accession number AB049811.

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