

Genetically Divergent Strains of Simian Immunodeficiency Virus Use CCR5 as a Coreceptor for Entry

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Entry of human immunodeficiency virus type 1 (HIV-1) requires CD4 and one of a family of related seven-transmembrane-domain coreceptors. Macrophage-tropic HIV-1 isolates are generally specific for CCR5, a receptor for the CC chemokines RANTES, MIP-1 α , and MIP-1 β , while T-cell line-tropic viruses tend to use CXCR4 (also known as fusin, LESTR, or HUMSTR). Like HIV-1, simian immunodeficiency virus (SIV) requires CD4 on the target cell surface; however, whether it also requires a coreceptor is not known. We report here that several genetically divergent SIV isolates, including SIVmac, SIVsmSL92a, SIVsmLib-1, and SIVcpz-GAB, can use human and rhesus CCR5 for entry. CXCR4 did not facilitate entry of any of the simian viruses tested, nor did any of the other known chemokine receptors. Moreover, SIVmac251 that had been extensively passaged in a human transformed T-cell line retained its use of CCR5. Rhesus and human CCR5 differed at only eight amino acid residues, four of which were in regions of the receptor that could be exposed, two in the amino-terminal extracellular region and two in the second extracellular loop. The human coreceptor was as active as the simian for SIV entry. In addition, HIV-1 was able to use the rhesus homologs of the human coreceptors, CCR5 and CXCR4. The SIV strains tested were specific for CCR5 regardless of whether they were able to replicate in transformed T-cell lines or macrophages and whether they were phenotypically syncytium inducing or noninducing in MT-2 cells. However, SIV replication was not restricted to cells expressing CCR5. SIV strains replicated efficiently in the human transformed lymphoid cell line CEMx174, which does not express detectable amounts of transcripts of CCR5. SIV also replicated in human peripheral blood mononuclear cells that were genetically deficient in CCR5. These findings indicated that, in addition to CCR5, SIV can use one or more unknown coreceptors that are expressed on human PBMCs and CEMx174 cells.

Simian immunodeficiency virus (SIV) was first identified in captive Asian macaques (14). The virus shares a variety of properties with human immunodeficiency virus (HIV), including similar genome structure, replication, and tropism for CD4⁺ cells (29). Because SIVmac is closely related to HIV type 2 (HIV-2) and causes AIDS in experimentally infected macaques (28), this virus has served as a useful model for understanding AIDS pathogenesis in humans. Infected animals have been used for testing antiviral therapeutics and for evaluating potential vaccines (17).

Phylogenetic analysis has shown that HIV-1 and HIV-2 cluster with SIV strains isolated from chimpanzees (SIVcpz) and sooty mangabeys (SIVsm), respectively (5, 21, 42). Multiple SIVsm isolates exist in North America and West Africa and are all widely different from SIVmac (5). HIV could have originated as a result of cross-species transmission of SIV from African apes or monkeys to humans. However, inoculation of African and Asian monkeys with HIV-1 does not generally result in infection (29). In contrast, some strains of HIV-2 will establish a persistent infection in baboons, but an AIDS-like disease has been observed in a limited number of infected animals (2).

HIV-1 infection is initiated by the high-affinity interaction of the gp120-gp41 envelope glycoprotein (Env) complex with CD4 on the target cell surface (34). Next, the virus envelope fuses to the cell membrane, releasing the virus core into the cytoplasm. Potentiation of the fusion reaction has been re-

cently shown to require a cell surface cofactor, or coreceptor (1, 7, 15, 18–20). The coreceptors belong to the seven-transmembrane-domain G-protein-coupled family of cell surface receptors. The majority of macrophage-tropic (M-tropic) isolates of HIV-1 appear to specifically use CCR5, a receptor for the CC chemokines RANTES, MIP-1 α , and MIP-1 β , while the transformed T-cell line-tropic (T-tropic) viruses predominantly use CXCR4 (also known as LESTR, fusin, or HUMSTR), a receptor for the CXC chemokine stroma-derived factor 1. Other chemokine receptors, such as CCR2 and CCR3, were also active in mediating entry of particular HIV-1 isolates (7, 18). Incubating target cells with the appropriate chemokine (RANTES, MIP-1 α , or MIP-1 β for M-tropic viruses or SDF-1 for T-tropic HIV-1) inhibits HIV-1 replication by blocking entry (3, 10, 43). This inhibition is likely to be due to competition between the virus Env glycoprotein and the chemokine for coreceptor binding, although alternative mechanisms such as ligand-mediated desensitization have not been ruled out.

Like HIV-1, SIV initiates infection by interacting with target cell CD4 (8, 9). Whether fusion then requires a coreceptor is not known. There is evidence suggesting that this is the case. Like HIV-1, SIV does not infect all cells expressing CD4. Mouse, rat, rabbit, and cat cells expressing human CD4 fail to support SIV replication (38), a finding that suggests a requirement for a specific primate cofactor. Also, like HIV-1, SIV isolates exhibit differential tropism that is controlled by *env*. For HIV-1, tropism is largely controlled by the V3 loop of gp120 and, to a lesser extent, other regions of the protein (44, 53). Similarly, SIV tropism can be controlled by the V3-like region of gp130 (24, 27), although this has not been as clear as in the case of HIV-1. Tropism could be explained by differential coreceptor specificity. However, it has been shown that

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SIVmac239, a T-tropic isolate, could efficiently enter macrophages, but does not replicate, indicating that cell tropism is regulated at a postentry step for SIVmac239 (40). While such considerations may blur the meaning of the term "tropism," it is used here to indicate the ability of a given virus to productively replicate in various cell types.

AIDS induced by either HIV or SIV is associated with a gradual depletion of the CD4⁺ T cells, an outcome that is likely to result primarily from direct cytotoxicity of the viruses. In addition, lentivirus infection of monocytes/macrophages and microglial cells also appears to be involved with viral pathogenesis (16, 52). In a large fraction of infected humans, HIV-1 tropism appears to change during the disease course (12, 13, 51). During the early phase of infection, viral isolates are typically M-tropic. Late in the disease, T-tropic viruses can be isolated. Presumably, this phenotypic switch results from a shift of coreceptor usage from CCR5 to fusin or other chemokine receptors. While isolates of SIV may be characterized as M- or non-M-tropic, animals can proceed to AIDS without the appearance of M-tropic virus (16, 26). Macaques infected with SIVmac239, a non-M-tropic virus, proceed through the entire course of disease without the appearance of M-tropic viruses. In some animals, however, M-tropic virus did appear and was associated with lung and nervous system disorders (52). Moreover, a phenomenon similar to the phenotypic switch that occurs in many patients with AIDS was found in infected pig-tailed macaques (48). Viruses isolated early after infection with the M-tropic virus SIVMneCL8 tended to be M-tropic and replicate poorly in T-cell lines. Those isolated at late stages of infection exhibited enhanced replication in T-cell lines, a reduced ability to replicate in macrophages, and an increased ability to form syncytia.

Here, we investigate the coreceptor usage of various SIVmac isolates. We find that, like M-tropic HIV-1, the SIV isolates that we tested used CCR5. These viruses include genetically divergent SIVmac, SIVsm, and SIVcpz. None of simian viruses tested could use the human or simian CXCR4. The simian viruses did not distinguish between human and simian CCR5. SIVmac was shown to use another yet to be identified coreceptor expressed on CCR5 cells.

MATERIALS AND METHODS

Virus stocks and cell lines. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll gradient separation, and macrophages were enriched by adherence to plastic as described previously (6, 40). Human osteosarcoma HOS.CD4 and glioma U87.CD4 cells that express human CD4 and chemokine receptors have been described previously (15). CEMx174, a T-B-cell hybrid cell line (49), and transformed T-cell lines MT-2 and PM1 have been previously described (33).

Seed stocks of SIVmac251, T-cell line-passaged SIVmac251, SIVmac239/nef-open, and SIVmac239/316EM were provided by R. C. Desrosiers, New England Regional Primate Research Center, Southborough, Mass.) SIVmac251 (lot 8/1/94) was derived by expanding the seed virus in human PBMC, infecting a rhesus monkey, reisolating the virus, and growing it in vitro in rhesus PBMC. Primate SIVmac239/nef-open was prepared from a seed stock derived by transfecting rhesus PBMC with the proviral clone. SIVmac239/nef-open/5593 was derived by infecting rhesus macaque Rh-5593 with SIVmac239/nef-open and 6 months later reisolating the virus by growing it in rhesus PBMC. The SIVmac239/316EM stock was prepared by expanding seed stock SIVmac239/316EM in rhesus PBMC. SIVsmLib-1 and SIVsmSL92a were isolated from sooty mangabeys in West Africa as previously described (6, 36). SIVsmLib-1 and SIVsmSL92a stocks were cultured in CEMx174. SIV stocks were quantitated by SIV p27 enzyme-linked immunosorbent assay (ELISA) (Cellular Products, Buffalo, N.Y.). The 50% tissue culture infective dose (TCID₅₀) for SIVmac was measured by limiting dilution in CEMx174 cells.

SIVcpzGAB was isolated by cocultivating Molt-4 clone 8 cells with PBMC from an SIV-infected chimpanzee from Gabon (46). SIVcpzGAB, HIV-1JR-FL, and HIV-1NL4-3 stocks were propagated and titers were determined in human PBMC.

Luciferase reporter viruses were prepared by cotransfecting 293T cells with pNL-Luc-env⁻ or pSIV-Luc-vpr⁻env⁻ and vectors expressing different SIV or

HIV-1 Envs. HIV-1-based virus stocks pseudotyped by various HIV-1, SIVmac, or amphotropic murine leukemia virus (A-MuLV) Envs were generated as described previously (11). Briefly, the HIV-1-based reporter virus, pNL-Luc-Env⁻, consists of NL4-3 proviral clone containing a luciferase gene in the nef position and a frameshift in env. The virus is produced as pseudotypes bearing HIV, SIV, or MuLV Env supplied in trans in the producer cells. Because the provirus is env⁻, it is competent for only a single cycle of virus replication. Thus, the intracellular amount of luciferase activity present is a direct reflection of virus entry. Similarly, SIVmac-based virus stocks were made by pseudotyping SIV particles produced by SIV-Luc-Vpr⁻ Env⁻ with SIVmac239 and SIVmac1A11 Envs. SIV-Luc-Vpr⁻ Env⁻ consists of mac239 proviral clone containing a luciferase gene in the position and frameshifts in vpr and env. SIV reporter isolates were produced by transfecting 293T cells with 10 μg each of pSIV-Luc-Vpr⁻ Env⁻ and pSM-mac1A11-env or pcMac239-env. Mac1A11-env confers an M-tropic phenotype (35), whereas mac239-env does not (40, 41). Because Rev is required for efficient expression of Env, cotransfection was also performed in the presence of Rev expression vector pSM-mac1A11rev-env. Supernatants were harvested 48 h postcotransfection and frozen at -80°C. Reporter viruses were quantified by HIV p24 (Abbott, Chicago, Ill.) or SIV p27 (Cellular Products) ELISA.

SIV replication in monocytes/macrophages. SIVmac251, SIVmac239/nef-open, and SIVmac239/316EM stocks were tested for growth in monocyte/macrophage cultures as described previously (32). The TCID₅₀ of the SIV stocks was determined in CEMx174 cells (54). PBMC were isolated by centrifugation of heparinized blood on lymphocyte separation medium (Organon Teknika, Durham, N.C.) (54). PBMC were washed with Ca-Mg-free phosphate-buffered saline (PBS) and resuspended in macrophage medium (32) at 2 × 10⁶ viable cells/ml. After overnight incubation at 37°C, the nonadherent cells were gently removed by washing three times with 5.0 ml of Ca-Mg-free PBS. Next, 5 ml of trypsin (catalog no. 4424; Sigma Corp., St. Louis, Mo.) was diluted with 5 ml of Ca-Mg-free PBS, added to each flask, and incubated horizontally at 37°C for 1 to 2 min. The cell suspension was removed and pelleted at 5°C, and cells were resuspended at 5 × 10⁵ viable cells/ml in macrophage medium. Cells (5 × 10⁵ per well) were placed into 24-well plates, using separate plates for each virus stock. The plates were incubated for an additional 5 days at 37°C in a CO₂ incubator or until macrophages appeared flat and well spread. After 5 days, the cell cultures were washed and given another trypsinization plus wash as described above to remove nonadherent cells. Infection was with 1,000 TCID₅₀ of each virus stock. Infection was monitored by SIV p27 antigen released into the culture medium as described previously (37).

Construction of plasmids containing simian CD4, CCR5, and CXCR4. RNA was extracted from concanavalin A-stimulated PBMC by using Triazol (GIBCO-BRL, Grand Island, N.Y.) and treated with 10 U of RNase-free DNase (Promega, Madison, Wis.). cDNA was prepared from 5 μg of RNA by using Superscript reverse transcriptase (GIBCO-BRL) and resuspended in 80 μl of TE (10 mM Tris [pH 8.0], 1 mM EDTA). Specific cDNAs were amplified as specified by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.) in 100 μl containing 5 μl of cDNA, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 200 μM each dATP, dGTP, dCTP, and dTTP, 20 pmol of each primer, and 2.5 U of EXPAND high-fidelity DNA polymerase (Boehringer Mannheim). Amplification cycles were 95°C for 2 min followed by 35 cycles of 95°C for 15 s, 42°C for 45 s, and 72°C for 2.5 min plus the last extension of 72°C for 8 min.

PCR primers hybridizing to the 5' and 3' untranslated regions of rhesus CD4 (Rh-CD4), human CCR5 (Hu-CCR5), and Hu-CXCR4 were the following: for CD4, 5'-TAA GGA TCC GCC CTG CCT CCC TCA GCA AGG CCA CAA TG-3' and 5'-TAG CTC GAG TGC AAT TGG GAT CTC CCT GGC CTC GTG CC-3'; for CXCR4, 5'-GGT GAA TTC ACC GCA TCT GGA GAA CCA GCG GTT ACC ATG-3' and 5'-GCG CTC GAG TAT CGT ATA AAA AAA AGT CTT TTA CAT CTG-3'; for CCR5, 5'-CCC CCA ACA GAG CCA AGC TCT CCA TCT AG-3' and 5'-CTG TGT ATG AAA ACT AAG CCA TGT GCA CAA C-3', (first round) and 5'-GCT GAG GAT CCT TTT ATT TAT GCA CAG GGT GGA ACA AGA TG-3' and 5'-GCG GAG CTC GAG GAC TGG GTC ACC AGC CCA CTT GAG TCC GTG-3' (second round). Underlines indicate inserted restriction site for ligating to plasmid DNA.

Amplified PCR products were purified by using a PCR-prep kit (Promega). Rh-CCR5 cDNA was digested with *Bam*HI and *Xho*I, and Rh-CXCR4 was digested with *Eco*RI and *Xho*I and cloned into the appropriately cleaved retrovirus expression vector pBABE-puro (39) and into the cytomegalovirus promoter-driven expression vector pcDNA-1/amp (Invitrogen Corporation, San Diego, Calif.). Nucleotide sequences of the cDNAs in both vectors were determined on both strands, using a T7 Sequenase version 2.0 DNA sequencing kit (Amersham Life Science, Cleveland, Ohio). Uncloned cDNA fragments were sequenced by using a T7 Sequenase PCR product sequencing kit (Amersham Life Science).

Sequences were aligned with MacVector software (International Biotechnology, Inc.), and amino acid homologies were calculated by using the Genetics Computer Group protein distance program without correction (22).

Env expression vectors for HIV-1 JR-FL, ADA, and HXB2 as well as A-MuLV and mac1A11 have been described previously (11, 15). pcMac239-env vector for expressing mac239 Env was constructed by amplifying the env gene from SIVmac239 proviral DNA with primers containing *Eco*RI (5'-T CGA GAA TTC ATG GGA TGT CTT GGG AAT CAG C-3') and *Xho*I (5'-T CGA

CTC GAG GTC CTG TTG GAT ATG GGT CTA-3') sites (underlined) hybridizing at the initiation codon on the 5' end and at the termination codon at the 3' end.

Expression of Rh-CD4, Rh-CCR5, and Rh-CXCR4. Virus stocks for the retrovirus vectors expressing Rh-CCR5 and Rh-CXCR4, pBabe/Rh-CCR5, and pBabe/Rh-CXCR4, respectively, were prepared by transfecting 293T cells with the MuLV *gag/pol* vector pSV- ψ -MuLV-*env*⁻ and pcVSV-G as described previously (30). Virus was harvested 48 h posttransfection and frozen in aliquots. HOS.CD4 and U87.CD4 cells were infected with 2 ml of the pBabe virus and were selected 48 h later in culture medium containing puromycin (1.0 μ g/ml; Sigma). Puromycin-resistant cells were then used in HIV-1 and SIV Env-mediated fusion assays and for infection with replication-competent or luciferase reporter virus.

For transient expression, 293T cells were transfected with cytomegalovirus-based expression vectors pcRh-CD4, pcRh-CCR5, and pcRh-CXCR4. Expression of Rh-CD4 was verified by flow cytometry of transfected 293T and HOS cells stained with 0.5 μ g of phycoerythrin-conjugated Leu3a. To test the function of Rh-CCR5 or Rh-CXCR4, HOS cells were cotransfected with pcRh-CCR5 or pcRh-CXCR4 (15 μ g) together with pcRh-CD4 (10 μ g). After 24 h, the cells (2×10^4) were seeded in 24-well plates. Luciferase reporter virus was added 24 h later.

Syncytium formation assay. 293T cells were transfected with pcDNA-I/Amp-based vectors encoding different SIV or HIV-1 Envs. After 48 h, the transfected cells (2×10^4) were mixed in 24-well dishes with an equal number of HOS.CD4 or U87.CD4 cells that express Rh-CCR5 or Rh-CXCR4. After 6 to 12 h of cocultivation, the cultures were photographed by phase-contrast microscopy.

Infectivity assays. CEMx174, MT-2, or PM1 cells (10^4) were infected with 0.5 ng (p27) of each SIV strain per ml or 500 TCID₅₀ of each HIV-1 strain. After 12 h, input virus was removed by replacing the culture medium three times. Syncytium formation was scored by counting multinucleated giant cells in each well.

To determine coreceptor usage of different viruses, HOS.CD4 or U87.CD4 cells (2×10^4) stably expressing human or rhesus coreceptors were seeded in 24-well plates. The next day, the cells were infected with 0.5 ng of SIV per ml or 500 TCID₅₀ of HIV-1 or SIVcpz. After 12 h, input virus was removed by replacing the medium three times. The cells were trypsinized 6 days later, and supernatant plus cells was transferred into six-well plates. Uninfected cells (5×10^4) of each were added to each culture. Virus production was measured by harvesting aliquots of each supernatant and measuring p27 or p24 by ELISA.

For luciferase reporter virus assays, cells (2×10^4) were seeded in 24-well dishes in culture medium and infected with luciferase reporter virus (50 ng p24 or p27) in a total volume of 500 μ l. After incubation overnight, 1 ml of medium was added to each well. After 3 additional days of culture, 100- μ l lysates were prepared and luciferase activity in 20 μ l was measured by using commercially available reagents (Promega).

Reverse transcription (RT)-PCR analysis of CCR5 transcripts. RNA was purified from CEMx174, MT-2, and PM1 cells by using Triazol (GIBCO-BRL) and used as the template (5 μ g) for a first-strand cDNA reaction using Superscript reverse transcriptase (GIBCO-BRL) and an oligo(dT) primer as described above. The cDNA was amplified over 35 cycles, using the second-round primers specific for full-length *ccr5* as shown above or primers 5'-CTC TGT YAC CCA YAT CTG CCG AGA-3' and 5'-CCT CAG AAT GAT ATT TGT CCT CAT GGT A-3' for mitochondrial cytochrome *b* gene to test the success of cDNA synthesis.

Nucleotide sequence accession numbers. The sequences of Rh-CCR5 and Rh-CXCR4 have been submitted to GenBank (accession numbers U73739 and U73740, respectively).

RESULTS

Rhesus and human coreceptors are highly conserved. CCR5 and CXCR4 genes were amplified by RT-PCR from rhesus PBMC RNA, using primers corresponding to the 5' and 3' untranslated regions of the human genes and cloned into plasmids. Nucleotide sequences were determined for the complete coding regions of two Rh-CCR5 and Rh-CXCR4 cDNAs derived from two different rhesus macaques (Rh-541 and Rh-1550). The two Rh-CCR5 and Rh-CXCR4 sequences were identical to each other and were highly homologous to their human counterparts. Rh-CCR5 and Rh-CXCR4 genes have up to 98.3% identity with the corresponding human genes (Table 1). Human and rhesus CCR5 differed by eight amino acids (Fig. 1A), and the majority of these were substitutions of similar amino acids (V to I, K to R, and I to M). The two CXCR4 sequences differed at six positions (Fig. 1B), three of which were conservative changes (M to I and K to R). Both coreceptors had amino acid changes in the extracellular amino termi-

TABLE 1. Percentages of sequence homology between human and rhesus CCR5 and CXCR4

Chemokine receptor	% Homology			
	Rh-CCR5		Rh-CXCR4	
	Amino acid	Nucleotide ^a	Amino acid	Nucleotide
Hu-CCR5	97.7	97.6	29.7	10.4
Hu-CXCR4	29.7	12.6	98.3	97.7

^a Based on Kimura's two-parameter formula.

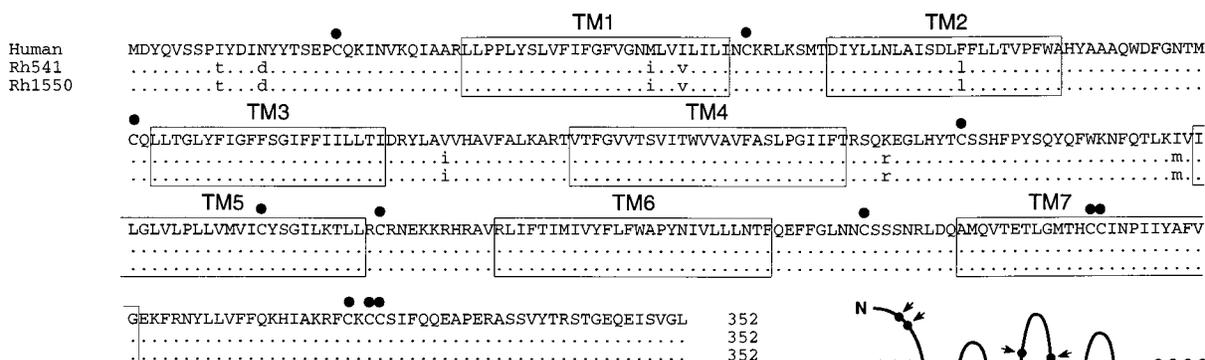
nus and in the second extracellular loop of the coreceptor that could conceivably play a role in chemokine binding or viral Env interaction. Thus, the simian genome encodes proteins corresponding to the two most active human coreceptors, ruling out the possibility that the inability of HIV-1 to infect rhesus monkeys is due to the absence of the coreceptor genes but leaving the possibility that amino acid differences play a role.

In humans, a 32-bp-deleted allele is common in some populations (30). We tested for a similar deletion might occur in monkeys; however, the sizes of the CCR5 RT-PCR products amplified from 85 additional rhesus macaques matched the Rh-CCR5 sequence. Polymorphisms may exist in Rh-CCR5, but detecting these will require sequencing of a larger number of alleles.

Rh-CCR5 but not Rh-CXCR4 mediates fusion with a panel of SIVmac Envs. To evaluate whether Rh-CCR5 and Rh-CXCR4 were active as coreceptors, we tested whether they would mediate membrane fusion with HIV-1 Env. Coreceptor-mediated fusion was measured by the ability of HOS or U87 cells stably expressing human CD4 and coreceptor to fuse to cells expressing Env following a brief cocultivation. HOS cells do not express detectable amounts of endogenous CCR5 but do express very low levels of CXCR4, whereas U87 cells express no detectable amounts of either human chemokine receptor. HOS.CD4-Rh-CCR5 and HOS.CD4-Rh-CXCR4 cells were cocultivated with 293T cells transiently transfected with an expression vector for HIV-1 or SIV Env. 293T cells expressing M-tropic HIV-1 Env JR-FL formed large multinucleated giant cells when mixed with HOS.CD4-Rh-CCR5 cells but did not fuse to HOS.CD4-Rh-CXCR4 cells (Fig. 2A). Conversely, 293T cells expressing the T-tropic HIV-1 Env HXB2 formed large syncytia with HOS.CD4-Rh-CXCR4 but not with HOS.CD4-Rh-CCR5. Similar results were obtained for U87.CD4 cells expressing the simian coreceptors (data not shown). Thus, both rhesus chemokine receptors were active for HIV-1, with a specificity similar to that of the analogous human coreceptors. This finding was consistent with the ability of SIV-HIV recombinants containing the env of HIV-1 to replicate in monkeys (23, 25, 32, 47).

We next tested whether the two rhesus chemokine receptors would mediate membrane fusion with SIVmac Env. 293T cells were transfected with vectors expressing mac239 or mac1A11 Env, and these cells were cocultivated with HOS.CD4-Rh-CCR5 or HOS.CD4-Rh-CXCR4 cells. HOS.CD4-Rh-CCR5 cells formed multinucleated syncytia with 293T cells expressing either of the SIVmac Envs (Fig. 2B). Rh-CXCR4-expressing cells did not form syncytia with cells expressing either SIV Env. Similar results were obtained with cultures containing U87.CD4-Rh-CCR5 or U87.CD4-Rh-CXCR4. Control cultures with parental HOS.CD4 or U87.CD4 cells or untransfected 293T cells did not show detectable syncytia, indicating that coreceptor and Env interaction was required for cell fusion.

A. CCR5



B. CXCR4

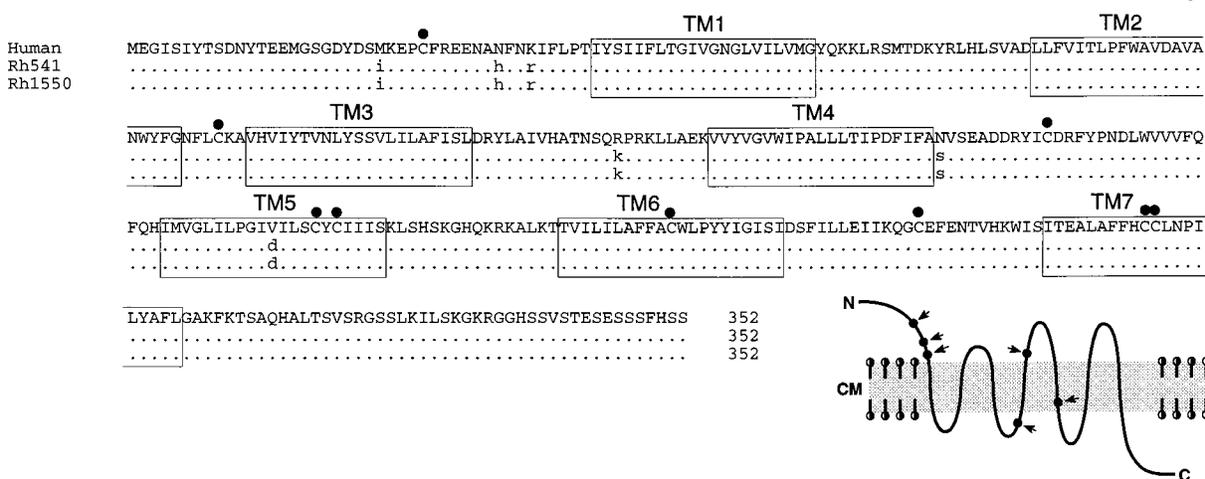


FIG. 1. Sequence alignment of human and rhesus CCR5 and CXCR4. Nucleotide sequences of CCR5 and CXCR4 cDNAs were derived from animals Rh-541 and Rh-1550 by RT-PCR. Rh-CCR5 (A) and Rh-CXCR4 (B) sequences were compared to the human sequences in GenBank. Cysteine residues are indicated by filled dots; the seven transmembrane domains (TM1 to TM7) (31, 50) are indicated by boxes; amino acid differences with the human sequences are shown in lowercase; insets show approximate locations of amino acid residues that differed between the two species. N, amino terminus; C, carboxy terminus; CM, cell membrane.

Rh-CCR5 mediates entry of SIVmac239 and SIVmac1A11.

CCR5 and CXCR4 function as coreceptors for entry of M- and T-tropic HIV-1, respectively. To test whether SIV also uses these two chemokine receptors, we used a luciferase reporter virus entry assay (11). For this assay HOS.CD4 cells expressing the different chemokine receptors were infected with single-cycle HIV-1- or SIVmac-based reporter viruses pseudotyped by various Envs. To control for postentry-related effects, cells were infected with A-MuLV pseudotyped viruses. In addition to the HIV-1-based luciferase reporter viruses, we used pseudotyped SIV-based reporter viruses to ensure that SIV Env function was not affected by the virus core. These viruses were produced by using pSIV-Luc-Vpr⁻Env⁻, a plasmid analogous to pNL-Luc-Env⁻ but based on an SIVmac239 provirus.

We demonstrated that pseudotypes bearing either the mac1A11 or mac239 Env efficiently infected HOS.CD4-Rh-CCR5 (Fig. 3A). They also infected HOS.CD4-Hu-CCR5 cells (Fig. 3A). In contrast, none of the SIVenv pseudotypes infected HOS.CD4 cells expressing human or simian CXCR4. Several controls confirmed the appropriate specificities of the

cells and viruses used. All cell types were infected to similar extents by the A-MuLV pseudotype, ruling out postentry phenomena. Reporter virus pseudotyped by M-tropic (JR-FL and ADA) Env-infected HOS.CD4 cells expressing human or rhesus CCR5 but did not infect HOS.CD4 cells expressing human or rhesus CXCR4 (Fig. 3A). In contrast, the T-tropic HIV-1 pseudotype-infected cells expressing human or rhesus CXCR4 but not those expressing human or rhesus CCR5. Cells not expressing a transfected coreceptor were efficiently infected only by the A-MuLV pseudotypes (Fig. 3A). The HXB2 pseudotype infected each cell type to a small extent, presumably as the result of low-level endogenous Hu-CXCR4 expression. SIVmac1A11 pseudotypes acted similarly regardless of whether they contained an HIV-1 or SIVmac core. Surprisingly, the mac239 Env was able to efficiently pseudotype HIV-1 cores but failed to form infectious pseudotypes with an SIVmac core (data not shown). This finding may be related to those of Zingler and Littman showing that the highly reduced infectivity of reporter viruses bearing mac239 Env compared to

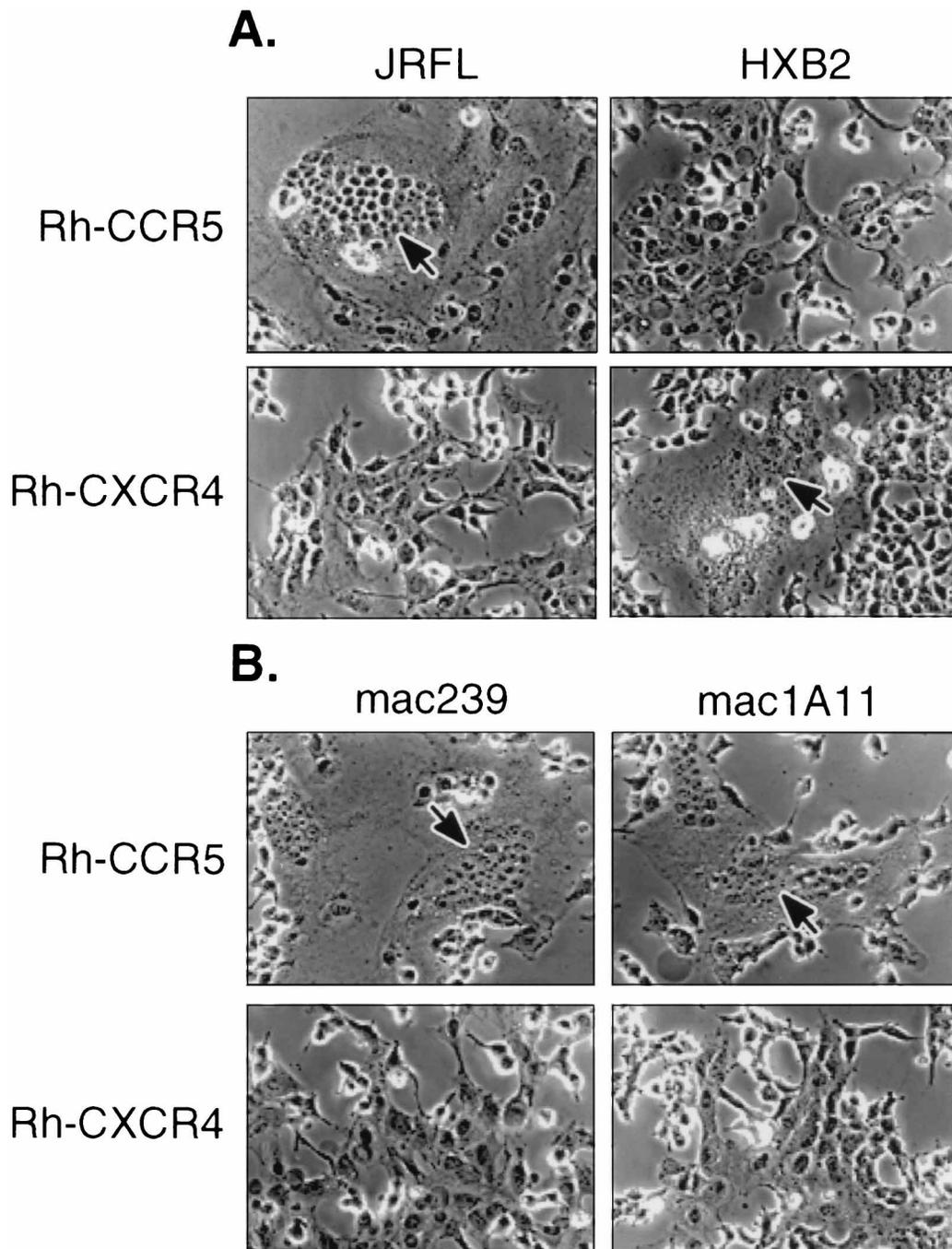


FIG. 2. Rh-CCR5 mediates cell fusion with SIV or HIV-1 Env, but Rh-CXCR4 mediates fusion only with HIV-1. (A) 293T cells were transfected with vector expressing M-tropic (JR-FL) or T-tropic (HXB2) HIV-1 Env. The cells were cocultivated overnight with HOS.CD4.Rh-CCR5 or HOS.CD4.Rh-CXCR4 cells. (B) The same panel A except that the 293T cells were transfected with vectors expressing SIV Env mac239 or mac1A11. Arrows indicate large multinucleated syncytia.

mac1A11 Env (56) may be due to different efficiencies of protein processing in human cells.

Because HOS.CD4 cells appear to express low amounts of endogenous CXCR4, it remained possible that this small amount of coreceptor could influence SIV Env-mediated entry (Fig. 3A). We therefore confirmed these results in assays using U87.CD4 cells. These cells do not express functional CXCR4 or detectable amounts of CXCR4 mRNA. The control U87.CD4.Babe cells and cells expressing CCR5 were highly

resistant to entry of HXB2 pseudotype yet remained fully infectable by the SIVmac pseudotypes (Fig. 3B). However, U87.CD4 cells expressing CXCR4 was infected by HXB2. Thus, mac239 and mac1A11 Envs use CCR5 and do not use CXCR4 to a significant extent.

Rhesus and human CD4 differ at 37 amino acid positions (4). It was possible, therefore, that the failure of CXCR4 to act as a coreceptor for SIV Env pseudotypes was due to an incompatibility between the rhesus coreceptor and Hu-CD4 on the

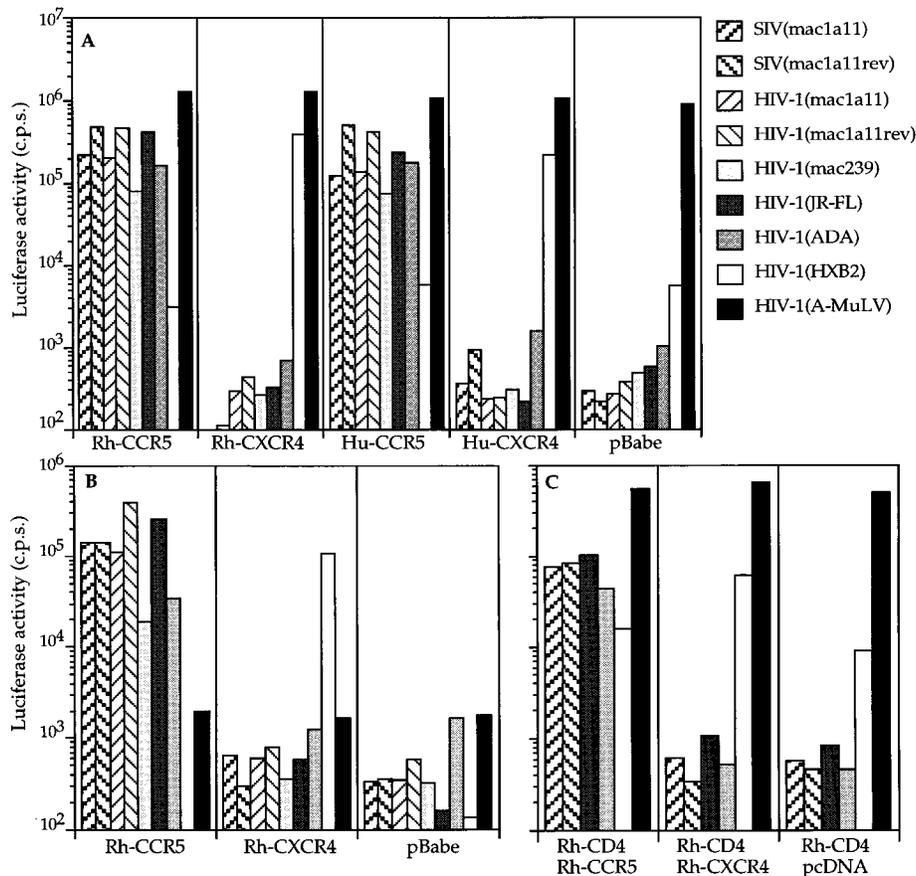


FIG. 3. Entry of luciferase reporter viruses into cells expressing human or rhesus CCR5 or CXCR4 were infected with HIV-1 or SIVmac239 luciferase reporter viruses pseudotyped by various SIV (mac1A11 or mac239), HIV-1 (JR-FL, ADA, and HXB2), or A-MuLV Envs (indicated in parentheses). Mac1A11rev contains the Rev coding sequence in addition to Env. Luciferase activity was measured on day 4. HOS.CD4 cells containing pBabe-puro (pBabe) were used for control of expression of endogenous coreceptors. (B) U87.CD4 cells stably expressing Rh-CCR5 or Rh-CXCR4 were infected with the reporter viruses as for panel A. A-MuLV pseudotypes reproducibly infected U87.CD4 cells inefficiently. Luciferase activity of HXB2 pseudotype on Rh-CCR5 cells was less than 100 cps. (C) HOS cells were transiently cotransfected with pcRh-CD4 and pcRh-CCR5 or pcRh-CXCR4. The cells were infected 2 days later with the luciferase reporter viruses and assayed as described for panel A. HOS cells supported some entry of T-tropic pseudotypes, possibly due to expression of endogenous CXCR4 or some other unidentified coreceptor. This experiment was repeated with similar results.

HOS.CD4 cells. To test this possibility, HOS cells were cotransfected with expression vectors for RhCD4 and coreceptor. The results were unchanged. Rh-CCR5 but not Rh-CXCR4 acted as a coreceptor for SIV Env pseudotypes (Fig. 3C). Therefore, both the human and simian CD4 molecules can function in concert with the relevant coreceptors to mediate SIV entry. Furthermore, Rh-CD4 does not permit use of Rh-CXCR4 as an SIVmac1A11 coreceptor.

Rhesus and human CCR5 support replication of diverse SIV strains. We next compared the abilities of various SIV strains to replicate in cells expressing CCR5 or CXCR4. To do this, the coreceptor-expressing HOS.CD4 and U87.CD4 cells were infected with different strains of SIV. The viruses tested included SIVmac239/nef-open; SIVmac239/nef-open/5593, a derivative of mac239 isolated after *in vivo* passage in an infected macaque for 6 months; SIVmac251, derived from SIVmac251 seed stock after *in vivo* passage in a macaque for 6 months; SIVmac239/316EM, a derivative of mac239 containing five amino acids in Env that allow it to replicate in macrophages; SIVsmSL92a and SIVsmLib-1, divergent viruses isolated from African sooty mangabeys (6, 36); and SIVcpzGAB, isolated from a chimpanzee in Gabon. M-tropic (JR-FL) and T-tropic (NL4-3) HIV-1 isolates were used to demonstrate appropriate coreceptor expression on each cell type.

Each of the SIV strains (Fig. 4), including the genetically divergent isolates SIVmac, SIVsm, and SIVcpz, replicated efficiently on HOS.CD4 and U87.CD4 cells expressing CCR5. Because the SIV strains tested had been grown in primary rhesus PBMC, we also determined the coreceptor specificity of SIVmac251 that had been extensively passaged in MT4, a transformed human T-cell line. Under the same culture conditions as used for Fig. 4, the T-cell-passaged SIV was found to retain its use of CCR5, yielding 5.3×10^3 and 3.2×10^5 pg of p27 per ml in HOS.CD4.CCR5 cells at days 4 and 7 postinfection, respectively. In contrast, p27 yields on HOS.CD4.CXCR4 were <10 pg/ml at both time points for the T-cell-passaged SIVmac251. None of the viruses distinguished between the human and rhesus coreceptors. They did not replicate in either cell type expressing CXCR4. Several of the viruses appeared to replicate slightly better in the U87.CD4 cells than in the HOS.CD4 cells. The reason for this was not clear, but it could have been due to differences in coreceptor expression or to a postentry phenomenon unrelated to CD4 or coreceptor.

Phenotypic characterization of genetically diverse SIV strains. Because all SIV strains tested used CCR5, it was not clear whether these viruses would show phenotypic differences in macrophage and T-cell lines. We used two methods to confirm the phenotypes of the viruses. First, the viruses were

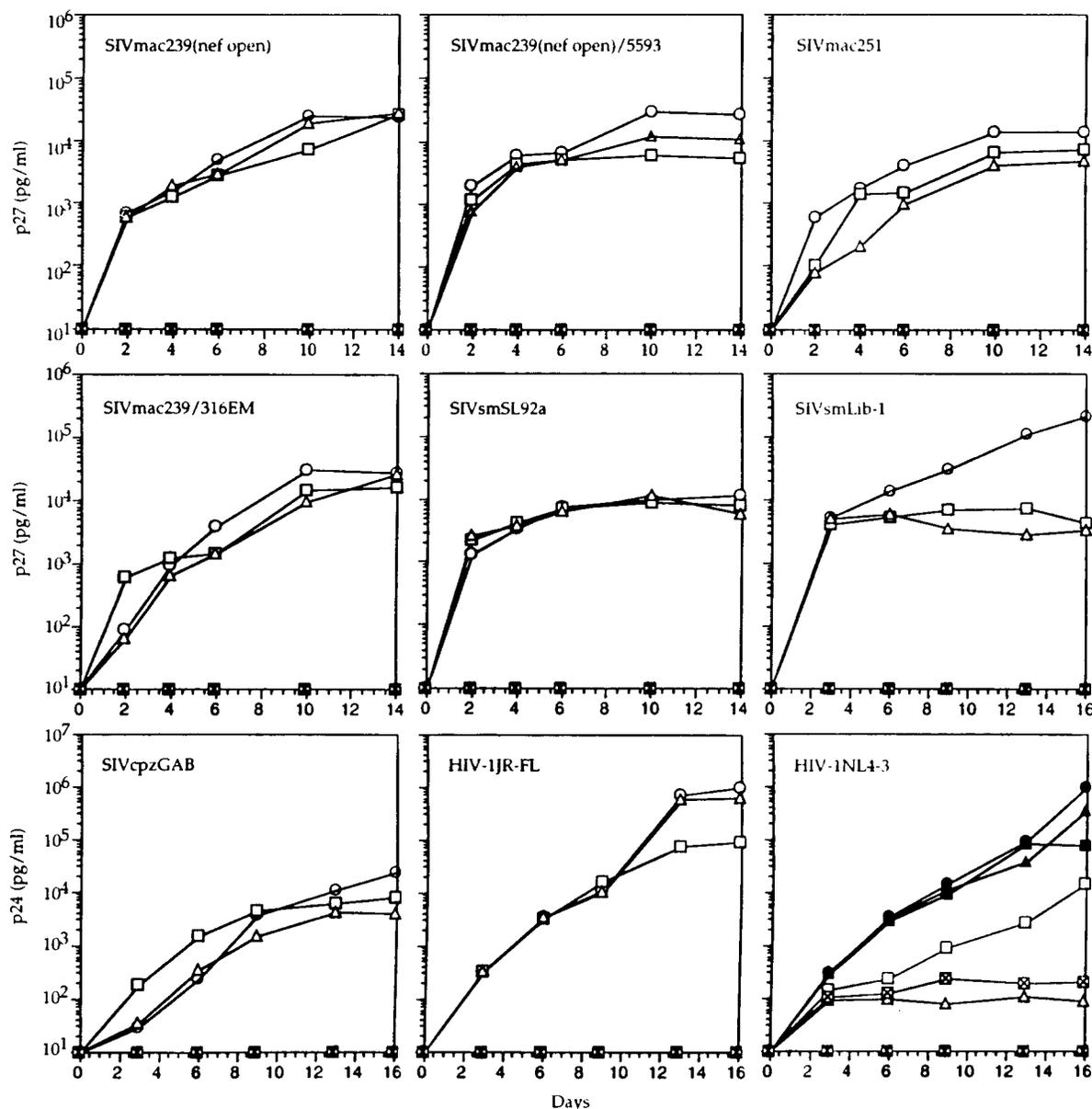


FIG. 4. Replication of SIV in HOS.CD4 and U87.CD4 stably expressing human or rhesus CCR5 or CXCR4. The cell lines tested include U87.CD4-Rh-CCR5 (○), U87.CD4-Rh-CXCR4 (●), U87.CD4.Babe (◆), HOS.CD4-Rh-CCR5 (□), HOS.CD4-Rh-CXCR4 (■), HOS.CD4-Hu-CCR5 (△), HOS.CD4-Hu-CXCR4 (▲), and HOS.CD4.Babe (■). Each virus was tested in all eight cell lines. ■ depicts cells that gave negative results. Supernatants were collected every 2 to 4 days after inoculation for p24 and p27 antigen ELISA.

tested for the ability to replicate in rhesus macrophages. The results showed that SIVmac251 and SIVmac239/316EM replicated in macrophages whereas SIVmac239/nef-open and SIVmac239/nef-open/5593 did not (Fig. 5). Thus, each of the viral stocks maintained single or dual tropism as described previously (14, 16, 40, 41), even after passage in vivo. SIVsmSL92a and SIVsmLib-1 also replicated on human macrophages (data not shown). Thus, SIV phenotype is probably controlled by factors in addition to coreceptor usage.

To further characterize the tropism of the viruses, each was then tested for the ability to replicate and form syncytia on three transformed T-cell lines (Table 2). An M-tropic non-syncytium-inducing (NSI) (HIV JR-FL) and a T-tropic syncytium-inducing (SI) (HIV NL4-3) isolate were used as controls.

The two HIV-1 isolates showed the expected tropism phenotype. We found that each of the SIV strains replicated to high titer on the three cell lines (Table 2). In addition, each strain formed syncytia on CEMx174 and PM1 cells. While each of the viruses replicated in MT-2 cells, only some of them formed visible syncytia. Interestingly, the non-M-tropic viruses (SIVmac239/nef-open and SIVmac239/nef-open/5593) tended to form syncytia more readily than the M-tropic viruses (SIVmac239/316EM, SIVmac251, SIVsmLib-1, and SIVsmSL92a). This situation is analogous to that of HIV-1, where M-tropism is generally associated with NSI phenotype while T-tropism is associated with SI phenotype. However, in the case of SIV, differences in syncytium-forming ability are not associated with differences in coreceptor usage. In addition, for HIV-1, M-

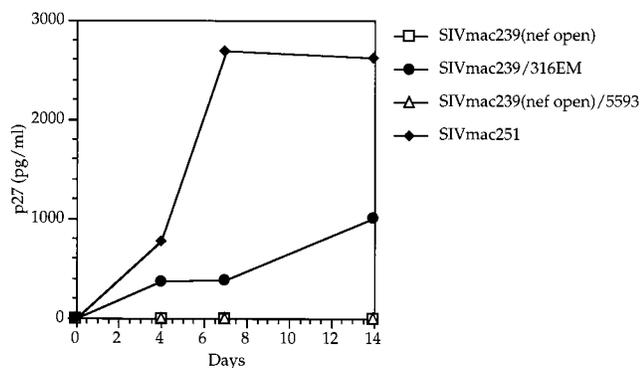


FIG. 5. Replication of SIVmac in rhesus macrophages. Cultures of rhesus macrophage were prepared and inoculated with the indicated SIV strain (3,000 TCID₅₀) as described in the Materials and Methods. A portion of the supernatant was collected on days 0, 4, 7, and 14 for p27 antigen measurement. Each point is the average of duplicate measurements.

tropism is generally associated with an inability to replicate in transformed T-cell lines. This is not the case for SIV, which regardless of phenotype and tropism for macrophages, grows efficiently in transformed T-cell lines.

Evidence for an additional SIV coreceptor. PM1 cells express CCR5 and as a result support replication of M-tropic strains of HIV-1 (15, 33). It was therefore expected that the SIV strains, each of which was used for CCR5, would replicate on PM1 cells. In general, MT-2 and CEMx174 cells do not support replication of M-tropic CCR-5-specific HIV-1 isolates such as JR-FL (Table 2). Thus, it is surprising that these two cell lines supported replication of SIVmac strains (Table 2), each of which was specific for CCR5 and four of which were M-tropic. CEMx174 cells are generally recognized as one of the most efficient producers of SIVmac and are routinely used for preparing high-titered stocks of this virus.

To test whether these findings could be due to low-level expression of CCR5 in the CEMx174 or MT-2 cells, we amplified CCR5 transcripts by RT-PCR from these cells and from PM1 cells. CCR5 transcripts could not be detected in CEMx174 or MT-2 cells but were readily detected in PM1 cells (Fig. 6). However, it was possible that SIV used another coreceptor besides CCR5 that is expressed in PM1 cells. To definitively rule out the possibility that replication in the cell lines could be due to low-level expression of CCR5, we tested the abilities of the viruses to replicate on human PBMC genetically defective for CCR5 expression. These PBMC were isolated

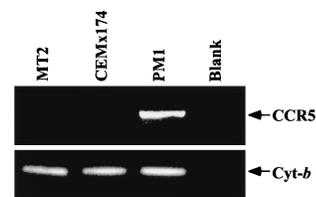


FIG. 6. RT-PCR analysis of CCR5 transcripts in MT-2, CEMx174, and PM1 cells. RNA isolated from the three cell lines was amplified with primers specific for Hu-CCR5 or for mitochondrial cytochrome b (Cyt-b) as a control. Blank, no mRNA added.

from individuals who are homozygous for a 32-bp deletion in CCR5 (ccr5⁻/ccr5⁻) (30). As expected, M-tropic HIV-1 did not replicate efficiently in these cells (Table 3). In contrast, SIVmac replicated in the CCR5⁻ cells, indicating a lack of requirement for this coreceptor.

Taken together, these findings suggested that SIV uses an additional, yet unidentified coreceptor present in CEMx174, MT-2, and CCR5⁻ cells. To test whether this coreceptor might correspond to a known chemokine receptor, SIVmac239, SIVmac251, and SIVsm isolates were tested for the ability to replicate on HOS.CD4 cells expressing CCR1, CCR2b, CCR3, and CCR4. None of these supported detectable levels of virus replication or entry of any of these viruses (data not shown). Thus, it is likely that SIVmac and SIVsm are able to use a second, yet unidentified coreceptor.

DISCUSSION

We show here that SIV, like HIV-1, requires a seven-transmembrane-domain G-protein-coupled chemokine receptor that acts in concert with CD4 to mediate entry. This was the case for the genetically divergent SIV strains SIVmac, SIVsm, and SIVcpz and may be a general feature of HIV and SIV entry. Importantly, all of the SIV strains tested here used CCR5, the major coreceptor for M-tropic HIV-1 isolates; CXCR4, the coreceptor for most T-tropic HIV-1 isolates, did not appear to mediate entry of any of the SIV strains tested, nor did any other chemokine receptors tested.

In the case of HIV-1, T-tropic and M-tropic viruses differ in their coreceptor usage (7, 15, 18–20). Coreceptor specificity, coupled with restricted cell-type expression of coreceptors, is likely to account for differential tropism of viruses specific for different coreceptors. For example, CCR5 but not CXCR4 may be present on macrophages, accounting for the known tropism of viruses specific for these two coreceptors. The fac-

TABLE 2. Replication and phenotypes of SIV and HIV-1 strains in human cell lines

Virus	CEMx174		MT2		PM1	
	Syncytia ^a	p27 or p24 ^b (pg/ml)	Syncytia	p27 or p24 (pg/ml)	Syncytia	p27 or p24 (pg/ml)
SIVmac239/nef-open/5593	++++	1.6 × 10 ⁵	++	1.8 × 10 ⁵	++++	2.9 × 10 ⁵
SIVmac239/nef-open	++++	1.8 × 10 ⁵	++	1.8 × 10 ⁵	++++	3.1 × 10 ⁵
SIVmac239/316EM	++++	2.6 × 10 ⁵	+	1.1 × 10 ⁴	++++	1.8 × 10 ⁵
SIVmac251	++++	2.6 × 10 ⁵	–	2.7 × 10 ⁵	++++	2.8 × 10 ⁵
SIVsmLib-1	++++	1.6 × 10 ⁵	–	6.3 × 10 ⁴	++++	2.8 × 10 ⁵
SIVsmSL92a	++++	2.7 × 10 ⁵	–	2.8 × 10 ⁵	++++	2.7 × 10 ⁵
HIV-1JR-FL	–	<10	–	<10	–	5.6 × 10 ⁵
HIV-1NL4-3	–	4.6 × 10 ⁵	++++	6.6 × 10 ⁵	++++	5.7 × 10 ⁵

^a Syncytium formation was visually scored in cell culture up to 16 days postinfection as >85% (++++), 25 to 50% (++), or <25% (+) cells with syncytia or no syncytium formation (–).

^b Assayed at day 16 postinfection. Multiple time points were sampled, and the time points shown are representative of those data.

TABLE 3. SIV replication in human cells genetically defective for CCR5

Virus (500 TCID ₅₀)	Supernatant p27 or p24 (pg/ml) ^a			
	ccr5 ⁻ /ccr5 ⁻ PBMC ^b		ccr5 ⁺ /ccr5 ⁺ PBMC	
	Day 6	Day 11/12 ^c	Day 6	Day 11/12
SIVmac239/nef-open/5593	4.4 × 10 ²	4.5 × 10 ³	2.6 × 10 ²	1.6 × 10 ³
SIVmac251	6.4 × 10 ²	4.2 × 10 ³	9.2 × 10 ²	4.4 × 10 ³
HIV-1JR-FL	<10	<10	2.1 × 10 ⁵	6.3 × 10 ⁴

^a Data are averages of two time points, and experiment was reproduced three times.

^b 2 × 10⁵ cells.

^c HIV-1 JRFL samples were collected on day 12 postinfection.

tors governing SIV tropism are less clear. All of the viruses that we used here were specific for CCR5, yet some replicated in macrophages whereas others did not. Thus, for SIV, factors other than differential coreceptor can contribute to tropism. Evidence supporting this possibility was provided by Mori et al., who showed that SIVmac239, a T-tropic virus, was competent for entry into macrophages but failed to replicate due to a restriction postentry (40). These findings do not rule out the possibility that differential coreceptor usage plays a role in SIV tropism; however, this will await identification of other coreceptors.

The ability of SIVmac to infect CCR5⁻ cells suggested that some strains could use coreceptors besides CCR5. The finding was that these same strains failed to infect HOS cells expressing CXCR4. Determining whether any HIV isolates also use this coreceptor awaits its identification.

Transmission and early replication of HIV-1 in infected individuals appears to be mediated by NSI M-tropic viruses that are largely specific for CCR5. Later in the course of disease, in about 50% of infected individuals, when symptoms appear, SI T-tropic viruses also appear (12, 13, 51, 55). A phenotypic switch has been reported in SIV-infected animals (48). Viruses isolated early in the disease tended to be M-tropic; late in the disease, virus isolates tended to lose M-tropism and gain ability to infect transformed cell lines. It is not known whether this change in phenotype is due to a switch in coreceptor usage. All of the viruses tested here were specific for CCR5 and not for CXCR4. However, it is possible that detecting CXCR4-specific SIV will require isolation of virus during later stages of disease. Furthermore, it is possible that coreceptor switching could occur with the unidentified coreceptor that we detected or with yet other undetected coreceptors.

The importance of CCR5 as an entry cofactor for HIV-1 in humans was indicated by the finding that a homozygous defect in the CCR5 gene appeared to convey resistance to infection (30, 45). The use of CCR5 by SIV lends further support for the central role played by this chemokine receptor both in human and simian AIDS. The finding that the majority of SIV and HIV-1 isolates share a requirement for the use of CCR5 for viral entry further strengthens the relevance of SIV as a model for AIDS. Furthermore, the human and rhesus coreceptors are highly conserved, differing at only eight (CCR5) or six (CXCR4) amino acid residues, and each simian protein is competent to mediate entry of either NSI M-tropic or SI T-tropic HIV-1 strains. The findings indicate that the coreceptor usage is not a species restriction for HIV-1 infection in macaques. Thus, the SIV system should prove useful for testing potential therapeutics that may be developed to target CCR5 and for understanding the pathogenesis and molecular biology

of AIDS. However, further study will be needed to first identify the coreceptors that are used in vivo by both HIV-1 and SIV.

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