Detection of Simian Immunodeficiency Virus in Diverse Species and of Human Immunodeficiency Virus Type 2 by Using Consensus Primers within the pol Region

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Received 13 March 2002/Accepted 18 May 2002

Human immunodeficiency virus type 2 (HIV-2) is the result of cross-species transmission of simian immunodeficiency virus (SIV) from sooty mangabey monkeys to humans. Primer pairs (intHIV-2/SIV) based on a region of integrate that has considerable homology across HIV-2 and SIV lineages were designed to develop a broadly cross-reactive molecular assay to detect lentivirus infection in primates. The intHIV-2/SIV primers detect HIV-2 and simian viruses SIVcpz, SIVsmm, SIVsyk, SIVagm, and SIVmnd. The primers are also capable of amplifying some HIV-1 strains. Additionally, sequences from the integrate amplicons were of sufficient genetic diversity to permit not only phylogenetic clustering of all simian viruses to their respective lineages but also HIV type and group classification. Thus, the primers described here provide a method to detect primate lentiviruses from diverse species of nonhuman primates, as well as from persons infected with HIV-1 and HIV-2.

Zoonotic transmission is an important factor in the emergence of retroviruses and other infectious agents in humans (2, 14, 29, 32, 33). At least 20 different nonhuman primate species in Africa have been shown to harbor simian immunodeficiency viruses (SIVs) (14). Thus, African primates represent an extremely large reservoir of lentiviruses that can potentially infect other species including humans (14, 29). Indeed, the extensive phylogenetic relatedness among many strains of human immunodeficiency virus type 1 (HIV-1) and HIV-2 and primate lentiviruses has elucidated the simian origin of AIDS (2, 7, 9, 12, 14, 29). These studies have further established that HIV-1 infection, the main cause of the worldwide AIDS pandemic (16), was the result of cross-species transmission of SIV from chimpanzees (Pan troglodytes troglodytes) to humans (12, 15, 31–33). Likewise, the HIV-2 epidemic seems to have emerged via cross-species transmission of SIV from sooty mangabey monkeys (Cercocebus sp.) (4, 8, 14).

Current evidence indicates that the SIV counterparts of HIV-1 and HIV-2 were introduced into the human population multiple times (at least seven transmission events have been suggested) (14, 29). Yet, the HIV-1 group M viruses appear to have arisen from just one such zoonotic transmission (12). Thus, while cross-species transmission of primate retroviruses to humans occurs relatively frequently, the subsequent spread of the retroviruses in the human population is rare (14, 29, 32, 33). Nevertheless, this scenario represents a unique opportunity to study both the emergence of new human retroviruses and the genetic diversities of these human and simian viruses. The primate lentiviruses for which full-length genomic sequences are available fall into five major equidistant phylogenetic lineages: (i) SIVcpz from chimpanzees, together with HIV-1; (ii) SIVsmm from sooty mangabey monkeys together with HIV-2; (iii) SIVagm from four species of African green monkeys; (iv) SIVsyk from Sykes monkeys; and (v) SIVmnd and SIVh0est from mandrills and l’Hoeest monkeys, respectively (3, 5, 7, 10, 12–15, 31). Recently, an additional SIV (SIVcol) from Guereza colobus monkeys (Colobus guereza), representing a sixth lineage of primate lentivirus, has been identified (8). Therefore, studies are needed to determine whether transmission of simian lentiviruses other than SIVcpz and SIVsmm to humans is occurring, particularly in regions where SIV infection in nonhuman primates is highly prevalent.

These studies require the development of molecular detection assays that can detect a wide range of lentivirus infections in both nonhuman primates and humans. We have recently described serologic and molecular diagnostic assays that permit detection of highly divergent HIV-1 strains and their simian counterpart, SIVcpz (21, 25, 35, 36). Here we report a sensitive and broadly reactive PCR-based molecular screening tool for detecting HIV-2 and SIVs from diverse species of primates. More importantly, sequences generated from these PCR products can be reliably used for phylogenetic classification within the HIV-2 and SIV lineages, thus permitting identification of the zoonotic source of infection.

MATERIALS AND METHODS

Primer design. Primers were designed based on the consensus sequences in the pol region of HIV-2 and SIV (20) and are designated intHIV-2/SIV primers. For reverse transcription (RT) and primary PCR, the primers were INT-F1 (forward: 5'-ATAGAACCCGCAAAAGGAAACAT; nucleotides 2241 to
Reference clones. The sensitivity of the intHIV-2/SIV primers was tested by using known copy numbers of cloned material representing HIV-1, HIV-2, and SIVcpz. For HIV-2, a cloned fragment of a Centers for Disease Control and Prevention HIV-2 isolate (GB122) was generated by directly cloning an integrase PCR fragment into the pCR-TOPO vector in accordance with the manufacturer’s protocol (Invitrogen, Carlsbad, Calif.). The following previously described molecular clones were used for SIVcpz (17) and HIV-1 (11): 92UG037.1 (subtype A), 93TH233.3 (CRF01-AE), 92RW009.6 (subtype A/C), 92NG083.2 (CRF02-AG), BC53G3 (subtype B), 93BR029.4 (subtype B/F), 92BR026.8 (subtype C), 94UG114.1 (subtype D), 93 BR020.1 (subtype F), and 92CR056.1 (subtype H). Known copy numbers of the partial GB122 clone, the infectious SIVcpz clone, and the HIV-1 molecular clones were used for sensitivity determination. For all samples, multiple dilutions were run in duplicate and each sample was subjected to PCR amplification at least twice.

Viral isolates and plasma specimens. HIV-1, HIV-2, and SIV isolates were generated by previously described coculture procedures (22, 23, 34). DNA or RNA extracted from cultured material were used to test these primers on a wide variety of primate lentivirus isolates. The SIV specimens included 7 SIVsm (sooty mangabey) specimens, 1 SIVst (stumptail macaque) specimen, 1 SIVcm (red-capped mangabey) specimen, 4 SIVagm (African green monkey) specimens, 1 SIVmd (mandrill) specimen, 1 SIVvyk specimen, 1 SIVcpz specimen, and SIVhu (an isolate derived from a human accidentally infected with SIVmac) (19). Additionally, 22 HIV-2 specimens, including uncultured peripheral blood mononuclear cells from 10 HIV-2-infected persons and 12 previously cultured isolates, were included in the study (22). A plasma panel from known HIV-1-seropositive individuals (Boston Biomedical Inc., Boston, Mass.), including 1 specimen each from Argentina, Canada, China, and Mozambique, 2 from the United States, 3 from Thailand, 5 from Zimbabwe, 6 each from Uganda and Ivory Coast, and 8 from Ghana, and an HIV-2-seropositive panel (Boston Biomedical Inc.), including 14 specimens from Ivory Coast, were also used for viral RNA detection by RT-PCR analysis using the intHIV-SIV primers.

PCR. DNA preparation was done by proteinase K digestion in a Tris-Triton buffer, and RNA was extracted by using the QiAamp viral RNA kit according to the manufacturer’s protocol (Qiagen Inc., Valencia, Calif.). Protocols for RNA extraction and conditions for RT-PCR and PCR are described elsewhere (35, 36), except that an annealing temperature of 50°C was used for both the primary and secondary intHIV-2/SIV primer sets. All reactions were carried out with appropriate negative controls to detect possible contamination.

Sequence and phylogenetic analysis. Selected nested-PCR products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced with Big-dye terminators (Perkin-Elmer, Foster City, Calif.) on an automated 377 DNA sequencer (Applied Biosystems, Foster City, Calif.). Sequences were aligned with CLUSTAL W (version 1.74) after editing, and phylogenetic trees were constructed by the neighbor-joining method using the PHYLIP, version 3.5c, package.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences obtained in this study are AF395546 to AF395571.

RESULTS AND DISCUSSION

Analysis of a cloned fragment from HIV-2 (GB122) revealed that the intHIV-2/SIV primers reliably amplified as few as 25 copies of HIV-2 per PCR (Fig. 1). Moreover, these primers also detected SIVcpz with the same sensitivity (Fig. 1). We next examined the cross-reactivity of these primers with HIV-1. Amplifications were performed with HIV-1 group M molecular clones with inputs of 100, 50, and 25 copies per PCR. All of the HIV-1 group M subtypes tested with the exception of subtypes C and D were amplified at 25 copies per reaction (Fig. 1); subtypes C and D could only be amplified at 100 copies per reaction. Thus, HIV-2, SIVcpz, and all HIV-1 subtype molecular clones were consistently amplified at 100 copies per reaction. In addition, RNA from the culture supernatant of an HIV-1 group N virus was amplified (Table 1).

We next tested the amplification efficiency of the primers for 12 HIV-2 primary isolates. The intHIV-2/SIV primers amplified all samples, which included nine HIV-2 subtype A specimens, two subtype B specimens, and one subtype A/B recombinant specimen (Table 1). As cultured isolates from most of the specimens were not available, we performed PCR amplification on DNA derived from uncultured peripheral blood mononuclear cells (PBMC). Analysis of primary PBMC from 10 HIV-2-infected individuals revealed that DNA of those from 8 was amplified by using these primers (Table 2), giving an amplification efficiency of 80%. These results are comparable to those for the primers identified in the protease region, which are highly specific for HIV-2 detection only (26).

The high sensitivity of HIV-2 DNA detection from primary PBMC led us to test the amplification efficiency of intHIV-2/SIV for detection of plasma viremia from HIV-2- and HIV-1-infected individuals. Results from duplicate experiments showed that the intHIV-2/SIV primers were able to amplify from RNA 5 of the 14 HIV-2 plasma samples tested (35.7%)
from HIV-2-infected donors from Ivory Coast. Specimens (Thailand), and 3 subtype G specimens (Ghana and Ivory Coast). (Mozambique and Zimbabwe), 5 subtype D specimens (Uganda), 3 subtype E subtype A specimens (Ghana and Ivory Coast), 6 subtype B specimens (Argentina with protease primers, previously shown to amplify as few as 10 (Table 2). Amplification with RNA from the same samples previously demonstrated for full-length integrase sequences. HIV-2 and SIV from sooty mangabeys formed a monophyletic cluster. Sequences from SIVrcm, SIVmnd, SIVagm, and SIVcpz were highly divergent and represented distinct lineages (2, 14). Similar phylogenetic analysis of sequences amplified from persons infected with HIV-1 group M or group O revealed clustering with their respective group M or group O sequences (Fig. 3) (20), although the subtype designation within the group M sequences was not reliable (data not shown). Thus, despite the small fragment size, the phylogenetic analysis of this region provides an adequate clustering pattern to identify the correct lineages of SIVs, as well as the HIV type and group.

The intHIV-2/SIV primers were able to amplify specimens from five major lineages of primate lentiviruses. Additionally, HIV-1 and HIV-2 sequences were correctly identified. How-

### Table 1. Amplification of HIV-2 and HIV-1 isolates with intHIV-2/SIV primers

| Reference clone or isolate | Country of origin | Subtype | Material tested | PCR amplification |
|---------------------------|-------------------|---------|-----------------|-------------------|
| HIV-2                     |                   |         |                 |                   |
| A1958                     | Senegal           | A       | DNA             | +                 |
| 60415K                    | Senegal           | A       | DNA             | +                 |
| A2270                     | Senegal           | A       | DNA             | +                 |
| 7923A                     | Guinea-Bissau     | A       | DNA             | +                 |
| SRLR3C                    | Guinea-Bissau     | A       | DNA             | +                 |
| GB87                      | Guinea-Bissau     | A       | DNA             | +                 |
| GB122                     | Guinea-Bissau     | A       | RNA             | +                 |
| 310248                    | Ivory Coast       | A       | DNA             | +                 |
| 77618                     | Ivory Coast       | A       | RNA             | +                 |
| 7312A                     | Ivory Coast       | A/B*    | DNA             | +                 |
| 310072                    | Ivory Coast       | B       | DNA             | +                 |
| 310319                    | Ivory Coast       | B       | DNA             | +                 |

### Table 2. Comparative sensitivities of intHIV-2/SIV and intM-Z primers for PBMC DNA and plasma RNA detection

| Specimen | Type | No. tested | No. positive (%) with: | intHIV-2/SIV | intM-Z |
|----------|------|------------|------------------------|-------------|--------|
| HIV-2    | PBMC DNA | 10 | 8 (80) | 0 (0) |
|          | Plasma   | 14 | 5 (36) | 0 (0) |
| HIV-1    | Plasma*  | 34 | 19 (55) | 34 (100) |

* Represents DNA derived from primary lymphocytes or plasma specimens derived from HIV-2-infected donors from Ivory Coast.

* Represents plasma specimens from HIV-1-infected persons, including 10 subtype A specimens (Ghana and Ivory Coast), 6 subtype B specimens (Argentina, Canada, China, Ghana, and United States), 7 subtype C specimens (Mozambique and Zimbabwe), 5 subtype D specimens (Uganda), 3 subtype E specimens (Thailand), and 3 subtype G specimens (Ghana and Ivory Coast).
ever, the integrase region was not sufficient to provide clade designation for either HIV-1 or HIV-2. In summary, the primers described here provide a quick method to amplify primate lentivirus genomic sequences from diverse species of primates, as well as PBMC from HIV-1- and HIV-2-infected persons. This assay should provide a useful tool for recognizing a wide range of lentivirus infections from nonhuman primates and humans, both for identification of HIV variants worldwide and for early detection of potential cross-species transmission.

ACKNOWLEDGMENT

This work was supported in part by NIH/NCRR Base Grant no. RR0016 to the Yerkes Primate Research Center.

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