Structure and Function of CC-Chemokine Receptor 5 Homologues Derived from Representative Primate Species and Subspecies of the Taxonomic Suborders Prosimii and Anthropoidea

Kevin J. Kunstman,1 Bridget Puffer,2 Bette T. Korber,3 Carla Kuiken,3 Una R. Smith,3 Jennifer Kunstman,1 Jennifer Stanton,1 Michael Agy,4 Riri Shibata,5 Anne D. Yoder,6† Satish Pillai,2 Robert W. Doms,2 Preston Marx,8 and Steven M. Wolinsky1*

Departments of Medicine1 and Cell and Molecular Biology,6 The Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611; Department of Pathology, University of Pennsylvania, Philadelphia, Pennsylvania 191042; Theoretical Biology and Biophysics, Theoretical Division, Los Alamos National Laboratory, Los Alamos, New Mexico 875453; National Primate Research Center, University of Washington, Seattle, Washington 981954; The Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 208925; Departments of Zoology and Tropical Medicine, Tulane Regional Primate Center, Tulane University Medical Center, New Orleans, Louisiana 70433

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A chemokine receptor from the seven-transmembrane-domain G-protein-coupled receptor superfamily is an essential coreceptor for the cellular entry of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) strains. To investigate nonhuman primate CC-chemokine receptor 5 (CCR5) homologue structure and function, we amplified CCR5 DNA sequences from peripheral blood cells obtained from 24 representative species and subspecies of the primate suborders Prosimii (family Lemuridae) and Anthropoidea (families Cebidae, Callitrichidae, Cercopithecidae, Hylotidae, and Pongidae) by PCR with primers flanking the coding region of the gene. Full-length CCR5 was inserted into pCDNA3.1, and multiple clones were sequenced to permit discrimination of both alleles. Compared to the human CCR5 sequence, the CCR5 sequences of the Lemuridae, Cebidae, and Cercopithecidae shared 87, 91 to 92, and 96 to 99% amino acid sequence homology, respectively. Amino acid substitutions tended to cluster in the amino and carboxy termini, the first transmembrane domain, and the second extracellular loop, with a pattern of species-specific changes that characterized CCR5 homologues from primates within a given family. At variance with humans, all primate species examined from the suborder Anthropoidea had amino acid substitutions at positions 13 (N to D) and 129 (V to I); the former change is critical for CD4-independent binding of SIV to CCR5. Within the Cebidae, Cercopithecidae, and Pongidae (including humans), CCR5 nucleotide similarities were 95.2 to 97.4, 98.0 to 99.5, and 98.3 to 99.3%, respectively. Despite this low genetic diversity, the phylogeny of the selected primate CCR5 homologue sequences agrees with present primate systematics, apart from some intermingling of species of the Cebidae and Cercopithecidae. Constructed HOS.CD4 cell lines expressing the entire CCR5 homologue protein from each of the Anthropoidea species and subspecies were tested for their ability to support HIV-1 and SIV entry and membrane fusion. Other than that of Cercopithecus pygerythrus, all CCR5 homologues tested were able to support both SIV and HIV-1 entry. Our results suggest that the shared structure and function of primate CCR5 homologue proteins would not impede the movement of primate immunodeficiency viruses between species.

The entry of primate immunodeficiency virus into a host target cell is mediated on the cell surface through sequential interactions of viral envelope glycoproteins with CD4 (10, 25, 44) and a chemokine receptor from the seven-transmembrane-domain G-protein-coupled receptor superfamily (1, 3, 6, 7, 11, 13, 14, 19, 40). Isolates of primate immunodeficiency virus that have different biological phenotypes use different subsets of chemokine receptors as cofactors for entry. Among the seven-transmembrane-domain G-protein-coupled receptors used for virus entry, the most physiologically relevant are CXC-chemokine receptor 4 (CXCR4) and CC-chemokine receptor 5 (CCR5), used by laboratory-adapted strains (X4 strains) and primary isolates of human immunodeficiency virus type 1 (HIV-1) (R5 and R5X4 strains), respectively (1, 2, 6, 8, 9, 39, 41, 45, 47). Unlike those of HIV-1, laboratory-adapted strains of simian immunodeficiency virus (SIV) do not use CXCR4 for virus entry, although CXCR4 derived from nonhuman primates is a functional coreceptor for X4 strains and some pathogenic simian-human immunodeficiency virus constructs (4, 5, 16, 32, 50, 51). Some SIV and human immunodeficiency virus type 2 (HIV-2) strains are not dependent on CD4 for virus entry and therefore can infect cells that lack CD4 but express CCR5 or CXCR4 (2, 17).

Whereas the envelope glycoproteins of the primate immunodeficiency viruses share many common features, including similar locations of cysteine residues, conserved and variable
regions, and CD4 binding domains, significant differences exist, especially between SIV and HIV-1 envelope glycoproteins (29). The external envelope glycoproteins of SIV and HIV-2 have approximately 70% amino acid sequence similarity to each other, and their nucleotide sequences have less than 40% similarity to those of selected HIV-1 envelope genes (29). Nonetheless, the divergent strains of the distantly related primate lentiviruses use a chemokine receptor from the seven-transmembrane-domain G-protein-coupled receptor superfamily for virus entry, and CCR5 use is indeed almost universal (4, 28, 49). Knowledge of CCR5 use by SIV per se is restricted to receptors that were derived from humans and selected nonhuman primates, however (4, 15, 49). Therefore, we cloned and analyzed CCR5 sequences derived from 24 representative primate species and subspecies of the suborders Prosimii and Anthropoidea. Phylogenetic reconstruction of the nonhuman CCR5 homologue sequences by maximum-likelihood and neighbor-joining methods recapitulated the evolutionary relationships for these representative primate species and subspecies. Despite a number of amino acid substitutions in multiple extracellular domains of the primate CCR5 homologues, many of which were highly conserved in primates within a given species, the primate CCR5 homologues supported SIV and HIV-1 entry.

MATERIALS AND METHODS
Peripheral blood cells from animals from representative species and subspecies of the primate suborders Prosimii and Anthropoidea. We obtained peripheral blood from individual members of 24 species in 17 genera of primates: 2 prosimians (Lemur catta and Varecia variegata rubra, both in the family Lemuridae), 4 New World monkeys (Alouatta caraya and Aotus trivirgatus, both in the family Cebidae; Callithrix jacchus and Saguinus nigricollis, both in the family Callitrichidae), 13 Old World monkeys (Cercopithecus diana, C. sabaeus, C. pygerythrus, Macaca fascicularis, M. fuscata, M. mulatta, M. tonkeana, M. nemestrina, and Colobus guereza), and 5 apes [Papio cynocephalus papio and Mandrillus (Papio) sphinx, both in the family Cercopithecidae; Hylabates concolor, in the family Hylabatidae; Gorilla gorilla, Pan troglodytes, and Pongo pygmaeus, all in the family Pongidae]. In phylogenetic reconstructions, this last family includes humans. Our use of animals for research purposes complied with all relevant federal guidelines and institutional policies.

PCR amplification of nonhuman primate CCR5 homologues. We isolated total cellular DNA by using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.). DNA pellets were resuspended in water, the nucleic acid content was quantified, and the DNA was used directly for in vitro amplification. To amplify a functional CCR5 gene from each species, we designed primers based on the conserved 5′ (CCR5A; 5′-GGAGGAGCACCATAAATACATTCTCA GG-3′) and 3′ (CCR5B; 5′-GACTGGTGCTACCCACCTTGAGTCC-3′) untranslated regions that flank the entire human CCR5 gene (GenBank accession number U57840). We were not able to amplify the entire CCR5 gene from cells obtained from primates of the families Lorisidae, Tarsiidae, and Lemuridae, a finding that suggests a nucleotide sequence mismatch within a region that flanks the gene. We were able to amplify the CCR5 DNA of the family Lorrisidae by using PCR with oligonucleotide primer pairs located within the CCR5 5′ (ATG GATTATCAAGTGTCGAAG) and 3′ (TCACAAGGCCCCACAGATTT) sequences. Five hundred nanograms of input cell DNA was used in each reaction.

PCR was performed as described previously (27) with a Perkin-Elmer Cetus 9600 automated thermal cycler programmed for 5 cycles at 98°C for 10 s, 55°C for 30 s, and 72°C for 45 s and then for 35 cycles at 98°C for 10 s, 60°C for 30 s, and 72°C for 45 s. Reagent controls were run in parallel. PCR product DNAs were resolved on a 1.0% agarose gel by electrophoresis. The appropriately sized band was excised, and the DNA was extracted by using the QIAquick gel extraction method (Qiagen, Valencia, Calif.). Purified PCR product DNA was inserted into vector pCR2.1 by the TA cloning method. All polynucleotide extractions and amplification reactions were done with appropriate negative controls to detect contamination throughout the process.

DNA sequencing. For each species, we obtained CCR5 sequences from at least one representative animal. Six clones containing the appropriately sized CCR5 homologue insert were randomly selected from each animal to ensure a 97% probability of analyzing both alleles [1/2^11n], where n is the number of independent clones]. The PCR product DNA inserts were sequenced in both directions by using ABI dye-terminator chemistry. Extension reactions were resolved on an ABI 377 automated sequencer and the sequences were assembled by using AutoAssembler (Applied Biosystems, Foster City, Calif.). Stop codons were found in two of the clones sequenced: 1 of 12 clones from two C. jacchus animals and 1 of 18 clones from six M. mulatta animals. Because these stop codons were not found in other clones, they likely represent PCR-associated errors. There were no frameshift mutations, in-frame insertions, or deletions in any of the cloned sequences. A consensus sequence was computed for each animal and was used in all DNA sequence analyses. We numbered the CCR5 homologue sequences according to the human CCR5 sequence under GenBank accession number U57840.

Phylogenetic analysis of primate CCR5 homologue sequences. Comparisons were based on various breakdowns of the available PCR product DNA sequence data. Both DNA and protein alignments were hand edited by using a multiple-alignment sequence editor (18). Analysis of the number of synonymous mutations per potential synonymous site and of the number of non synonymous mutations per potential non synonymous site was performed by using the formula of Nei and Gojobori (34). Because the CCR5 nucleotide sequences of primates in the same species proved to be very similar or identical to each other, we selected the representative CCR5 sequence for analysis to amplify the full-length CCR5 homologue sequence from primates of the taxonomic suborder Prosimii; we therefore used the 1,019 nucleotides that we could evaluate for all nonhuman primates for all phylogenetic analyses.

The CCR5 sequence alignments were subjected to three different tree-building methods: maximum parsimony, neighbor joining (a distance method), and maximum likelihood. These methods were implemented with the computer programs PAUP, version 3.1.1 (48); neighbor in PHYLIP, version 3.5c (48), and RevDnArates, a modification of DNArates code that incorporates a general reversible base substitution model, and RevDnAml, a modification of fastDNAnaml code that also incorporates a reversible model for rapid implementation of PHYLIP maximum-likelihood code (26, 35). We used the exhaustive branch-and-bound search method to find all possible shortest trees under parsimony. We compared 57 models of nucleotide substitution for neighbor-joining distance by using hierarchical likelihood ratio tests (22, 23). Of these 57 models, 56 are implemented in Modeltest, version 3.06 (37). The general time-reversible model with site-specific rates is implemented in PAUP, RevDnAml, and RevDNArates; in Modeltest, it is computed by hand. We tested all 57 models by using a single neighbor-joining tree based on the assumptions and methods implemented in Modeltest. This strategy includes the Jukes-Cantor model (equal base frequencies, equal rates of substitution), minimum evolution, no invariant sites, negative branch lengths set to zero, and random breaking of ties. We used RevDnArates to optimize parameters for the model with the general time-reversible model with site-specific rates that include base frequencies, substitutable rates, and four site categories of rates. After selecting a best model, we used RevDnAml to search for the best tree. For the final tree, we used PAUP with 500 neighbor-joining search replicates to compute bootstrap percentages for each branch.

Expression vector constructs. We selected a representative CCR5 homologue from each species to transfer into mammalian expression vector pCDNA3.1. Briefly, clone pCR2.1 was digested with the appropriate flanking restriction enzymes to remove the CCR5 insert. The insert was inserted into pCDNA3.1 and then sequenced in its entirety to ensure that no mutations had been introduced in the cloning process. We performed in vitro transcription-translation reactions (Promega, Madison, Wis.) by using the subcloned genes to ensure that a protein product of the accurately correct molecular weight could be made from each clone. Translatable clones were used to assess the role of primate CCR5 genes as cofactors for virus entry. Luciferase reporter viruses were prepared by cotransfecting 293T cells with pNL-Luc-Env or pSIV-Luc-R-E and vectors expressing different SIV or HIV-1 envelopes as described previously (11, 14).

Primary SIV strains. Primary SIV strains (SIVsmSL92b and SIVsmFNO) were isolated from cells obtained from two sooty mangabeys by cocultivation with uninfected cells from the native host species as described previously (4).

Virus entry assay. HOS.CD4 cells were transiently transfected with pCDNA3.1-CCR5 constructs containing the CCR5 homologues of the various primate species by using a CellPhect kit (Pharmacia Biotech). To test for virus entry, cells (2 × 10^5 per well) in culture medium (Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum) were seeded in 24-well plates 24 h posttransfection. After an additional 24 h of culturing, cells were infected with luciferase reporter viruses (40 ng of p24 or
we prepared 100 ml per well) after overnight incubation. After an additional 3 days of culturing, medium from the reporter virus-infected cells was replaced with fresh medium (1 p27) in a total of 300 ml of culture medium containing 1 ng of Polybrene/ml. The medium from the reporter virus-infected cells was replaced with fresh medium (1 ml per well) after overnight incubation. After an additional 3 days of culturing, we prepared 100 ml of cell lysate and measured the luciferase activity in 20 ml by using commercial reagents (Promega).

Cell-cell fusion assay. The cell-cell fusion assay has been described in detail elsewhere (41). Briefly, effector QT6 cells were infected with vaccinia virus expressing T7 polymerase (vTF1.1) and then transfected with plasmid pcDNA3.1 containing the designated envelope gene via the calcium phosphate transfection method. Target QT6 quail cells were transfected with plasmids expressing CD4, coreceptor, and T7 luciferase. At 24 h after infection or transfection, effector cells were added to target cells, and fusion was quantified 8 h after mixing by lysing cells with 0.5% Triton X-100. An aliquot of the lysate was mixed with an equal volume of luciferase assay reagent (Promega), and luminescence was read in a Luminometer (42).

Nucleotide sequence accession numbers. After rigorous quality control testing (30), the 169 human and nonhuman CCR5 homologue sequences were submitted to GenBank under accession numbers AF161887 to AF162055.

RESULTS

Phylogenetic analysis of primate CCR5 sequences. Figure 1 shows a representative phylogenetic tree for the primate CCR5 homologues created by using the maximum-likelihood method with the F84 model and 10 jumble replicates. One representative CCR5 sequence per species (Table 1) was used to generate the maximum-likelihood tree. The only difference between the phylogenetic reconstructions created by using the maximum-likelihood and neighbor-joining methods was the location of C. guerza, which was located basal to the C. aethiops cluster in the maximum-likelihood tree but basal to all cercopithecines in the neighbor-joining tree (data not shown). Since bootstrapping a maximum-likelihood tree with 25 taxa was not computationally feasible, we bootstrapped the equivalent neighbor-joining tree, which yielded approximately the same branching order, 500 times. Values greater than or equal to 50% are shown on the maximum-likelihood tree. Despite the inherent limitations of this approach, it does provide some indication of the stability of the tree. The full sequence alignment has 31 taxa and 1,019 nucleotide sites in the CCR5 homologues from all nonhuman primates that we could evaluate with no missing data, gaps, or ambiguous nucleotides. Of the 1,019 sites, 776 are constant and (under parsimony) 81 are uninformative and 162 are informative. For this alignment, there are 588 shortest parsimony trees, each 330 steps long (data available on request). The large number of shortest trees reflects poor resolution in a few parts of the tree, primarily within species and between closely related species. This result is to be expected. Of the 57 models of nucleotide substitutions that we tested, 8 models include a parameter to estimate the transition/transversion ratio, which ranged from 3.7 to 4.2.

Deduced amino acid sequences of primate CCR5 homologues. Figure 2 shows the alignments of the deduced amino acid sequences of the CCR5 homologues from the nonhuman and human primate species. We observed length conservation of the predicted CCR5 protein sequences for all primates of the suborder Anthropoidea, each of which was 353 amino acids long. Because we able to amplify the CCR5 DNA of members of the family Lorisidae by using PCR with oligonucleotide primer pairs for the CCR5 coding region, the peptide sequences of the CCR5 homologues of L. catta and V. variegata rubra were 339 amino acids long. Nevertheless, among all the primate species and subspecies of the taxonomic suborders Prosimii and Anthropoidea, the 12 cysteine residues in CCR5 were highly conserved.

Among the diverse species and subspecies, greater amino acid differences were found for primates of the suborder Prosimii, family Lorisidae, than for primates of the suborder Anthropoidea, families Callitrichidae, Cebidae, Cercopithecidae, Hylobatidae, and Pongidae (Fig. 2). We found that amino acid substitutions in specific positions were highly conserved among the different primate species and subspecies of the taxonomic suborders Prosimii and Anthropoidea compared with the hominid CCR5 consensus sequence. While amino acid substitutions in positions 13 (N to D) and 129 (V to I) were conserved among all nonhuman primate species and subspecies, greater differences were found among the evolutionarily more divergent species. In particular, greater amino acid differences were found for CCR5 homologues from primates of the suborder Prosimii, families Lorisidae, Tarsiidae, and Lemuridae, than for those from primates of the suborder Anthropoidea, families Callitrichidae, Cebidae, Cercopithecidae, Hylobatidae, and Pongidae. Because of the distant relationship between prosimians and New World and Old World monkeys, we anticipated this result.
Among the species of the superfamily Hominoidea, we found an additional amino acid substitution at position 9 (I to T) for *H. leucogenys*, *G. gorilla*, and *P. pygmaeus* but not for *P. troglodytes*. Among the species of the superfamily Cercopithecoidea, we commonly found amino acid substitutions in the amino terminus (position 9 [I to T]), the first intracellular loop (positions 58 [M to I] and 61 [I to V]), the second transmembrane domain (position 78 [F to L]), and the second extracellular loop (position 171 [K to R]). We found an additional conserved substitution in the second extracellular loop (position 198 [I to M]) for *M. fascicularis*, *M. fuscata*, *M. mulatta*, *M. tonkeana*, and *M. nemestrina*. Among the species of the superfamily Cebioidea, we found amino acid substitutions in the amino terminus (positions 15 [Y to G], 16 [T to A and T to P], 24 [N to D], 27 [I to M], 28 [A to G], and 30 [R to H]), the first transmembrane domain (positions 38 [L to M and L to I] and 40 [I to L]), the first intracellular loop (position 61 [I to V]), and the second transmembrane domain (position 81 [L to F]). Additional amino acid substitutions were found in the first extracellular loop (position 92 [A to G]), the third transmembrane domain (position 103 [L to F]), the second extracellular loop (positions 174 [L to Y], 180 [S to P], 184 [Y to F], 194 [O to E], and 198 [I to M]), the sixth transmembrane domain (position 260 [F to Y]), the seventh transmembrane domain (position 292 [I to V]), and the carboxy terminus (positions 329 [E to K] and 335 [S to N]). Last, among the species of the suborder Prosimii, from which we were able to obtain representative clones of the CCR5 homologue with species-specific amino and carboxy termini, we found additional amino acid substitutions in positions throughout the molecule. The limited number of species of the suborder Prosimii precludes a formal analysis of deduced amino acid sequence conservation. Nevertheless, analysis of the number of synonymous mutations per site and the number of nonsynonymous mutations per site (24) suggests that the nonhuman CCR5 homologues underwent purifying selection, as witnessed by the high ratio of nonsynonymous to synonymous substitutions per site (average over all species, 6.62), especially in the carboxy-terminal third of the protein, the region beyond codon 200.

### TABLE 1. List of species (or subspecies which taxonomists may treat as species) studied

<table>
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<tr>
<th>Zoological name</th>
<th>Common name</th>
<th>$n^a$</th>
<th>GenBank accession no.</th>
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<td>Orangutan</td>
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<td>Crab-eating macaque</td>
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<td>Japanese macaque</td>
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<td>Red-ruffed lemur</td>
<td>3</td>
<td>AF162009–AF162013, AF162014</td>
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</table>

$a$ Number of sequences reported for each species out of a total of 169 sequences; each sequence is the consensus sequence from six sequenced clones obtained from a single animal.

$b$ Shown in alignments and trees.

Diverse primate CCR5 homologues support membrane fusion by virus strains. To determine whether the diverse primate CCR5 homologues function as coreceptors for SIV, we tested the recombinant proteins in a cell-cell fusion assay by using SIVmac Env 239.2 and SIVmac Env 316. No fusion was observed when Env-negative cells were used. Figure 3 shows that expression of the diverse nonhuman primate CCR5 homologues in QT6 cells along with human CD4 allowed fusion with QT6 effector cells expressing either of the two different envelope proteins.

Primate CCR5 homologues support pseudovirus entry. Envelope-mediated cell-cell fusion assays generally reflect the ability of a virus to enter cells; however, differences between cell-cell fusion and virus infection assays are sometimes observed. We therefore tested the ability of CCR5 homologue-transfected HOS.CD4 cells to support infection by luciferase reporter viruses. The HIV-1NL4-3 and SIVmac239 luciferase reporter viruses have a nonfunctional *env* gene and a luciferase gene inserted into *nef*. These features permit the single-round
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>Function</th>
<th>Expression</th>
<th>Localization</th>
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<tr>
<td>Actin</td>
<td>Filament</td>
<td>Structure</td>
<td>High</td>
<td>Cytoplasm</td>
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<tr>
<td>Myosin</td>
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<td>Structure</td>
<td>Low</td>
<td>Extracellular</td>
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<td>Vimentin</td>
<td>Filament</td>
<td>Structure</td>
<td>Medium</td>
<td>Endoplasm</td>
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<td>Tubulin</td>
<td>Filament</td>
<td>Structure</td>
<td>High</td>
<td>Cytoplasm</td>
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</table>

**Gene Expression Levels:**
- Actin: High
- Myosin: Low
- Vimentin: Medium
- Tubulin: High

**Localization Sites:**
- Cytoplasm
- Extracellular
- Endoplasm

**Function:**
- Structure

**Regulation:**
- Expression levels vary with different cellular processes.

**Analysis:**
- Comparative analysis of gene expression in different cell types.

**Conclusion:**
- Actin and Tubulin are dominant in the cytoplasm, indicating primary roles in cell structure and dynamics.

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**Table 1: Comparative Gene Expression**

<table>
<thead>
<tr>
<th>Species</th>
<th>Actin</th>
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<th>Vimentin</th>
<th>Tubulin</th>
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<tr>
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<td>Low</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>Mouse</td>
<td>High</td>
<td>Low</td>
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<td>High</td>
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<tr>
<td>Yeast</td>
<td>Low</td>
<td>Medium</td>
<td>Low</td>
<td>Low</td>
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</table>

**Remark:**
- Yeast has a simpler cellular structure, limiting the expression levels of structural proteins.

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**Figure 2: Gene Localization Map**

- Actin: Cytoplasmic filaments
- Myosin: Extracellular matrix
- Vimentin: Endoplasmic reticulum
- Tubulin: Cytoplasmic microtubules
quantification of virus infection after complementation with the desired envelope protein. We created HOS.CD4 cell lines expressing each of the primate CCR5 homologues, except for those of the family Lorisoidea, since the full-length construct required the use of oligonucleotide primer pairs for the CCR5 coding region per se. These cell lines were infected with HIV-1NL4-3 or SIVmac239 luciferase reporter viruses pseudotyped with Env proteins that can use CCR5 (SIVmac1A11, HIV-1 JR-FL, and ADA) or an Env protein that uses only CXCR4 (HXB2). Reporter viruses bearing an amphotropic murine leukemia virus Env were used as a control. All but one of the representative primate CCR5 homologue constructs supported HIV-1 and SIV entry into their respective transfected cell lines (16, 28). The single exception was CCR5 of C. pygerythrus, which contained a polymorphism in the amino terminus, as reported previously (17) (data not shown).

 DISCUSSION

In this study, we analyzed the structure and function of CCR5 derived from representative primate species and sub-species of the Prosimii superfamily Lemuroidea and the Anthropoidea superfamilies Cebioidea, Cercopithecoidae, and Hominoidea. We identified amino acid changes in the CCR5 homologues specific to each of the primate species and sub-species. Further, many amino acid changes were conserved among primates species and subspecies and shared between primates of the same family. Each nonhuman primate CCR5 homologue was used as an entry cofactor by HIV-1 regardless of the viral phenotype. The genetically divergent SIV strains tested here, SIVmac239, SIVmac1A11, SIVsmNSL92b, and SIVsmFNI, also used each of the nonhuman primate CCR5 homologues as an entry cofactor. Thus, despite the significant difference between SIV and HIV-2 strains and HIV-1 strains in the range of primate CD4+ cells that they productively infect and the efficiencies with which they do so, all of the primate immunodeficiency viruses tested used the genetically diverse receptors for virus entry.

Alignment with and comparison to the human CCR5 amino acid sequence of the deduced nonhuman primate CCR5 homologue amino acid sequences showed species-specific changes among the diverse species, with greater amino acid differences for prosimians than for Old World monkeys, New World monkeys, and apes. Specifically, we found conserved amino acid substitutions at positions 33 (N to D) and 129 (V to I) among all nonhuman primate CCR5 homologue sequences relative to the human CCR5 consensus sequence. Additionally, we found highly conserved amino acid substitutions at position 9 (I to T) for apes and at positions 9 (I to T), 58 (M to I), 61 (I to V), 78 (F to L), and 171 (K to R) for Old World monkeys and an additional conserved substitution at position 198 (I to M) for macaques. Similar amino acid changes were observed in limited data sets of nonhuman primate CCR5 homologue sequences derived from representative species of the superfamilies Cebioidea and Cercopithecoidae (33). We observed larger numbers of relatively conserved amino acid substitutions among New World monkeys and prosimians.

CCR5 polymorphisms were also found within a given species, suggesting that, like humans (3, 38), nonhuman primates have polymorphisms (5, 28). Most of these chemokine receptors functioned as coreceptors for SIV and HIV-1 entry. Other than the stop codon found in two alleles, we identified only one mutation, an N13D change for the vervet CCR5 homologue, that adversely affected virus entry (17). Although we did not find any deletions within the nonhuman primate CCR5 homologue coding regions sequenced here, a 32-bp deletion allele exists in the human population (31, 38) and a 24-bp deletion allele exists in the C. torquatus torquatus population (36). Such polymorphisms may play a role in the limited pathogenicity of SIV in naturally and experimentally infected C. alyi hosts.

Each of the nonhuman primate CCR5 homologues tested here permitted the entry of genetically diverse SIV and HIV-1 Env constructs, including SIVmac1A11 and HIV-1 laboratory strains JR-FL, ADA, and HXB2. Thus, whereas simian-human immunodeficiency virus constructs allow HIV-1 envelope fusion and entry in primate cells expressing CD4, the SIV accessory genes overcome the restrictions to replication at the postentry level. These findings indicate that the nonhuman primate CCR5 homologue is not a species-specific restriction for primate immunodeficiency virus entry and support the hypothesis that HIV-1 originated as a cross-species transmission event (4, 20).

The topology of the phylogenetic tree based on the nonhuman primate CCR5 homologues sequenced here agrees with that constructed by others using limited numbers of nonhuman primate CCR5 sequences from phylogenetically distinct species of Anthropoidea and Hominoidea (33, 43, 46). We generally found excellent agreement between the phylogenetic reconstructions of nonhuman primate CCR5 homologue sequences and available COI and CD4 gene sequences (12, 21, 52) created by using the maximum-likelihood method (data not shown). The branching patterns of the nonhuman primate CCR5 homologue sequence sets estimated by the maximum-likelihood and neighbor-joining methods revealed distinct genetic lineages, with quantitatively greater branch lengths for prosimians than for Old World monkeys, New World monkeys,

FIG. 2. Alignment of deduced human and nonhuman primate CCR5 homologue amino acid sequences. Directly below the human CCR5 consensus sequence are the nonhuman primate CCR5 homologue sequences for apes (P. troglodytes, G. gorilla, P. pygerythrus, and H. concolor), Old World monkeys (P. cynocephalus papio, M. sphinx, M. fascicularis, M. fascata, M. mulatta, M. nemestrina, C. guereza, C. diana, C. aethiops sabaeus, C. pygerythrus, C. mona, C. nictitans, and E. patas), New World monkeys (A. caraya, A. triivirgatus, Saguinus sp., and C. jacchus), and prosimians (L. catta and V. variegata rubra). Each nonhuman primate CCR5 homologue sequence is the consensus sequence of six clones obtained from each animal to ensure a 97% probability that both alleles were represented. Amino acids in the alignment that share similarity with the human CCR5 consensus sequence shown at the top are indicated by a dash. The truncated 5’ and 3’ ends of the prosimian CCR5 homologues are indicated by asterisks. The amino acid alignment is annotated for the amino terminus, extracellular loops (ECL), intracellular loops (ICL), transmembrane regions (TM), and carboxy terminus. Apes are highlighted in blue, Old World monkeys are highlighted in magenta, New World monkeys are highlighted in green, and prosimians are highlighted in red.
and apes. The branching order was also consistent with the presumed historical diversification of the nonhuman primates (21, 46, 52). Specifically, closer linkages were observed among species representing Cebioidea, Cercopithecoida, and Hominoidea. The only difference between the phylogenetic reconstructions created by using the maximum-likelihood and neighbor-joining methods was the location of Colobus, which clustered in an ancestral location relative to the C. aethiops cluster in the maximum-likelihood tree but in an ancestral location relative to all cercopithecines in the neighbor-joining tree (data not shown). This discrepancy, noted by others (52), suggests that the nonhuman primate CCR5 homologue may not be a suitable marker for low-level phylogenetic analysis. Analysis of the number of synonymous mutations per site and the number of nonsynonymous mutations per site (24, 34) suggests that the nonhuman primate CCR5 homologues underwent purifying selection. Furthermore, a difference in the number of synonymous mutations per site and the number of
nonsynonymous mutations per site for cercopithecines and Colobus implies a difference in selective pressures for these two lineages (52).

In summary, nonhuman primate CCR5 homologue sequences are highly homologous, with amino acid sequence variations in the amino-terminal and second extracellular domains showing evolutionary conservation among the different species. Except for an N133 change for the C. aethiops CCR5 homologue (17), all other nonhuman primate CCR5 homologues supported SIV and HIV-1 entry. The topologies of the nonhuman primate CCR5 homologue phylogenetic trees recapitulated the presumed evolutionary relationship among the nonhuman primates, suggesting that this phylogenetic information is useful for resolving the relationships among the taxonomic subdivisions.

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