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# Generation of a replication-competent chimeric simian-human immunodeficiency virus carrying *env* from subtype C clinical isolate through intracellular homologous recombination

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ABSTRACT

A new simian-human immunodeficiency virus (SHIV), carrying *env* from an uncloned HIV-1 subtype C clinical isolate (97ZA012), was generated through intracellular homologous recombination, a DNA repair mechanism of the host cell. PCR fragments amplified from an existing SHIV plasmid (a 7-kb fragment from the 5′ end and a 1.5-kb fragment from the 3′ end) and a 4-kb fragment amplified from 97ZA012 cDNA containing *env* were co-transfected to human lymphoid cells. The resulting recombinant was subjected to serial passage in rhesus peripheral blood mononuclear cells (RhPBMCs). The resulting SHIV 97ZA012 was replication competent in RhPBMCs and monkey alveolar macrophages, and possessed CCR5 preference as an entry co-receptor. Experimental infection of rhesus macaques with SHIV 97ZA012 caused high titers of plasma viremia and a transient but profound depletion of $CD4^+$ T lymphocytes in the lung. Animal-to-animal passage was shown to be a promising measure for further adaptation of the virus in monkeys.



## Introduction

Human immunodeficiency virus (HIV) infections have been a major global public health issue since their initial recognition in the 1980s. Globally, approximately 33 million individuals are living with HIV, 1.8 million people die of HIV-related complications, and 2.6 million people newly acquired the virus in 2009 (UNAIDS, 2010). Establishment of effective preventive measures is urgently needed to control the epidemic.

Extensive genomic diversity is a characteristic trait of HIV. HIV type 1 (HIV-1), the major genotype of the virus, comprises four subgroups: M, N, O, and P. Subgroup M further comprises numerous subtypes and circulating recombinant forms (CRFs), which are recombinant viruses among subtypes. Among the subtypes, subtype C plays a leading role in the epidemic, accounting for nearly 50% of global HIV infections (Hemelaar et al., 2011). Greater numbers of viral particles are detected in the vaginal secretions of pregnant individuals infected with subtype C than from persons infected with subtypes A or D (John-Stewart et al., 2005), potentially making subtype C more transmissible than others and rendering it predominant in the current epidemic. A compact V1/V2 loop and threonine at 316 located in the V3 loop of Env, distinct features shared by many subtype C isolates, may contribute to preferential replication of these viruses in the genital tract (Walter et al., 2009).

The humoral immune reaction directed against subtype C virus is unequal to that directed against subtype B virus. Virus-neutralizing antibodies mounted in individuals infected with subtype C are directed against the alpha-2 helix in the Env C3 region. This region is rarely immunogenic in subtype B virus infection (Moore et al., 2008), indicating a conformational difference in Env between these subtypes. The development of a tractable animal model for subtype C is thus necessary to establish a strategy for effective induction of neutralizing antibodies directed against the protein of this particular subtype.

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Simian-human immunodeficiency virus (SHIV) carrying Env derived from subtype C would be an especially vital tool because it would allow for evaluation of the effectiveness of vaccine-induced immunity in the context of virus infection *in vivo*.

However, only a few subtype C SHIVs are available, and none reproducibly replicates to high titers and induces disease in monkeys. In addition, limited numbers of SHIVs utilize the CCR5 molecule as an entry co-receptor. The scarcity of available SHIV strains is attributed to the difficulty in generating an infectious chimeric virus. SHIVs have been generated through recombinant DNA techniques involving implantation of a chunk of genes, such as *tat*, *rev*, *vpu*, and *env*, from the molecular clone of parental HIV-1 into the backbone of the SIV239 molecular clone. This method does not always lead to successful generation of infectious SHIV. Two presumable reasons may explain this difficulty: (1) incompatibility of a particular clone from the parental HIV-1 swarm with the SIV backbone and (2) inadequate employment of "breakpoints," sites of recombination, for the given parental clones of HIV-1 and SIV. The generation of SHIVs by the conventional technique (*i.e.*, recombination of HIV-1 genes from a molecular clone verified to be infectious to human cells with an SIV backbone at breakpoints that are reasonably assumed to be appropriate) may represent a major bottleneck for the development of new SHIV strains.

Intracellular homologous recombination (IHR) is a cellular mechanism for the restoration of DNA double-strand breaks. It also takes place when exogenously introduced DNA fragments share "homologous sequences" (Srinivasan et al., 1989). The mechanism has been attributed to the generation of infectious HIV-1 particles from cell lines carrying multiple defective provirus genomes (Inoue et al., 1991). IHR also causes generation of infectious HIV-1 through co-transfection of truncated viral cDNA clones into the cells (Kalyanaraman et al., 1988; Srinivasan et al., 1989) or through recombination between exogenous sequences and integrated chromosomal HIV sequences (Clavel et al., 1989; Srinivasan et al., 1989). It is then utilized as a measure to readily generate recombinant HIV-1 (Cheng-Mayer et al., 1990; Hertogs et al., 1998; Kellam and Larder, 1994).

We reasoned that generation of SHIV through IHR could circumnavigate the above-mentioned issues and accelerate the process as follows: (1) DNA fragments prepared by polymerase chain reaction (PCR) with cDNA from an uncloned HIV-1 genome would provide a continuum of heterogeneous sequences that potentially contain competent clone(s) in the context of infection in monkey cells *in vitro* and monkeys *in vivo* when combined with an SIV backbone, and (2) random occurrence of IHR within "homologous sequences" would likely produce multiple SHIV genomes with breakpoints at various sites, increasing the chance for emergence of a virus with favorable fitness. In addition, co-transfection of DNA fragments into cells susceptible to viral infection would subject the generated recombinant virus to multi-round replication, causing selection/evolution of a replication-competent virus. Based on this reasoning, we embarked on IHR-mediated generation of SHIV to investigate the utility of these potential advantages.

## Results

### *Generation of recombinant virus through IHR*

To generate a novel SHIV carrying the *env* gene derived from a clinical isolate of subtype C HIV-1 through IHR, we prepared three DNA fragments by PCR as depicted in Fig. 1. Approximately 1100 bps of overlapping sequence (where IHR was expected to take place) were shared by Fragments I and III, 1400 bps were shared by Fragments II-a and III, and 1200 bps were shared by Fragments II-b and III (Fig. 1).

Although we envisioned that recombination between the two DNA fragments could theoretically take place at any base within these overlaps, potentially resulting in generation of multiple sets of recombinant genomes, only replication-competent recombinant(s) would emerge as representative following transfection with these fragments into susceptible cells for lentiviral replication and multi-round replication cycles. To test this hypothesis, mixtures of Fragments I, II-a, and III (Transfection #1) or Fragments I, II-b, and III (Transfection #2), 0.2 μg of each DNA preparation, were co-transfected to human T-lymphoid cell line C8166-CCR5 cells. The cultures were maintained for 3 weeks to monitor emergence of recombinant virus by microscopic observation because the parental HIV-1 97ZA012, which contributed the *env* gene to the transfection, was known to induce syncytia in the cells (data not shown). Transfection #1 produced syncytia on day

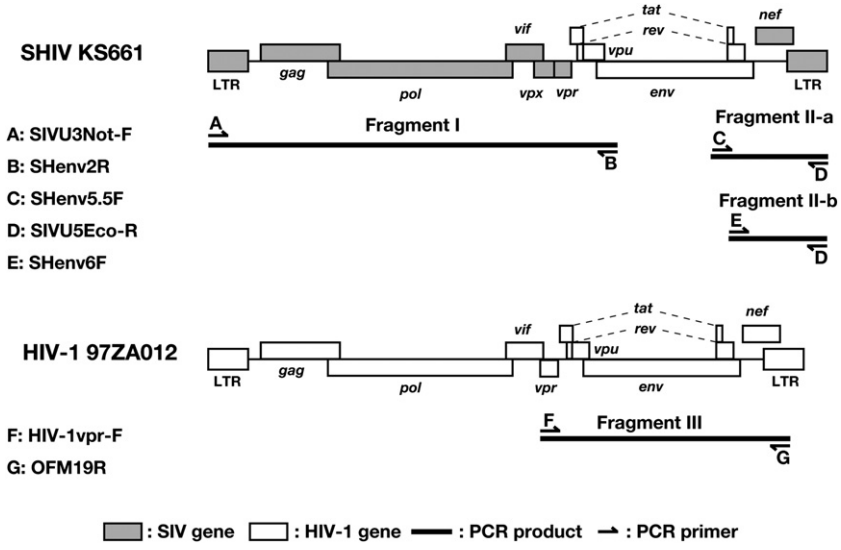


**Fig. 1.** Schematic representation of HIV-1/SHIV genome organizations and PCR fragments employed for co-transfection. Filled boxes represent genes derived from SIV. Open boxes represent genes derived from HIV-1. SHIV KS661, existing SHIV, carries *tat*, *rev*, *vpu*, and *env* genes from subtype B HIV-1 89.6. Broad lines represent PCR fragments; Fragments I and II-a/II-b were amplified using plasmid DNA of SHIV KS661 as a template. Fragment III was amplified from cDNA of the HIV-1 97ZA012 genome as a template. Arrows represent PCR primers whose identifiers are depicted in the figure.