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Polymorphisms in the CCR5 Genes of African Green Monkeys and Mice Implicate Specific Amino Acids in Infections by Simian and Human Immunodeficiency Viruses

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CCR5, a receptor for the CC chemokines RANTES, Mip1α, and Mip1β, has been identified as a coreceptor for infections by macrophage-tropic isolates of human immunodeficiency virus type 1 (HIV-1). To study its structure and function, we isolated cDNA clones of human, African green monkey (AGM), and NIH/Swiss mouse CCR5s, and we quantitatively analyzed infections by macrophage-tropic HIV-1 and SIV_{mac251} after transfecting human HeLa-CD4 cells with the CCR5 expression vectors. The AGM and NIH/Swiss mouse CCR5 proteins are 97.7 to 98.3% and 79.8% identical to the human protein, respectively. In addition, we analyzed site-directed mutants and chimeras of these CCR5s. Cell surface expression of CCR5 proteins was monitored by using a specific rabbit antiserum and by binding the chemokine [^{125}I]Mip1β. Our major results were as follows. (i) Two distinct AGM CCR5 sequences were reproducibly found in DNA from CV-1 cells. The AGM clone 1 CCR5 protein differs from that of clone 2 by two substitutions, Y14N in the amino-terminal extracellular region and L352F at the carboxyl terminus. Interestingly, AGM clone 1 CCR5 was inactive as a coreceptor for all tested macrophage-tropic isolates of HIV-1, whereas AGM clone 2 CCR5 was active. As shown by chimera studies and site-directed mutagenesis, the Y14N substitution in AGM clone 1 CCR5 was solely responsible for blocking HIV-1 infections. In contrast, both AGM CCR5 clones were active coreceptors for SIV_{mac251}. Studies of DNA samples from other AGMs indicated frequent additional CCR5 polymorphisms, and we cloned an AGM clone 2 variant with a Q93R substitution in the extracellular loop 1 from one heterozygote. This variant CCR5 was active as a coreceptor for SIV_{mac251} but was only weakly active for macrophage-tropic isolates of HIV-1. In addition, SIV_{mac251} appeared to be dependent on the extracellular amino terminus and loop 2 regions of human CCR5 for maximal infection. Our results suggest major differences in the interactions of SIV_{mac251} and macrophage-tropic HIV-1 isolates with I9, N13, and Y14 in the amino terminus; with Q93 in extracellular loop 1; and with extracellular loop 2 of human CCR5. (ii) The NIH/Swiss mouse CCR5 protein differs at multiple positions from sequences recently reported for other inbred strains of mice. This CCR5 was inactive as a coreceptor for HIV-1 and SIV_{mac251}. Studies of chimeras that contained different portions of NIH/Swiss mouse CCR5 substituted into human CCR5, as well as the reciprocal chimeras, indicated that the amino-terminal region and extracellular loops 1 and 2 of human CCR5 contribute to its coreceptor activity for macrophage-tropic isolates of HIV-1. Specific differences with previous CCR5 chimera results occurred because the NIH/Swiss mouse CCR5 contains a unique substitution corresponding to P183L in extracellular loop 2 that is nonpermissive for coreceptor activity. We conclude that diverse CCR5 sequences occur in AGMs and mice, that SIV_{mac251} and macrophage-tropic HIV-1 isolates interact differently with specific CCR5 amino acids, and that multiple regions of human CCR5 contribute to its coreceptor functions. In addition, we have identified naturally occurring amino acid polymorphisms in three extracellular regions of CCR5 (Y14N, Q93R, and P183L) that do not interfere with cell surface expression or Mip1β binding but prevent infections by macrophage-tropic isolates of HIV-1. In contrast to previous evidence, these results suggest that CCR5 contains critical sites that are essential for HIV-1 infections.

Fusion of the viral membrane with the cell surface membrane during infections by human immunodeficiency virus type 1 (HIV-1) involves collaboration between the primary receptor CD4 and a coreceptor (1, 14–17, 20). The recently identified coreceptors are seven transmembrane G protein-coupled receptors for proinflammatory chemokines (1, 14–17, 20). The major coreceptor for macrophage-tropic isolates of HIV-1 is CCR5, which binds the CC chemokines RANTES, Mip1α, and Mip1β (1, 14–17), whereas the major coreceptor for T-cell-tropic HIV-1 isolates is CXCR4 (fusin/LESTR), which binds the CXC chemokine SDF-1 (6, 9, 20). Approximately 10% of Caucasians have a truncated nonfunctional form of CCR5, and 0.3% are homozygous for this variant (3, 30, 39). The resistance of these homozygous individuals to infection by HIV-1 suggests that CCR5 is a critical coreceptor for HIV-1 transmission (30, 36, 39). Recent evidence suggests that the HIV-1 gp120 glycoprotein forms a ternary complex with CD4 and coreceptors (29, 41, 43). Several CCR5 mutants incapable of G-protein-mediated signal transduction are active as coreceptors for HIV-1 (18, 19, 22).

It is not known whether coreceptors serve as attachment sites to strengthen virus adhesion or whether their roles are more complex, nor is it known whether CCR5 polymorphisms occur in nonhuman primates such as African green monkeys (AGMs) (*Cercopithecus aethiops*) that appear to have been infected with immunodeficiency viruses since ancient times (2, 7, 24, 25, 28, 32). Recent studies of CCR5 chimeras have

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suggested that multiple extracellular regions contribute to infections by macrophage-tropic isolates of HIV-1 and that no single region is essential (4, 8, 34, 38). If correct, this would imply that mutations of single amino acids in CCR5 would not block coreceptor activity unless they prevented processing to cell surfaces or caused global changes in folding. However, as discussed elsewhere (8), the conclusion that all regions of human CCR5 are expendable would be unwarranted if portions of the reference proteins used in the chimera constructions were able to contribute to the coreceptor activities that were observed. To address these issues, we isolated CCR5 cDNAs from human, AGM, and NIH/Swiss mouse cells and we quantitatively analyzed their coreceptor activities in infections of human HeLa-CD4 cells by macrophage-tropic isolates of HIV-1 and by the simian immunodeficiency virus SIV_{mac251}. In addition, we analyzed coreceptor activities and cell surface expression levels of CCR5 site-directed mutants and interspecies chimeras. We found a surprising diversity of CCR5 sequences in DNA samples from different AGMs. Studies of these CCR5 clones suggested that SIV_{mac251} interacts differently than all tested macrophage-tropic HIV-1 isolates with position 14 in the amino terminus of CCR5, with position 93 in extracellular loop 1, and with extracellular loop 2. Our chimera results differ in several respects from those of other studies (4, 8, 34, 38), in part because the NIH/Swiss mouse CCR5 contains a unique amino acid substitution in extracellular loop 2 that is nonpermissive for HIV-1 infections. We have identified amino acids in three extracellular regions of CCR5 that appear to be critical for HIV-1 coreceptor function.

MATERIALS AND METHODS

Cells and viruses. HeLa and HeLa-CD4 cells (clone HI-J) were described previously (26). AGM cell lines CV-1, BS-C-1, Vero, and COS-7 as well as NIH/Swiss mouse NIH 3T3 fibroblasts were from the American Type Culture Collection (Rockville, Md.). HeLa, HeLa-CD4, CV-1, and NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). COS-7 cells were maintained in the same medium supplemented with glucose (4.5 g/liter). BS-C-1 and Vero cells were maintained in minimum essential medium with 10% FBS and 0.1 mM MEM nonessential amino acids (Life Technologies, Inc., Grand Island, N.Y.). The macrophage-tropic SF162, JR-FL, ADA, and Ba-L isolates of HIV-1 were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and were contributed by Jay Levy, by Irvin Chen, by Howard Gendelman, and by Suzanne Gartner, Mikulas Popovic, and Robert Gallo, respectively. HIV-1 viruses were passaged in phytohemagglutinin-stimulated human peripheral blood mononuclear cells (PBMCs). The medium was harvested at times of peak reverse transcriptase release, passed through a 0.45-μm-pore-size filter, aliquoted, and stored at −80°C. SIV_{mac251}, which was generously donated by Jay Nelson, was propagated in CEMX174 cells, and titers were determined in sMAGI cells as described previously (12).

PCR cloning of CCR5 genes from human, AGM, and NIH/Swiss mouse cells. All custom oligonucleotides were synthesized by Oligos, etc. (Stamford, Conn.), and all restriction enzymes were obtained from New England Biolabs (Beverly, Mass.). Genomic DNAs from human HeLa cells; AGM cell lines CV-1, BS-C-1, and Vero; and NIH/Swiss mouse NIH 3T3 cells were prepared as described elsewhere (5). In addition, genomic DNA was prepared from the liver, spleen, and kidneys of a C57BL/6 mouse by standard methods (5). Two DNA samples, one from a vervet AGM and the other from a sabaeus AGM, were generously donated by Jon Allan (Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Tex.). PCR was performed with cloned Pfu polymerase (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. Reactions were performed in a 100-μl volume containing 1× cloned Pfu buffer, 0.1 mM each deoxynucleoside triphosphate, 0.5 to 1.5 μg of genomic DNA, 50 pmol each of forward and reverse primers, and 5 U of cloned Pfu polymerase. Thermal cycling was performed by heating to 94°C for 45 s; followed by 25 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 2 min 30 s; and a final extension step of 72°C for 10 min. The primers used for human and AGM CV-1 DNAs were CKR5/5′ (forward) (5′ GGGGATCCGGTGGAACAAGATGGAT 3′) and CKR5/3′ (reverse) (5′ CCCTCGAGCCACTTGAGTCCGTGTCACA 3′). The primers used for NIH/Swiss and C57BL/6 mouse DNAs were MC5/5′ (forward) (5′ CCGGATCCCAGGATGGATTTTCAAGGG 3′) and MC5/3′ (reverse) (5′ GGCGCTCGAGTCAACCAGGTCATAAACCAGT 3′). Design of the primers was based on the reported sequences of human and mouse CCR5s (10, 37). The primers overlap the presumed start and stop codons of these genes (underlined). The ~1.1-kbp specific amplification products were subcloned into pBluescriptII(KS+) (Stratagene) by using the *Bam*HI and *Xho*I restriction sites engineered into the primers, and in the plasmid multiple cloning site. The inserts were sequenced by fluorescent DNA sequence determination which was performed by the Microbiology and Molecular Immunology Core Facility on the PE/ABD 377 DNA sequencer using dye terminator cycle sequencing chemistry (PE Applied Biosystems, Foster City, Calif.). Primers for sequencing were M13 universal (−20) and M13 reverse (United States Biochemical Corporation, Cleveland, Ohio), which anneal to the vector 5′ and 3′ of the insert, respectively, and the internal primers CKR1 (5′ TCAT CATCCTCCTGACAATCG 3′) (for human and AGM CV-1 clones) and CKR2 (5′ CTTCTTCATTATCCTCCTGAC 3′) (for NIH/Swiss mouse cells). The human sequence agreed with the reported sequence (37). Although the NIH/Swiss mouse sequence differed from the reported mouse sequences (8, 10, 31), sequencing of multiple PCR products confirmed that our sequence was correct. The sequence of the C57BL/6 mouse CCR5 differed from the reported sequence for strain 129/SvJ at one nucleotide, resulting in a coding change at the equivalent of human codon 206. The reported sequence has Ser at this position (10), whereas our newly cloned sequence codes for Pro. Since this residue is Pro in the human, AGM, and NIH/Swiss mouse CCR5s and since this change lies outside the extracellular loop 2 region which was of interest for this study, we used the cloned sequence in our study. Two different sequences of AGM CCR5 were obtained. These differ from each other in 3 nucleotides, two of which result in coding changes: clone 1 has an Asn residue at position 14 whereas clone 2 has a Tyr, and clone 1 has a Phe at position 352 whereas clone 2 has Leu. Both sequences were isolated from multiple PCRs, suggesting that both are present in the genomic DNA of CV-1 cells. The *Bam*HI-to-*Xho*I fragments were then subcloned into pcDNA3 (Invitrogen Corp., San Diego, Calif.) cut with the same enzymes.

The primers used for vervet AGM, sabaeus AGM, BS-C-1, and Vero DNAs were AGMF (forward) (5′ GGGTGGAACAAGATGGATTATC 3′) and AGMR (reverse) (5′ ACTGTATGGAAAATGAGAGCTGC 3′). The 567-bp PCR products were sequenced directly by using the AGMF primer. The 567-bp PCR product from the sabaeus AGM was digested with *Cla*I and subcloned into the same sites of the AGM clone 2 expression vector to give the AGM clone 2(Q93R) expression construct.

Construction of chimeric and mutant CCR5s. The human/mouse chimeras MMHH, HMMM, MHMM, MMHM, MMMH, HHMM, MHHH, HMHH, HHMH, and HHHM were created by using conserved sites for the restriction endonucleases *Msc*I, *Bgl*II, and *Eco*RI (see Fig. 1 and 2). They were constructed in pBluescriptII(KS+) except for HHHM, which was constructed in pcDNA3. The human or NIH/Swiss mouse *Bgl*II-to-*Bsa*BI fragments, containing the CCR5 second extracellular loop, were cloned into the wild-type human CCR5, wild-type mouse CCR5, or HMMM chimera to produce HHHHloop2M, MMMMloop2H, and HMMMloop2H. The HHHHloop2M(L183P) CCR5 was made by subcloning the *Bgl*II-to-*Bsa*BI fragment from C57BL/6 mouse CCR5 into the same sites in the human CCR5 cDNA. In the extracellular loop 2 region, NIH/Swiss and C57BL/6 mouse CCR5s differ only at the equivalent of codon 183 in human CCR5; thus, this construct differs from HHHHloop2M by the change of codon 183 from Leu to Pro.

The AGM clone 1/human chimeras were constructed in pBluescriptII(KS+) by using the conserved *Bgl*II site. Both plasmids were digested with either *Bgl*II and *Bam*HI or *Bgl*II and *Xho*I. The fragments from AGM clone 1 CCR5 were then ligated into the vector containing human CCR5 digested with the same enzymes. A chimera was constructed by using the amino-terminal half of AGM clone 1 CCR5 and the carboxyl-terminal half of AGM clone 2 CCR5. The result is a CCR5 which is identical to that of AGM clone 2 except for the Y14N mutation in the amino-terminal extracellular region. This chimera is referred to as AGM clone 2(Y14N). All CCR5 chimeras constructed in pBluescriptII(KS+) were excised with *Bam*HI and *Xho*I and subcloned into the pcDNA3 expression vector.

Site-directed mutagenesis was performed with the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mutagenesis was performed on AGM clone 2 or human CCR5 in pBluescriptII(KS+) by using the mutagenic primers T9I (5′ GTCAAGTCCAATCTATGACATCG 3′), D13N (5′ CCAACCTATGACATCAATTATTATACATCGGAGCC 3′), and Y14N (5′ CCAATCTATGACATCAATAATTATACATCGGAGCCC 3′). The T9I mutagenic primer introduces a C-to-T mutation (underlined) which mutates codon 9 of the AGM clone 2 CCR5 from Thr (ACC) to Ile (ATC). Likewise, the D13N primer mutates codon 13 of AGM clone 2 CCR5 from Asp (GAT) to Asn (AAT), and the Y14N primer mutates codon 14 of human CCR5 from Tyr (TAT) to Asn (AAT). The mutations were confirmed by sequencing, and the mutated CCR5s were then excised with *Bam*HI and *Xho*I and ligated into pcDNA3 cut with the same enzymes.

Assay for coreceptor function. Coreceptors were transiently expressed in HeLa-CD4 (clone HI-J) cells by the calcium phosphate transfection method (5) using 20 μg of pcDNA3-CCR5 plasmid DNA per 25-cm^2 flask seeded 24 h previously with 5×10^5 cells. Forty-eight hours posttransfection the cultures were trypsinized and plated at 1.5×10^4 to 2×10^4 cells per 2-cm^2 well of a 24-well cluster plate for HIV-1 infection. Infectivities by macrophage-tropic HIV-1 isolates were determined by the focal infectivity assay as described previously (27). Briefly, 72 h posttransfection cells were pretreated with DEAE-dextran (8 μg/ml)

in serum-free DMEM at 37°C for 20 min. The cells were washed with serum-free DMEM and then incubated with 0.2 ml of virus diluted in DMEM plus 0.1% FBS at 37°C. After 2 h the cells were fed with 1 ml of DMEM plus 10% FBS and incubated at 37°C for 3 days. The cells were then fixed in ethanol, and infected foci were visualized by an immunoperoxidase assay (13). For this purpose, the 0.45-μm-filtered supernatant from the anti-p24 hybridoma 183-H12-5C (AIDS Research and Reference Reagent Program; contributed by Bruce Chesebro and Hardy Chen) was used at a 1:5 dilution. Staining of cells infected with SIV_{mac251} was done by using a monoclonal antibody to the viral p27 protein (generously donated by Jay Nelson) purchased from Immunodiagnostics (Boston, Mass.). The cells were then sequentially incubated with 1:400 peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) serum (Organon Teknika Corp., Durham, N.C.) and a substrate solution of 3-amino-9-ethyl-carbazole (Sigma, St. Louis, Mo.).

Anti-CCR5 antibody production. A synthetic peptide corresponding to the amino-terminal 26 amino acids of human CCR5 (NH_4^+-MDYQVSSPIYDIN YYTSEPCQKINVK-CO_2^-) (Microchemical Facility, Emory University, Atlanta, Ga.) was coupled to soluble keyhole limpet hemocyanin (KLH) (Sigma) via the free Cys residue by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Sigma) by standard methods (23). It was determined that 4.2 mg of peptide was coupled to 5 mg of KLH to give an estimate of ~108 peptides coupled per M_r ~400,000 KLH monomer. Four hundred sixty micrograms of the KLH-peptide conjugate was injected per rabbit per injection. Rabbits were initially injected with the coupled peptide in 50% complete Freund's adjuvant (Life Technologies, Inc.) and then boosted 14 days later with the conjugate in 50% incomplete Freund's adjuvant (Life Technologies, Inc.). Subsequent boosts were ~28 days apart and in phosphate-buffered saline (PBS) (0.14 M NaCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, 2.6 mM KCl) only. Test bleeds were taken, and serum was prepared, 12 to 14 days after each boost. After the second boost, the serum of one of three rabbits was positive as demonstrated by Western immunoblotting of COS-7 cell membranes expressing human CCR5. The bleed following the third boost was used in this project.

Membrane isolation and Western immunoblotting. Total cellular membranes were isolated from ~1×10^6 COS-7 cells 48 h after being transfected (by using the DEAE-dextran method [5]) with the pcDNA3 (mock) or pcDNA3-CCR5 DNA construct. Monolayer cells were washed twice with PBS and scraped into 10 ml of PBS. The cells were pelleted at $150 \times g$ for 5 min, resuspended in 4 ml of swelling buffer (20 mM HEPES [pH 7.2], 5 mM KCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride [Sigma] [added fresh]), and swelled on ice for 10 min. Cells were homogenized in a Dounce homogenizer to ~90% breakage. Nuclei were pelleted at $250 \times g$ for 10 min. The membranes were pelleted from the supernatant at ~$27{,}000 \times g$ for 15 min (20 krpm in Beckman 70.1 Ti rotor) and stored at −20°C. The solubilized membrane proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis as described elsewhere (5) and transferred to NitroPure nitrocellulose membranes (Micron Separations, Inc., Westboro, Mass.). Membranes were sequentially incubated with the anti-CCR5 serum, used at a 1:250 dilution, and then with protein A-horseradish peroxidase conjugate (Bio-Rad) at a 1:10,000 dilution. Membranes were washed, and horseradish peroxidase was visualized with chemiluminescence reagents (Dupont NEN Research Products, Boston, Mass.) used according to the manufacturer's instructions.

Cell surface expression of CCR5s. Cells were plated in four-well chamber slides (Nunc, Inc., Naperville, Ill.) 24 h prior to assay for CCR5 expression by immunofluorescence. Viable cells were incubated with anti-CCR5 serum diluted 1:25 to 1:50 in complete medium (DMEM plus 10% FBS) for 1 h at 37°C. After being washed with complete medium, the cells were incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated affinity-purified antibody to rabbit IgG (Organon Teknika Corp., West Chester, Pa.) diluted 1:100 in complete medium. Cells were then washed in complete medium, rinsed with PBS, fixed with cold methanol for 5 min, and mounted over drops of 50% glycerol in PBS, and slides were viewed by fluorescence microscopy. A similar procedure was used to measure relative quantities of CCR5 antibody bound to the cells, except that the cells were incubated in 0.2 ml of complete medium with [^{125}I]protein A (0.4 μCi/ml, 2 to 10 μCi/μg; DuPont NEN Research Products) instead of the FITC-conjugated secondary antibody and the cells were plated in 24-well cluster plates. The cells were washed, solubilized in 0.1 N NaOH, and counted in a gamma counter, and the protein concentrations were determined by the Coomassie dye method (Bio-Rad Laboratories). In addition, expression of CCR5 proteins on cell surfaces was analyzed by binding of 0.5 nM [^{125}I]Mip1β (2,200 Ci/mmol; DuPont NEN Research Products) for 2 h at 37°C. The cells were washed, solubilized, and counted, and protein levels were determined as described above. Table 1 summarizes the abbreviations, structures, and evidence for cell surface expression of CCR5 proteins used in this investigation. As discussed below, several additional CCR5 proteins were analyzed and appeared to be negative for cell surface expression and for coreceptor activity [HHMM, HHHM, MHMM, MHMMloop2H, and MHMM(L183P)]. They are not included in Table 1 because they were not informative.

Nucleotide sequence accession numbers. The GenBank accession numbers for the AGM clone 1, AGM clone 2, human, NIH/Swiss mouse, and C57BL/6 mouse CCR5 nucleotide sequences are U83324, U83325, U83326, U83327, and AF022990, respectively.

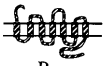
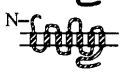
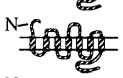
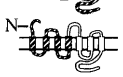
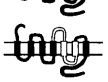
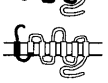
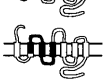
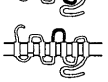
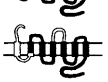
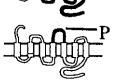
RESULTS

Structures of human, AGM, and NIH/Swiss mouse CCR5s and construction of their chimeras and site-directed mutants. Because the CCR5 DNA coding sequence lacks introns (37), we were able to isolate these sequences by a DNA-PCR method (see Materials and Methods). Figure 1 shows a comparison of the amino acid sequences of human, AGM, and NIH/Swiss mouse CCR5 proteins. Two AGM CCR5 sequences (clones 1 and 2) were reproducibly isolated from the DNA of CV-1 cells. The AGM and mouse CCR5 sequences are 97.7 to 98.3% and 79.8% identical to the human sequence, respectively. The AGM clone 1 sequence differs from that of AGM clone 2 by only two substitutions, Y14N, which creates an NYT consensus site for potential asparagine-linked glycosylation, and L352F, at the carboxyl terminus. The NIH/Swiss mouse CCR5 amino acid sequence differs at multiple positions from recently described sequences isolated from the 129/SvJ (10), B6CBA (31), and BALB/c (8) strains of mice. These four mouse CCR5 sequences differ from each other at a total of 14 positions, and all contain a two-amino-acid insertion in the amino terminus compared to human CCR5 (all amino acid numbering in this paper corresponds to the human CCR5). Figure 1 also indicates the locations of the seven presumptive transmembrane sequences A to G. Figure 2 shows a topological model of human CCR5 that highlights the positions of amino acids that differ among the human, AGM, and mouse proteins. It is important to note that extracellular loop 3 is identical in these CCR5s; thus, our studies were not informative about the potential role of this region in coreceptor function. In addition, this model shows the locations in the coding region of the *Msc*I, *Bgl*II, and *Eco*RI restriction enzyme cleavage sites in the DNA sequences that were used to construct most of the interspecies CCR5 chimeras, as well as the *Bsa*BI site that was used to construct additional chimeras (see below).

It was unclear from the above results whether the diversity of AGM CCR5 sequences was unique to CV-1 cells. To address this issue, two DNA samples were analyzed that had been prepared from PBMCs of different AGMs. Investigation of a vervet AGM DNA suggested that this monkey was heterozygous for a D13N substitution in the wild-type AGM clone 2 CCR5. Similarly, sequence analysis of the DNA sample from a sabaeus AGM suggested heterozygosity for a Q93R substitution in the wild-type AGM clone 2 sequence (Fig. 3A). In this case the mutation generated a novel *Nci*I restriction enzyme cleavage site in the CCR5 gene. Reproducibly, PCR amplification of CCR5 sequences from this DNA revealed the presence of this *Nci*I site in the product (Fig. 3B). A *Cla*I fragment from the PCR product encoding this Q93R substitution was cloned and used to prepare a pcDNA3-AGM2(Q93R) expression vector for functional analysis (see below). Southern blot studies of AGM DNA samples suggested the presence of a single CCR5 gene with multiple alleles and the absence of a CCR5 pseudogene (results not shown). Based on these results, we examined portions of the CCR5 sequences amplified from the AGM cell lines Vero and BS-C-1. Analysis of the Vero DNA sequence was consistent with homozygosity for the AGM clone 2 allele. In contrast, analysis of the BS-C-1 DNA from a grivet AGM (40) suggested heterozygosity for an AGM clone 2 Q93K substitution (results not shown). Thus, among the five AGM DNA samples that we examined, four appeared to have derived from heterozygotes with variant CCR5 alleles.

Studies of human CCR5 structure and expression using a rabbit antiserum specific for the amino-terminal extracellular sequence. The antiserum made to the amino-terminal hydrophilic region of human CCR5 (amino acids 1 to 26) showed a

TABLE 1. Properties of CCR5 proteins

CCR5	Structure	Immunofluorescence (% positive)[a]	[^{125}I]protein A immunoassay (cpm/μg of protein)[b]	[^{125}I]Mip1β binding (cpm/μg of protein)[c]
Human		15	125	15
AGM clone 1		9.8	21	61
AGM clone 2		6.6	10	111
AGM2(Q93R)		9.9	21	94
NIH/Swiss mouse		—	—	28
AGM2(Y14N)		22	40	60
AGM2(T9I)		13	ND	22
AGM2(D13N)		7.6	ND	16
AGM1/human		20	67	67
Human/AGM1		24	134	15
Human(Y14N)		12	33	1.6[d]
MMHH		—	—	15
HMMM		18	57	0[d]
MMHM		—	—	31
MMMH		—	—	28
MHHH		—	—	131
HMHH		17	111	7.3[d]
HHMH		5.0	13	2.1[d]
HHHHloop2M		19	100	0[d]
MMMMloop2H		—	—	16
HMMMloop2H		9.6	47	4.5[d]
HHHHloop2M(L183P)		15	ND	0[d]

[a] See Fig. 4. The results shown are averages for independent experiments. For human CCR5, the average transfection efficiency determined by this method was 15% (n = 9). —, CCR5 protein not detected by the antiserum due to the presence of the NIH/Swiss mouse N terminus.

[b] The average background in mock-transfected cells was 7.1 cpm/μg of protein (n = 9). Each assay was done in duplicate, and the counts per minute per microgram of protein above background were recorded. The results are averages of multiple assays. The average protein value for the samples was approximately 50 to 100 μg in independent assays. —, CCR5 protein not detected by the antiserum due to the presence of the NIH/Swiss mouse N terminus; ND, assay not performed.

[c] The [^{125}I]Mip1β binding assays were done in triplicate by using 0.5 nM [^{125}I]Mip1β at 37°C for 2 h. The data are average levels above background. The average backgrounds in the assays were 6.7 cpm/μg of protein (n = 7). The average protein value for the samples was approximately 50 to 100 μg in independent assays.

[d] Not significantly above background; however, the CCR5 protein was highly expressed on cell surfaces as detected by the antibody methods.

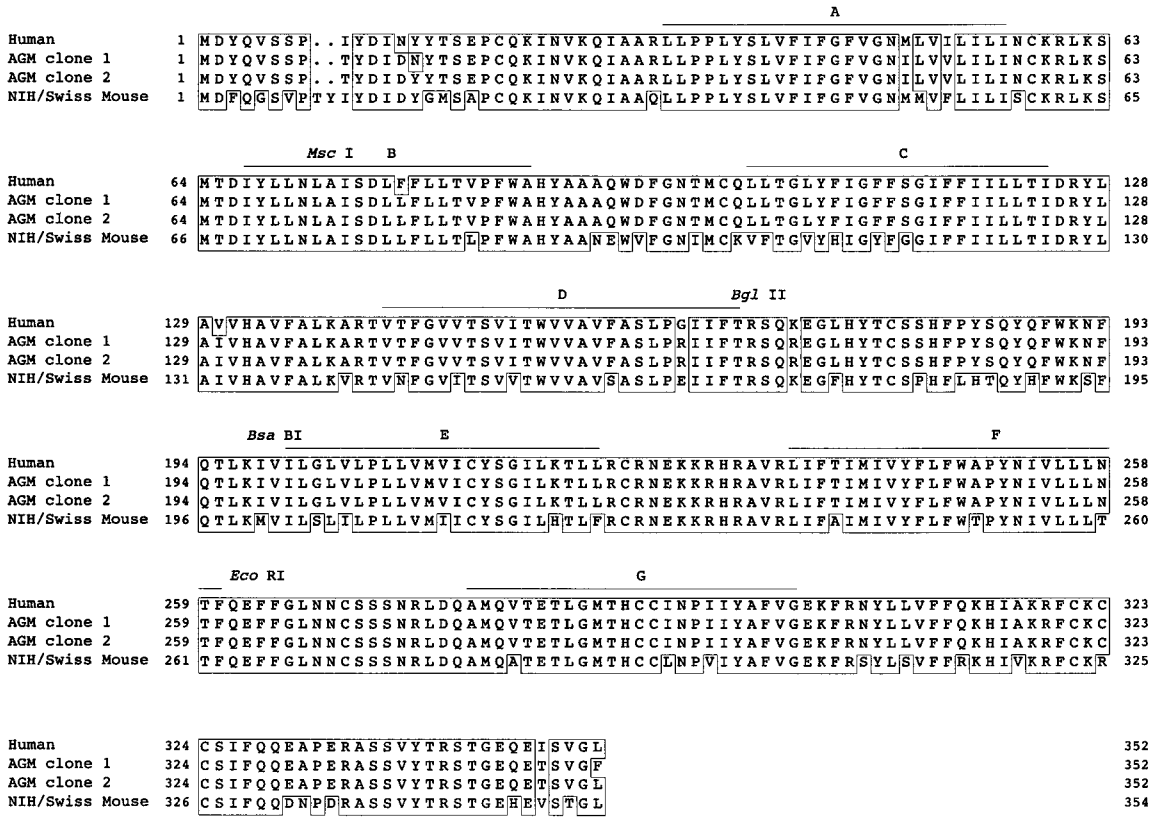


FIG. 1. Sequence alignment of human, AGM, and NIH/Swiss mouse CCR5 proteins. Several clones were isolated from each PCR and were sequenced to ensure reproducibility and accuracy for the cloning. The two distinct AGM clones contained three nucleotide substitutions that resulted in substitutions of two amino acids. The AGM clone 2 sequence, which is more homologous to the CCR5 sequences of other species, is considered to represent the main evolutionary lineage. The amino acid substitutions in AGM clone 1 are Y14N and L352F. The NIH/Swiss mouse sequence differs from previously reported sequences from the 129/SvJ (10), B6CBA (31), and BALB/c (8) strains by multiple amino acid substitutions. The seven presumptive transmembrane regions (A to G) and the common restriction enzyme cleavage sites that were used to generate chimeric CCR5 proteins are indicated.

strong specific reactivity with surfaces of viable HeLa-CD4 cells that stably express human CCR5 (Fig. 4). The background immunofluorescence was negligible for control HeLa cells or for cells incubated with preimmune serum rather than antiserum. The patching of CCR5 in Fig. 4 occurred when the antibody bound to the surfaces of viable cells at 37°C, whereas uniform immunofluorescence occurred when the cells were fixed with 3.7% paraformaldehyde prior to addition of the antiserum (results not shown). This suggests that CCR5 is able to diffuse in the cell surface membrane. Although this antiserum did not react with cells that expressed NIH/Swiss mouse CCR5, substantial immunofluorescence was observed with cells that expressed AGM CCR5. In addition, strong specific radioactive labeling with very low backgrounds occurred when [^{125}I]protein A was incubated with the cells instead of the FITC-conjugated secondary antibody (Table 1).

The rabbit antiserum was also active in Western immunoblotting as seen by specific reactivity with an M_r ~40,000 protein that occurred in membranes from COS-7 cells that had been transfected with pcDNA3-CCR5 but not in membranes from mock-transfected cells (Fig. 5). A strong specific component of this size was also seen when we used membranes from HeLa-CD4/CCR5 cells that stably synthesize human CCR5 or membranes from *Xenopus laevis* oocytes that had been injected with a synthetic mRNA that encodes CCR5 (results not shown). The size of human CCR5 as revealed by our antiserum to the native receptor appears to be compatible with that determined in a previous study that used epitope-tagged CCR5 (38). Also in agreement with the latter study, we found that the native human CCR5 protein lacks asparagine-linked oligosaccharides susceptible to cleavage with peptide–*N*-glycosidase F. In addition, neither AGM clone 1 nor AGM clone 2 CCR5 protein contained an asparagine-linked oligosaccharide susceptible to cleavage with this enzyme (results not shown).

Coreceptor activities of human, AGM, and NIH/Swiss mouse CCR5 proteins for SIV$_{mac251}$ and macrophage-tropic isolates of HIV-1. The coreceptor activities and cell surface expression levels of the human, AGM clone 1 and clone 2, and mouse CCR5 proteins were assayed after transient transfection of HeLa-CD4 cells (clone HI-J) with the pcDNA3-CCR5 expression vectors. Cultures were infected with either SIV$_{mac251}$ or macrophage-tropic HIV-1, and the foci of infected cells were stained 48 to 72 h later by using antibodies to viral proteins and peroxidase-conjugated secondary antibodies (13). Because no foci occurred in the absence of exogenous CCR5 expression, we could distinguish between low-level activity and inactivity of any CCR5 protein with a high degree of confidence. This is an advantage compared to nonfocal or syncytial assays that have been used to study coreceptors. Moreover, we believe that this assay method is as reproducible as other quantitative methods that require transient transfection of cells. In five independent transfection and infection assays using the expression vector for human CCR5, the numbers of foci per well seen with the HIV-1 isolates SF162, JR-FL, Ba-L, and ADA had standard

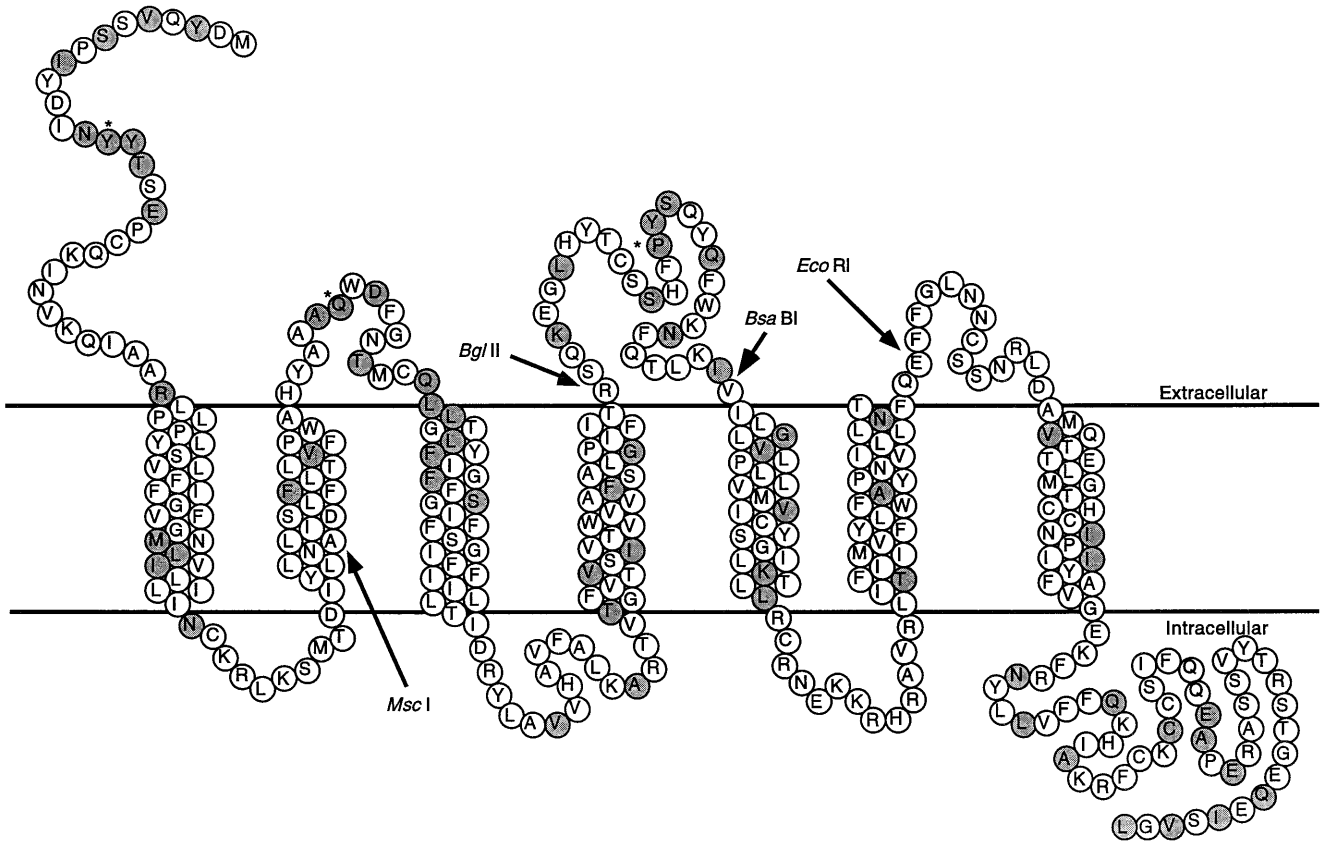


FIG. 2. Topological model of human CCR5 in the membrane. The extracellular membrane face is above the membrane (parallel lines), whereas the intracellular face is below. The splice sites used to make CCR5 chimeras are indicated. Amino acids that differ among the human, AGM, and NIH/Swiss mouse CCR5 proteins (Fig. 1) are shaded. Asterisks at Y14, Q93, and P183, sites that appear to be critical for infections by macrophage-tropic isolates of HIV-1.

deviations of ±16.6, ±32.5, ±10.4, and ±23.7%, respectively. Generally, the titers observed in our transient transfection assays (ca. 500 to 1,000 foci/well for cells expressing human CCR5) were approximately 10% of the total-virus titers assayed in HeLa-CD4/CCR5 cells that stably express CCR5 in all of the cells. This corresponded approximately with our efficiencies of transient transfection (Table 1). Moreover, the titers in our clones of HeLa-CD4/CCR5 cells were as large as the tissue culture infective doses in cultures of human PBMCs (35).

As shown in Fig. 6B, the SIV_{mac251} virus was highly infectious for HeLa-CD4 cells that had been transfected with expression vectors for human, AGM clone 1, and AGM clone 2 CCR5s but was noninfectious for cells that expressed NIH/Swiss mouse CCR5. Infectivity for cells that expressed AGM clone 1 was approximately threefold lower than that for cells expressing AGM clone 2 CCR5, and this difference was highly significant by the paired-comparison t test ($P \leq 0.001$; $n = 4$). In contrast to SIV_{mac251}, all of the macrophage-tropic HIV-1 isolates that we tested were infectious for cells that expressed human or AGM clone 2 CCR5 but were completely noninfectious for cells that expressed AGM clone 1 CCR5. This was verified in four independent assays, each using four different isolates of macrophage-tropic HIV-1 (i.e., SF162, JR-FL, Ba-L, and ADA), and was further substantiated by the experiments described below. Thus, the SIV_{mac251} and HIV-1 viruses were differently affected by the amino acid substitutions that distinguish the AGM clone 1 and AGM clone 2 CCR5 proteins. Studies were also done with the AGM clone 2(Q93R) CCR5 variant that was cloned from a sabaeus AGM DNA sample. This CCR5 was highly expressed on cell surfaces as determined by immunoassays and by binding of [^{125}I]Mip1β (Table 1). As shown in Fig. 6, it was almost completely inactive as a coreceptor for macrophage-tropic isolates of HIV-1 but was highly active for SIV_{mac251}. Specifically, in six independent assays, the titers of the SF162, JR-FL, Ba-L, and ADA HIV-1 isolates were reduced by 88.2% ± 2.2% for cells with the Q93R variant compared with the wild-type AGM clone 2 CCR5 protein. In contrast, in four independent assays, the Q93R variant was 110% ± 18% as active as AGM clone 2 for infections by SIV_{mac251}. Interestingly, the AGM clone 2 and AGM clone 2(Q93R) CCR5s were as active as human CCR5 for SIV_{mac251} infections. In contrast, AGM clone 2 CCR5 was much less active (ca. 20%) than human CCR5 for infections by HIV-1 ($P < 0.001$; $n = 22$). Thus, AGM clone 2 CCR5 is only partially active as a coreceptor for HIV-1.

Coreceptor activities of human and AGM CCR5s analyzed by using chimeras and site-directed mutants. To learn which of the amino acid substitutions in AGM clone 1 CCR5 are responsible for its inability to mediate infections by macrophage-tropic isolates of HIV-1, we analyzed human/AGM clone 1 CCR5 chimeras for their coreceptor activities in HIV-1 infections. As shown in Fig. 7, the AGM clone 1/human CCR5 chimera with the AGM clone 1 amino terminus was inactive in HIV-1 infections, whereas the reciprocal human/AGM clone 1 chimera was fully active. This suggested that the Y14N substitution was fully responsible for the lack of HIV-1 coreceptor activity of AGM clone 1 CCR5 and that the L352F substitution

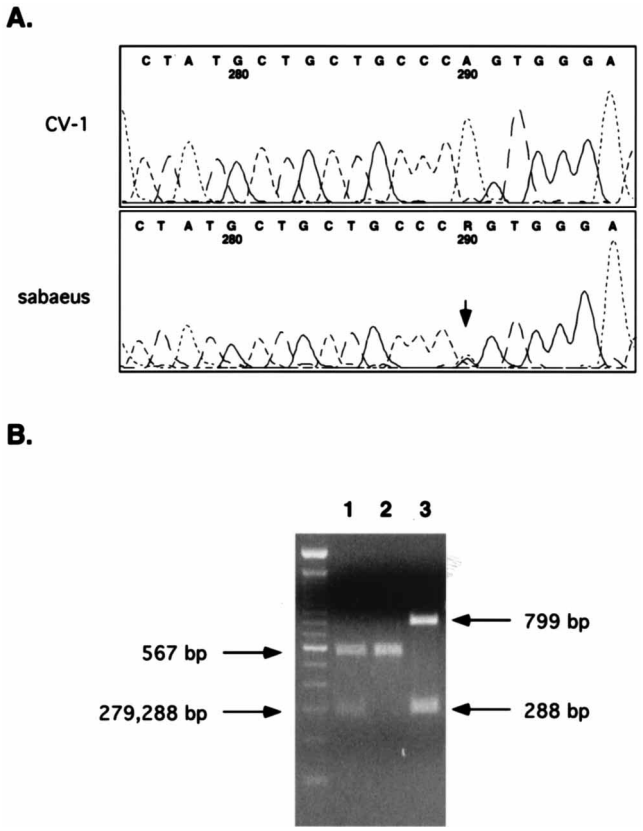


FIG. 3. Identification of a CCR5 polymorphism in the DNA of a sabaeus AGM. (A) Electropherograms from automated thermal cycle sequencing analysis of DNA samples amplified from the CCR5 genes of the sabaeus monkey (lower section) compared with the corresponding sequence from the AGM CV-1 cell line. The control CV-1 sample contains a homogeneous sequence in this region, with an A at position 290 (numbered from the first base of the PCR primer AGMF [see Materials and Methods]). In contrast, the sabaeus AGM sequence was identical except for an ambiguity (R = A or G) at this position. The same change was seen with CCR5 sequences that were independently amplified from the sabaeus AGM DNA sample. (B) Restriction enzyme digestion analysis of CCR5 DNAs that were obtained in an independent PCR amplification reaction using the CV-1 and sabaeus DNA preparations. Restriction fragments generated by digestion with *Nci*I were separated on a 2% agarose gel. The mutation implicated in the sabaeus CCR5 gene by the A290G substitution (panel A) adds a novel *Nci*I restriction enzyme cleavage site that reproducibly cleaves a fraction of the 567-bp sabaeus PCR amplification product to give fragments of predicted sizes 279 and 288 bp which are not resolved on this gel (lane 1). Digests of the CV-1 DNA amplification product show no cleavage fragments (lane 2). The 567-bp fragment is only partially cleaved in lane 1 because the sabaeus monkey was heterozygous (see panel A). The PCR product containing the mutant sabaeus CCR5 sequence was cut with *Cla*I, and the fragment spanning codons 12 through 124 of the AGM clone 2 coding sequence was cloned into the pcDNA3-AGM clone 2 plasmid that had also been cut with *Cla*I as described in Materials and Methods. In lane 3 the full coding sequence of the AGM clone 2(Q93R) mutant CCR5 [1,087 bp excised from the pcDNA3-AGM clone 2(Q93R) plasmid with *Bam*HI and *Xho*I] was digested with *Nci*I, showing that the mutant also gives the predicted 288-bp fragment as well as a fragment with a predicted size of 799 bp. The wild-type AGM clone 2 sequence is not digested with *Nci*I (results not shown).

had no significant effect. This conclusion was substantiated by studies of the human(Y14N) CCR5 mutant and the AGM clone 2(Y14N) CCR5 mutant. In both cases, the single Y14N mutations eliminated coreceptor activity for macrophage-tropic isolates of HIV-1 (Fig. 7). Thus, the Y14N mutations eliminated coreceptor activity for macrophage-tropic isolates of HIV-1 not only in the context of AGM CCR5 but also in the context of human CCR5. In addition, the AGM clone 2(Q93R) variant CCR5 was almost completely inactive as a coreceptor for all tested isolates of HIV-1 (Fig. 6). As shown in Table 1, all of the CCR5s in Fig. 6 and 7 were well expressed on cell surfaces as determined by three assays. These results suggest that different macrophage-tropic isolates of HIV-1 are highly dependent on amino acids at positions 14 and 93 of CCR5. In contrast, SIV_{mac251} infections are much less impaired by the Y14N mutation and not significantly affected by the Q93R mutation. As mentioned above, AGM clone 2 CCR5 is only approximately 23% as active for HIV-1 infections as human CCR5. In contrast, the human/AGM clone 1 chimera is as active as human CCR5 (Fig. 7), and similar results were obtained with a human/AGM clone 2 chimera (results not shown). This suggests that sequences in the amino-terminal half of AGM clone 2 CCR5 inhibit but do not prevent HIV-1 infections. The only amino acid substitutions on the extracellular surface in this region that distinguish the human and AGM clone 2 CCR5 proteins are I9T and N13D (Fig. 1). This raised the possibility that HIV-1 infections may be partially inhibited by the I9T and N13D substitutions. To test this hypothesis, we constructed the T9I and D13N mutations in the AGM clone 2 context. As shown in Fig. 7, these substitutions both appeared to increase the efficiencies of infections by macrophage-tropic isolates of HIV-1 approximately twofold. However, the increases varied among the HIV-1 isolates, and the ADA isolate appeared to be unaffected by the T9I substitution. These results suggest that these sequence differences contribute significantly to the reduced coreceptor activity of AGM clone 2 CCR5 compared to human CCR5 for HIV-1 infections.

Coreceptor activities of human/mouse CCR5 chimeras in infections by macrophage-tropic HIV-1. In parallel with the above investigations, we used our human and NIH/Swiss mouse CCR5 clones to construct and analyze human/mouse CCR5 chimeras. For this purpose we initially analyzed chimeras constructed by using the *Msc*I, *Bgl*II, and *Eco*RI sites (Fig. 2). Chimeras were made by substituting mouse sequences into human CCR5 (to give MMHH, HHMM, MHHH, HMHH, HHMH, and HHHM) as well as reciprocally substituting human sequences into mouse CCR5 (to give HMMM, MHMM, MMHM, and MMMH). In addition, we made the HHHHloop2M chimera by using the *Bgl*II and *Bsa*BI sites to specifically substitute only the extracellular loop 2 sequence of NIH/Swiss mouse CCR5 into the human protein (Fig. 2). MMMMloop2H and HMMMloop2H chimeras were made by substituting the coding sequence for extracellular loop 2 from human CCR5 into NIH/Swiss mouse CCR5 or the HMMM chimera, respectively (Fig. 2 and Table 1). After the HeLa-CD4 cultures were transfected with the pcDNA3-CCR5 plasmids, multiple subcultures were prepared for analyzing cell surface expression of each CCR5 protein and infections by four macrophage-tropic isolates of HIV-1. These CCR5 constructs and viruses were analyzed together in three to five independent experiments, which fully supported the same conclusions (Fig. 8A).

In considering these results, it is important to understand the three methods that were used to detect cell surface expression of CCR5 proteins (see Materials and Methods). The first method employed rabbit antiserum that detects the amino terminus of human CCR5 but not of mouse CCR5 to measure the percentage of transfected cells that immunofluoresce (Fig. 4). The second method used the same antiserum followed by binding of [^{125}I]protein A to quantitatively measure the relative levels of antibody adsorbed onto the cell surfaces. The third method analyzed the binding of 0.5 nM [^{125}I]Mip1β to the cells and was capable of detecting all of the CCR5 proteins on cell surfaces that could bind this chemokine. Because 0.5

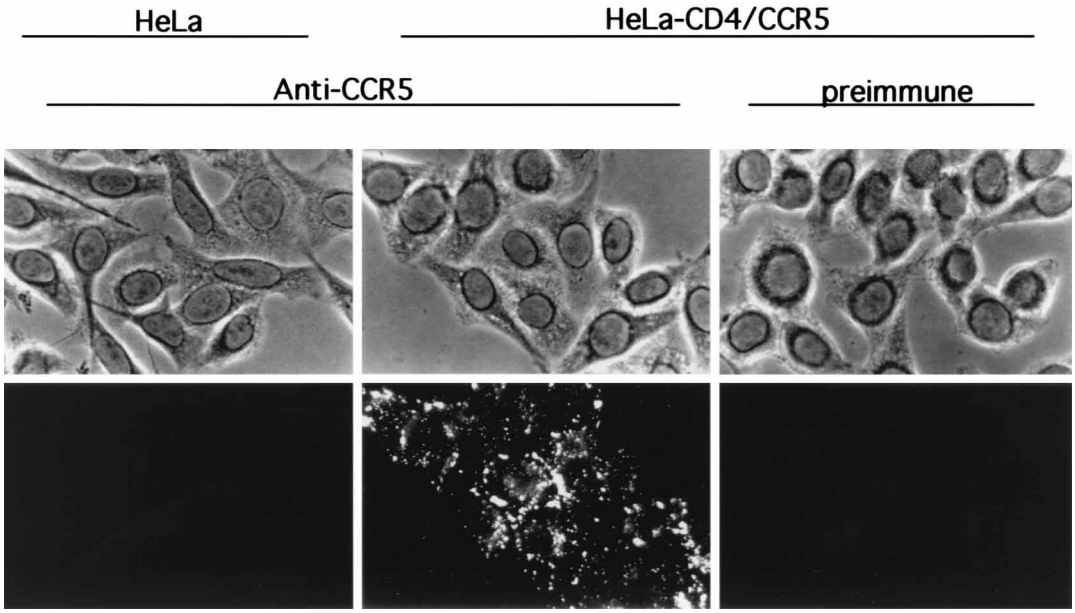


FIG. 4. Detection of human CCR5 on surfaces of viable cells by immunofluorescence microscopy. Upper panels, phase contrast; lower panels, fluorescence. HeLa-CD4/CCR5, a clone of the HeLa-CD4 cell line HI-J which stably expresses human CCR5 encoded by the retroviral expression vector pSFF-CCR5 (27, 35) in all of the cells. Anti-CCR5, rabbit anti-CCR5 serum; preimmune, preimmune serum from the same rabbit. The patching of fluorescence is caused by antibody-induced cross-linking of the cell-surface CCR5. Labeling occurs only when the specific antibody binds to cells that express CCR5 and not with preimmune serum or with HeLa cells that lack CCR5.

nM Mip1β is below the K_d for the binding of this chemokine to CCR5 proteins (37), because the K_d values differ for the CCR5 proteins used in this study (see below), and because slight variations in K_d between different CCR5 proteins would result in large differences in binding of 0.5 nM Mip1β, the results obtained with this method provided only a qualitative indication of cell surface expression of the CCR5s. Saturating the receptors by using very high concentrations of [^{125}I]Mip1β was impractical due to limitations in our resources and because this would have lowered the ratio of specific saturable labeling in comparison to the negative controls. Complete Mip1β binding analyses were done by using a competition method (37) for the CCR5s of humans, AGMs, and mice. These assays confirmed that the CCR5s had distinct apparent affinities for human Mip1β and indicated that the K_d for human CCR5 in HeLa-CD4 cells was approximately 17 nM (35). A previous analysis indicated an apparent 50% inhibitory concentration of 7.4 nM for human CCR5 expressed in human 293T cells in a competitive binding assay (37).

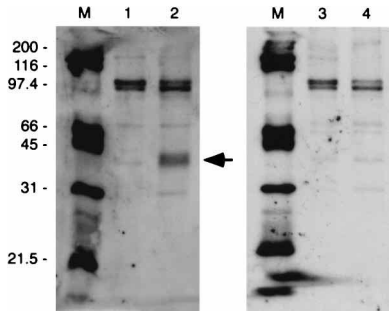


FIG. 5. Protein immunoblot (Western) analysis of human CCR5 in membrane preparations from COS-7 cells that express this chemokine receptor. Membrane preparations from COS-7 cells transfected with the pcDNA3-CCR5 expression construct (lanes 2 and 4) or with pcDNA3 alone (lanes 1 and 3) were separated on sodium dodecyl sulfate–10% polyacrylamide gel and transferred to nitrocellulose. Transferred proteins were then blotted with the anti-CCR5 antibody prepared as described in the text (lanes 1 and 2) or with the preimmune serum from the same rabbit (lanes 3 and 4). Bound rabbit IgG was detected by standard chemiluminescent Western blotting techniques. The molecular weights of size standards (lanes M) are indicated (in thousands). The band at M_r ~40,000 (arrow) is detected only in CCR5-transfected cells with the serum from the immunized rabbit. The intense bands near the top represent nonspecific cross-reaction with one of the chemiluminescence reagents, since they were absent when blots were visualized with [^{125}I]protein A.

Three of our CCR5 chimeras (HHMM, HHHM, and MHMM) did not appear to be expressed on HeLa-CD4 cell surfaces in our assays. Consequently, their inabilities to function as coreceptors were not informative, and they are not included in Table 1 or Fig. 8. Similarly, derivatives of MHMM that contain the second extracellular loop of human CCR5 or C57BL/6 mouse CCR5 [i.e., MHMMloop2H and MHMM (L183P)] were not expressed on cell surfaces. Interestingly, these results are perfectly concordant with recent findings of Bieniasz et al. that chimeras with human sequences in the second region and mouse sequences in the fourth are incompatible with expression on cell surfaces (8). This supports the validity of our methods. The HHMH chimera was weakly expressed on cell surfaces, but the HHHHloop2M chimera that supported the same conclusions was highly expressed. Surprisingly, several of the chimeras that were well expressed as detected by our antiserum did not significantly bind [^{125}I]Mip1β or bound only a low level of this chemokine (e.g., HMMM, HMHH, HHMH, and HHHHloop2M). Presumably, these chimeric CCR5s must fold into a conformation with a lower affinity for Mip1β than other CCR5s tested. Since HMHH is an active coreceptor (Fig. 8), ability to bind Mip1β with a high affinity is not a prerequisite for coreceptor function. Studies of G-protein-coupled receptors have indicated that minor changes in folding can have large effects on affinities for agonists or antagonists (11, 21, 42).

The results in Fig. 8A indicate that the MHHH and HMHH chimeras were partially active as coreceptors for macro-

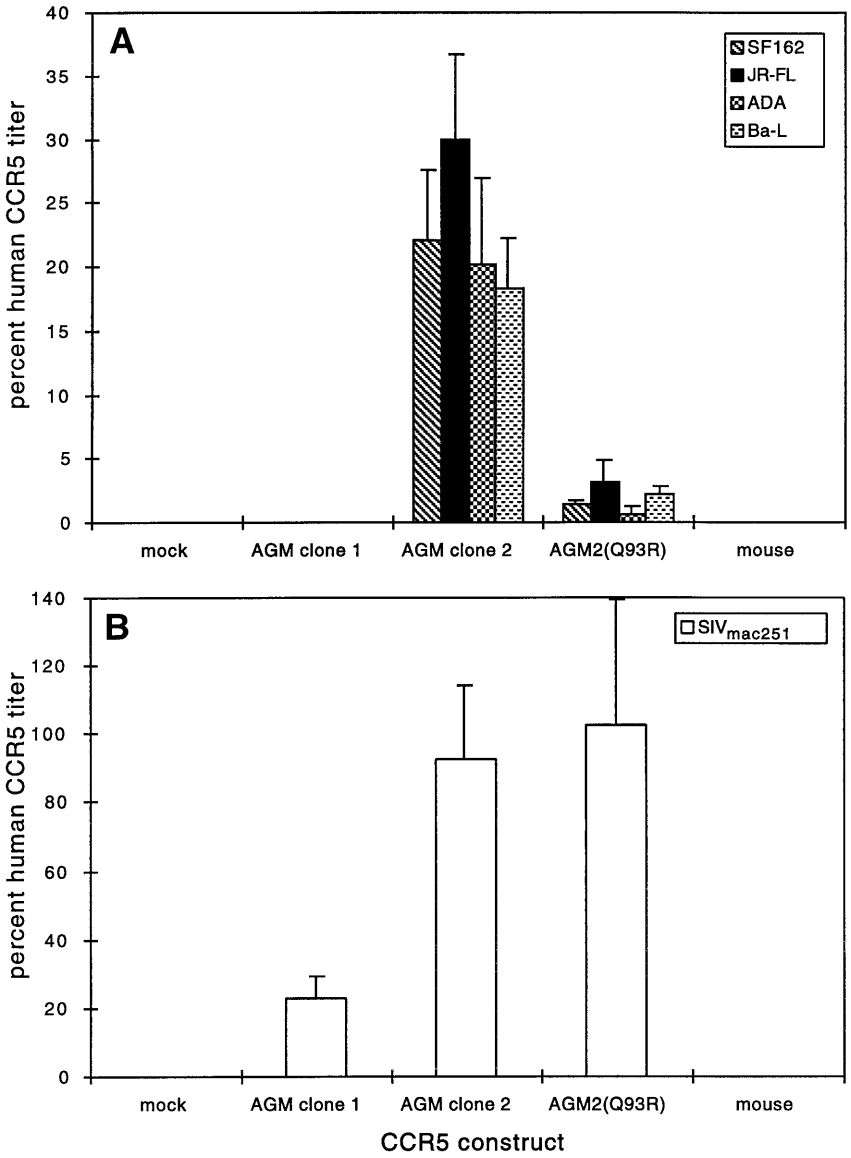


FIG. 6. Coreceptor activities of naturally occurring CCR5 proteins encoded by humans, AGMs, and mice. The coreceptor activities were all normalized relative to the activity of human CCR5 in the same experiment. (A) Activities for infections by the macrophage-tropic HIV-1 isolates SF162, JR-FL, ADA, and Ba-L; (B) activities for infection by SIV_{mac251}. Error bars represent standard errors of the means except for $n = 2$, where they represent ranges. $n = 4$ for JR-FL except with AGM clone 2, where $n = 6$; $n = 2$ for ADA except with AGM clone 2, where $n = 4$; $n = 6$ for SF162 and Ba-L except with AGM clone 2(Q93R), where $n = 7$, and AGM clone 2, where $n = 8$. For SIV_{mac251}, $n = 3$ except with AGM clone 2(Q93R), where $n = 4$.

phage-tropic isolates of HIV-1. This is intriguing because both of these mouse substitutions caused many nonconservative changes in the protein. In contrast, the MMHH chimera was completely inactive as a coreceptor, although it was expressed on cell surfaces as determined by [^{125}I]Mip1β binding. Comparison of the MHHH and HMHH chimeras, which are active coreceptors, with MMHH, which is inactive, suggests that the amino-terminal and extracellular loop 1 regions of human CCR5 both contribute to coreceptor function. The fact that the MHHH and HMHH chimeras are significantly less active than human CCR5 for all tested isolates of HIV-1 ($P < 0.001$; $n = 20$) also suggests that human sequences in these substituted regions contribute to coreceptor activity. Interestingly, the titers of SF162 were reduced by approximately 65% on cells expressing HMHH compared to cells expressing MHHH (Fig. 8), and this difference was highly significant ($P \leq 0.002$; $n = 5$). Additionally, Ba-L titers were reduced by approximately 30% on HMHH compared to MHHH. While this difference is less striking, it is also statistically significant ($P \leq 0.006$; $n = 5$). The titers of JR-FL and ADA were not significantly different on HMHH compared to MHHH by the paired-comparison *t* test. Taken together, these results suggest that the contributions to coreceptor activity of the amino terminus and extracellular loop 1 of CCR5 may differ depending on the HIV-1 strain.

In addition, the results in Fig. 8 clearly indicate that the HHMH and HHHHloop2M chimeras are completely inactive as coreceptors for HIV-1. These results could imply that extracellular loop 2 of human CCR5 is essential for coreceptor activity and/or that loop 2 of NIH/Swiss mouse CCR5 is non-

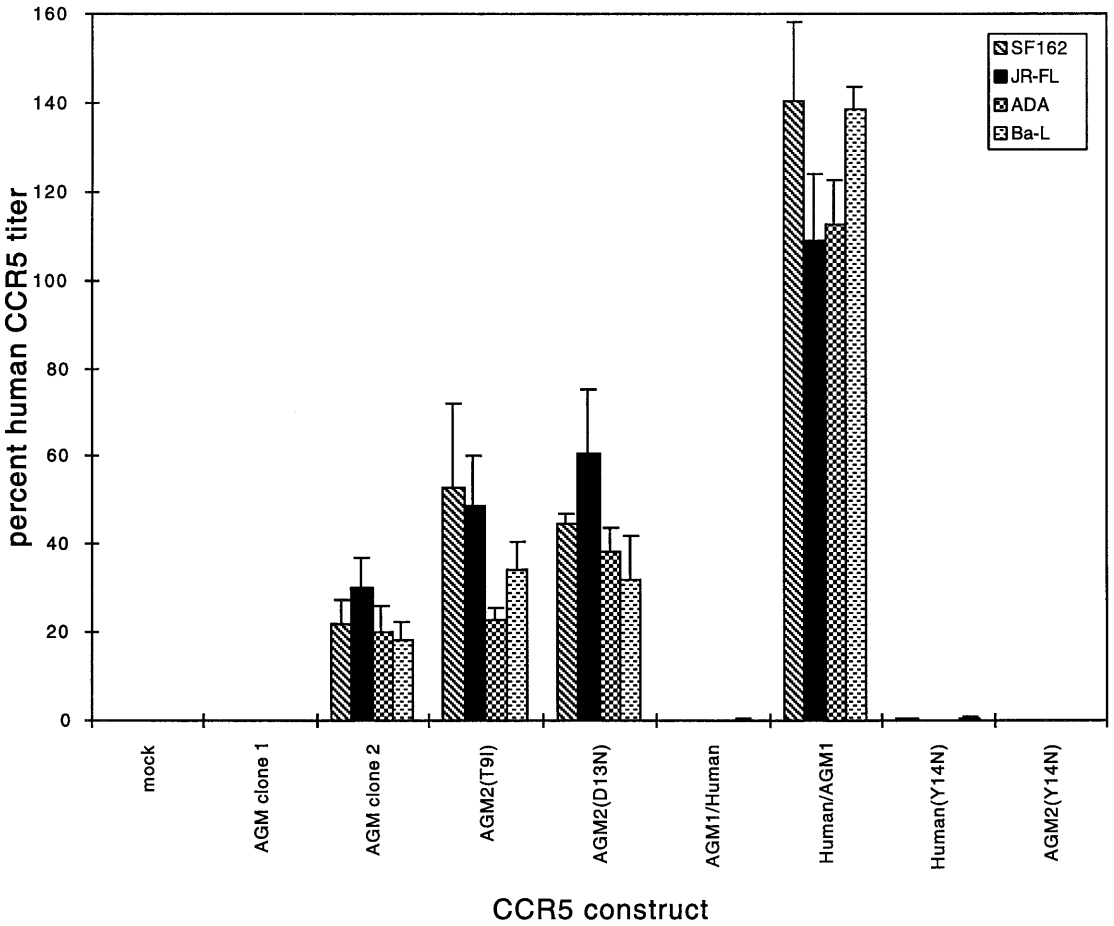


FIG. 7. Coreceptor activities of human/AGM CCR5 chimeras and of human(Y14N), AGM clone 2(Y14N), AGM clone 2(T9I), and AGM clone 2(D13N) mutants. The coreceptor activities were all normalized relative to the activity of human CCR5 in the same experiment, using the macrophage-tropic HIV-1 isolates SF162, JR-FL, ADA, and Ba-L. Error bars represent standard errors of the means except for $n = 2$, where they represent ranges. $n = 2$ for AGM clone 1/human, human/AGM clone 1, and AGM clone 2(Y14N); $n = 4$ for human(Y14N). For SF162 and Ba-L, $n = 6$ with AGM clone 1 and $n = 8$ with AGM clone 2. For JR-FL and ADA, $n = 4$ and $n = 2$ with AGM clone 1, respectively, and $n = 6$ and $n = 4$ with AGM clone 2, respectively. $n = 4$ for AGM clone 2(T9I) and AGM clone 2(D13N).

permissive for HIV-1 infections. Either of these interpretations would be compatible with the fact that our HMMM and MMMH chimeras were also inactive. In addition, the MMHM and MMMMloop2H chimeras were inactive as coreceptors for HIV-1 isolates, suggesting that the second extracellular loop of human CCR5 is insufficient for coreceptor function in the context of the NIH/Swiss mouse protein. In contrast, the MMMMloop2H chimera was partially active as a coreceptor for SIV_{mac251} (see below). The inactivity of HHHHloop2M as a coreceptor for HIV-1 was surprising because Atchison et al. recently presented evidence that the loop 2 region of 129/SvJ mouse CCR5 is compatible with coreceptor activity for HIV-1 (4). For example, they reported that 129/SvJ mouse chimeras with only amino-terminal sequences or extracellular loop 1 sequences of human CCR5 were active coreceptors for the Ba-L isolate of HIV-1. Inspection of the second extracellular loop sequences of the NIH/Swiss and 129/SvJ mouse CCR5 proteins indicated that they differ at only one amino acid, a nonconservative P183L substitution in the NIH/Swiss mouse protein. Previously described CCR5 proteins that have coreceptor activity including human, rhesus macaque, and AGM clone 2 CCR5s, as well as human CCR2b, contain a Pro residue at the corresponding position. Moreover, the BALB/c mouse CCR5 extracellular loop 2 region also contains a Pro at the corresponding position and was recently shown by Bieniasz et al. to allow cell fusion mediated by the *env* proteins of the ADA and Ba-L isolates of HIV-1 using a chimera that was otherwise identical to our HHHHloop2M (8).

These considerations strongly suggested that NIH/Swiss mouse CCR5 contains a P183L substitution that interferes with HIV-1 coreceptor activity but not with Mip1β binding. To test this interpretation, we cloned the CCR5 sequence from the genomic DNA of a C57BL/6 mouse, confirmed that it contained a P183 residue, and used this loop 2 region to construct additional chimeras. As indicated in Fig. 8, the HHHHloop2M(L183P) chimera was partially active as a coreceptor for macrophage-tropic isolates of HIV-1, in contrast to the HHHHloop2M chimera, which differs only at position 183. The C57BL/6 mouse CCR5 protein and a chimera containing loop 2 of C57BL/6 mouse CCR5 in the NIH/Swiss mouse CCR5 background were both inactive as coreceptors for HIV-1 (results not shown). These results indicate that a Leu at the position equivalent to amino acid 183 of human CCR5 is not compatible with HIV-1 infection.

Coreceptor activities of human/mouse CCR5 chimeras in infections by SIV_{mac251}. We also studied a limited number of the human/NIH/Swiss mouse chimeras as coreceptors for SIV_{mac251} (Fig. 8B). The MMMMloop2H and MMHH chime-

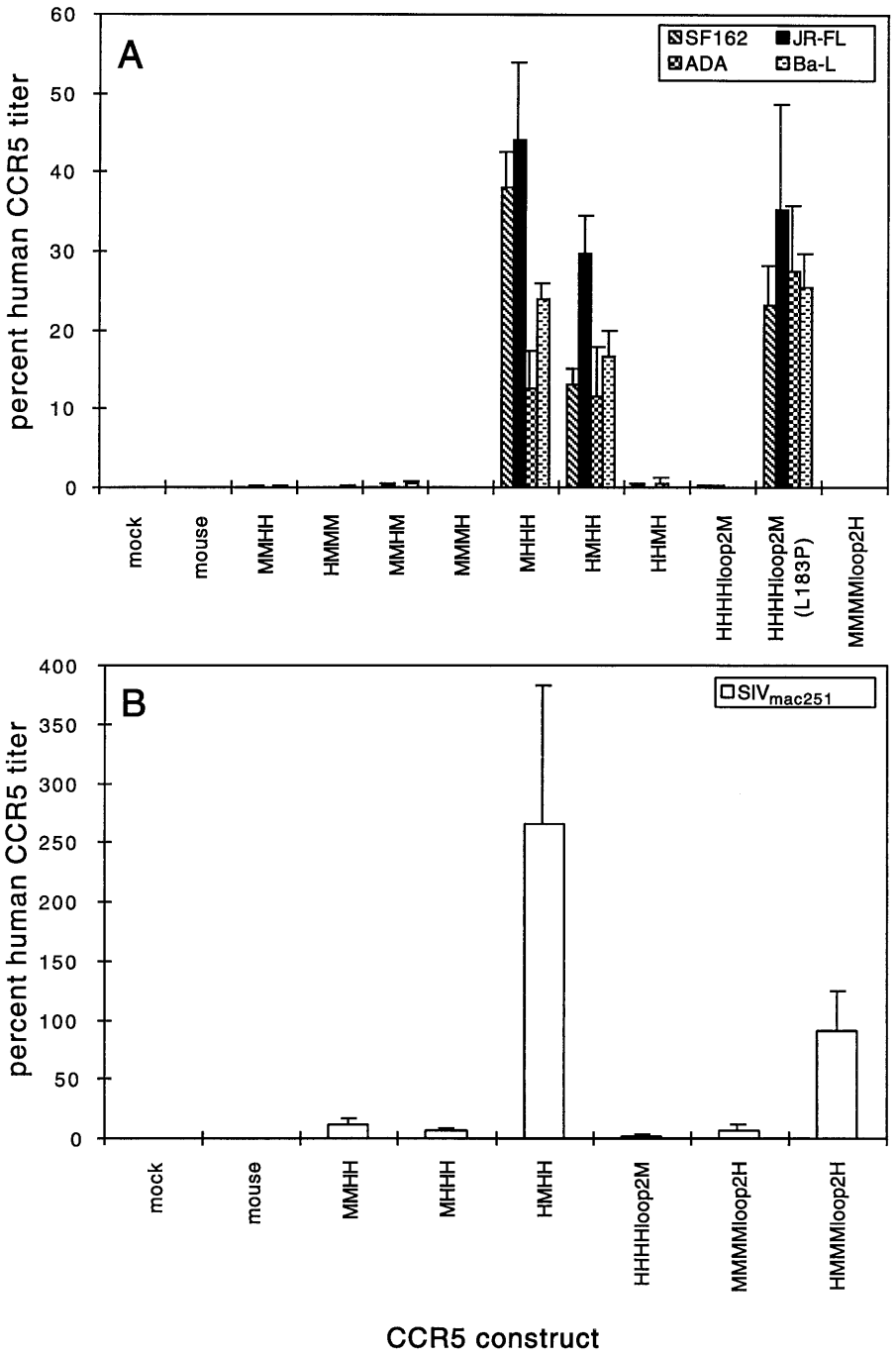


FIG. 8. Coreceptor activities of human/NIH/Swiss mouse CCR5 chimeras for infections of macrophage-tropic HIV-1 (A) or SIV_{mac251} (B). The coreceptor activities were all normalized relative to the activity of human CCR5 in the same experiment. Error bars represent standard errors of the means except for $n = 2$, where they represent ranges. In panel A, $n = 5$ except for HMMM, MMMH, and HHMH, where $n = 3$, and for HHHHLoop2M(L183P), where $n = 4$ for the SF162, ADA, and Ba-L isolates and $n = 3$ for the JR-FL isolate. In panel B, $n = 3$ for the mock, mouse, MMMMloop2H, and HMMMloop2H CCR5 constructs and $n = 2$ for MMHH, MHHH, HMHH, and HHHHloop2M.

ras are weakly active (approximately 6 and 11% of human CCR5, respectively), suggesting that extracellular loop 2 of the human CCR5 protein contributes to infections by SIV_{mac251} but that it is insufficient for full activity. These low levels of coreceptor activity are significant because our assay has zero background in mock-transfected cells. Additionally, the coreceptor activity of the MHHH chimera was similar to those of the MMMMloop2H and MMHH chimeras, suggesting that addition of the human extracellular loop 1 sequence did not substantially increase coreceptor activity. In contrast to these chimeras, the HMHH chimera was approximately twofold more active than the human CCR5 protein for SIV_{mac251}. Additionally, the HMMMloop2H chimera, which has the same presumptive extracellular surface as the HMHH chimera, was

approximately as active as human CCR5 in mediating infection of HeLa-CD4 cells by SIV_{mac251}. These results suggest that the amino acid residues in the amino terminus and extracellular loop 2 regions of human CCR5 that differ from NIH/Swiss mouse CCR5 are important in coreceptor function for SIV_{mac251}. Finally, the HHHHloop2M chimera was only about 2% as active as the human CCR5, suggesting that while extracellular loop 2 from a species compatible with infection is not sufficient for full SIV_{mac251} coreceptor activity (i.e., MMHH and MMMMloop2H), it is probably required.

DISCUSSION

Functional diversity of CCR5 sequences in AGMs. We reproducibly cloned two CCR5 sequences from the DNA of the AGM cell line CV-1 (Fig. 1). The AGM clone 1 protein contains Y14N and L352F in positions that are otherwise conserved in the human, AGM clone 2, and mouse CCR5 proteins. These AGM CCR5 proteins differ dramatically in their coreceptor activities. Both are active coreceptors for SIV_{mac251}, but only AGM clone 2 protein is active for macrophage-tropic isolates of HIV-1 including SF162, JR-FL, Ba-L, and ADA (Fig. 6 and 7). By using chimera constructions and site-directed mutagenesis, we found that the inability of AGM clone 1 CCR5 to function as a coreceptor for HIV-1 is solely due to the Y14N substitution and that the human(Y14N) mutant CCR5 is also inactive (Fig. 7). Thus, the Y14N mutation prevents infections by macrophage-tropic isolates of HIV-1 but does not block infections by SIV_{mac251}. Similarly, the AGM clone 2(Q93R) variant that we isolated from a sabaeus AGM DNA sample was fully active as a coreceptor for SIV_{mac251} but was only weakly active for all tested isolates of macrophage-tropic HIV-1 (Fig. 6). Studies using human/NIH/Swiss mouse CCR5 chimeras also indicated that SIV_{mac251} could weakly use MMMMloop2H (containing only the human extracellular loop 2 in an NIH/Swiss mouse background), whereas this chimera was unable to mediate infections by macrophage-tropic isolates of HIV-1 (Fig. 8). However, SIV_{mac251} uses HMMMloop2H much more efficiently than MMMM loop2H, suggesting that SIV_{mac251} also requires sequences in the amino-terminal region of human CCR5 for maximal infectivity. These results suggest that the HIV-1 isolates are more dependent than SIV_{mac251} on Y14 and Q93 sites in human CCR5, whereas SIV_{mac251} is more reliant on extracellular loop 2. Moreover, we found that HIV-1 isolates use human CCR5 approximately five times more efficiently than the wild-type AGM clone 2 CCR5, whereas SIV_{mac251} uses these CCR5s equally well. This reduction in HIV-1 infections is at least partially caused by the I9T and N13D substitutions in AGM clone 2 compared to human CCR5 (Fig. 1 and 7). Recent findings by Edinger et al. are consistent with these conclusions (18).

To learn whether the Y14N mutation is common in AGMs or unique to the CV-1 cell line, we initially examined the first ~560 coding bp of two DNA samples that had been prepared from PBMCs of AGMs. Our investigation of a vervet AGM DNA sample suggested that it contained two distinct CCR5 sequences, one with a D13N mutation and one that appeared identical in the predicted amino acid sequence to AGM clone 2. The D13N mutation in the AGM clone 2 context enhanced infections by macrophage-tropic isolates of HIV-1 approximately twofold, in accordance with the presence of Asn at position 13 in human CCR5 (Fig. 7). The sabaeus AGM DNA sample also appeared to have derived from a CCR5 heterozygote, and in this case one allele encoded a variant with a Q93R substitution in extracellular loop 1. This mutation added a novel *Nci*I restriction enzyme cleavage site that was reproducibly detected in CCR5 DNA amplified from this monkey but not in DNA amplified from CV-1 cells (Fig. 3). As mentioned above, this AGM clone 2(Q93R) protein was almost completely inactive as a coreceptor for multiple isolates of macrophage-tropic HIV-1 but was fully active for SIV_{mac251} (Fig. 6). Based on these results, we analyzed the DNAs from the two AGM cell lines Vero and BS-C-1. The Vero analysis was consistent with homozygosity for the AGM clone 2 CCR5 sequence. In contrast, sequencing analysis of CCR5 amplified from BS-C-1 cells, which were derived from a grivet AGM (40), suggested that this cell line is heterozygous in the coding sequence of its genomic DNA for a novel point mutation which encodes a Q93K substitution in the AGM clone 2 CCR5 protein. Thus, among the five AGM DNA samples that we examined, four appeared to have derived from CCR5 heterozygotes and all of the amino acid changes were distinct. This frequent heterozygosity implies that substantial CCR5 polymorphism must occur within single breeding populations of different AGM subspecies. In contrast, approximately 10% of North American Caucasians have a CCR5 variant with a 32-base deletion (3, 30, 39). The frequency of polymorphisms is substantially lower in other races (3, 30, 39). We conclude that AGMs have a higher prevalence of CCR5 polymorphisms than humans ($P < 0.001$).

There is evidence that AGMs have been infected with SIV_{agm} since ancient times and that SIV_{agm} viruses have become highly diverse (2, 7, 24, 25, 28, 32). Indeed, AGMs have been classified into four geographically distinct subspecies—vervets (*Cercopithecus aethiops pygerythrus*), tantalus (*Cercopithecus aethiops tantalus*), grivets (*Cercopithecus aethiops aethiops*), and sabaeus (*Cercopithecus aethiops sabaeus*)—and each subspecies is naturally infected at a very high prevalence by a distinct strain of SIV_{agm} without apparent immunodeficiency (2, 7, 32, 33). Moreover, SIV_{agm} isolates are approximately equally divergent from SIV_{mac251} and HIV-1 (24, 25). This evidence implies that AGMs and SIV_{agm} viruses have coevolved since a time preceding divergences of AGM subspecies. These considerations suggest that extensive CCR5 polymorphism in AGMs might have been selected by their prolonged exposure to a large reservoir of SIV_{agm} viruses and that the polymorphisms in AGM CCR5 might limit pathogenesis or inhibit emergence of more pathogenic SIV_{agm} variants. If this were correct, the specific amino acid polymorphisms in AGM CCR5s would often occur at sites that are important for viral infection and pathogenesis. In agreement with this hypothesis, four of the amino acid substitutions that we have detected in AGM CCR5s are clustered at two sites on the extracellular surface (D13N and Y14N at the first site and Q93R and Q93K at the other site) that appear to be important for infections by macrophage-tropic HIV-1. In contrast, these amino-terminal and extracellular loop 1 regions of mouse CCR5 contain many nonconservative differences from human CCR5, yet the MHHH and HMHH chimeric proteins are relatively active coreceptors for HIV-1 (Fig. 1 and 8). In addition, polymorphisms in human CCR5 are infrequent and are dispersed throughout the protein (3). Further evidence about these issues will require analyses of additional AGM CCR5 genes and studies of multiple SIV_{agm} isolates using suitable focal infectivity assays. As more AGM CCR5 variants are identified, it will also be important to analyze their chemokine receptor activities. Currently, we are investigating these issues using novel and sensitive assays for chemokine receptor signaling in *Xenopus* oocytes and in mammalian cells (results not shown).

CCR5 polymorphisms in mice and analyses of human/NIH/Swiss mouse CCR5 chimeras. CCR5 of NIH/Swiss mice differs

at 10, 8, and 14 positions from the recently described CCR5 sequences of 129/SvJ (10), B6CBA (31), and BALB/c (8) mice, respectively. The NIH/Swiss mouse CCR5 protein is 79.8% identical to human CCR5 (Fig. 1) but is completely inactive as a coreceptor for SIV_{mac251} or macrophage-tropic HIV-1. Consequently, to identify regions of human CCR5 that are important for interactions with HIV-1 but are absent from NIH/Swiss mouse CCR5, we employed human/mouse CCR5 chimeras. After this work was substantially initiated, Atchison et al. described an independent study using the 129/SvJ mouse sequence as the reference for their chimera constructions (4), and analyses of human/CCR2b chimeras were also reported (4, 18, 38). A study of human/BALB/c mouse CCR5 chimeras was also reported during revision of our manuscript (8). Although our conclusions are compatible with those studies (see below), the chimeras that we made and the results that supported our conclusions were somewhat different. A major difference derived from the presence in the NIH/Swiss mouse CCR5 of a P183L substitution (human CCR5 numbering) at a site in extracellular loop 2 that is otherwise conserved in all CCR5 proteins that are known to function as coreceptors for human or animal immunodeficiency viruses. This substitution did not prevent cell surface expression or Mip1β binding but was nonpermissive for infections. Accordingly, all chimeras with this sequence were inactive in our coreceptor assays (Fig. 8). In contrast, the HHHHloop2M(L183P) chimera which contains a Pro residue at position 183 was an active coreceptor for HIV-1 (Fig. 8).

The MHHH and HMHH chimeras were partially active as coreceptors for macrophage-tropic isolates of HIV-1, whereas MMHH was inactive, although it was expressed on cell surfaces (Fig. 8). Comparison of these active MHHH and HMHH coreceptors with MMHH suggests that the extracellular loop 1 and amino-terminal regions of human CCR5 both contribute positively to coreceptor function. The fact that the MHHH and HMHH chimeras are significantly less active than the human CCR5s ($P \leq 0.02$; $n = 5$) also supports this conclusion. In contrast, the HHMH and HHHHloop2M chimeras were inactive as coreceptors as were all other CCR5 chimeras that contained the NIH/Swiss mouse loop 2 sequence. This suggested that the human loop 2 sequence was essential and/or that the NIH/Swiss mouse sequence was nonpermissive for infections. In either case, this would imply that HIV-1 interacts closely with CCR5 loop 2. The fact that our MMHM, MMHH, and MMMMloop2H chimeras were inactive as coreceptors for HIV-1 suggested that the human loop 2 sequence was insufficient for coreceptor function in the context of the NIH/Swiss mouse CCR5 protein. In contrast, SIV_{mac251} was able to use MMHH and MMMMloop2H as coreceptors at low efficiencies, and addition of the human amino terminus (i.e., HMMMloop2H and HMHH chimeras) restored full activity (Fig. 8B). Because other evidence clearly indicated that the human loop 2 sequence is not essential for coreceptor activity in the context of chimeras made with the 129/SvJ CCR5 or human CCR2b protein (4, 8, 38), we inferred that the NIH/Swiss mouse CCR5 loop 2 must be nonpermissive for infections, and we observed that it differs from the permissive 129/SvJ sequence only by a nonconservative P183L substitution. To test this idea, we cloned the CCR5 coding sequence from a C57BL/6 mouse and we made the HHHHloop2M(L183P) chimera that contained this sequence. This chimera was active as a coreceptor for HIV-1, in contrast to the identical chimera that has a Leu residue at position 183 (Fig. 8). This evidence suggests that the P183L substitution that occurs in NIH/Swiss mice causes an absolute block in infections by all tested macrophage-tropic isolates of HIV-1.

We believe that the results of our analyses of human/NIH/Swiss mouse CCR5 chimeras are fully compatible with related evidence from other laboratories which have also implicated the amino terminus and extracellular loops 1 and 2 of human CCR5 in infections by macrophage-tropic HIV-1 (4, 8, 34, 38). However, as discussed elsewhere (8, 34), the earlier studies did not give identical results, presumably because the reference proteins, viruses, cell lines, assay methods, and chimera splice sites were distinct. For example, several of these studies used syncytium rather than infection assays, epitope-tagged rather than native CCR5s, or coreceptor overexpression in COS-7 cells.

Based on our results, we believe that a major source of error in chimera analyses derives from the assumption that the reference protein sequences used in the chimeras have neutral rather than positive or negative effects on the activity being measured. As mentioned by Bieniasz et al. (8), this assumption of neutrality is unlikely to be valid for coreceptors because the reference proteins that have been used were all closely related to human CCR5 and because many HIV-1 isolates can promiscuously employ different chemokine receptors such as CCR2b and CCR3 (14, 16, 38). Such promiscuity implies that substantial variations in coreceptor sequences are compatible with productive interactions with HIV-1. Our evidence clearly demonstrates that the assumption of neutrality is incorrect for the NIH/Swiss mouse loop 2 sequence, which is nonpermissive for coreceptor activity. One possible interpretation is that the P183 residue in human CCR5 and at the corresponding positions in the reference proteins used in previous chimera studies contributed positively to the coreceptor activities that were observed. Alternatively, the Leu residue at this site in NIH/Swiss mouse CCR5 might prevent infections, perhaps by a steric mechanism. In either case, effects of reference protein sequences are clearly very difficult to detect or to control for, yet they can profoundly influence results of chimera analyses.

Evidence for active sites in human CCR5. In addition to the chimera approach, which has a limited resolution and is complicated by certain assumptions as described above, we have employed an alternative method to identify active sites in CCR5. Specifically, we have compared closely related CCR5 proteins that differ only in several amino acids yet have substantial differences in their coreceptor activities. This approach has proven to be advantageous because it does not require assumptions about the ability of HIV-1 to interact with a reference protein and because it has enabled us to rapidly identify specific CCR5 amino acids that are important for HIV-1 infections. By studying CCR5 polymorphisms in AGMs and mice, we have found that the Y14N, Q93R, and P183L substitutions severely inhibit infections by macrophage-tropic isolates of HIV-1 (Fig. 6 to 8). Similarly, by comparing the coreceptor activities of the closely related human and AGM clone 2 proteins, we obtained evidence that I9T and N13D substitutions significantly reduce but do not prevent HIV-1 infections (Fig. 7).

To the best of our knowledge, Y14N, Q93R, and P183L are the first amino acid substitutions on the extracellular surface of CCR5 that allow processing to cell surfaces and chemokine binding but appear to block HIV-1 infections. Additional mutagenesis studies of the Y14, Q93, and P183 amino acids are in progress and may help to more precisely determine the roles of these residues in HIV-1 infections. In contrast, chimera studies have indicated that many nonconservative substitutions and deletions at nearby positions are compatible with CCR5 coreceptor activity. Based on these considerations, we propose that macrophage-tropic HIV-1 isolates interact broadly with different regions of CCR5 but that certain amino acids in these

regions are nevertheless critical for infections by independent isolates of macrophage-tropic HIV-1. These key amino acids might be important for conformations of the contact regions, or they might directly comprise the contact sites. As discussed above, the existence of critical sites in CCR5 was unexpected because of earlier evidence that all regions of human CCR5 may be expendable for infections by macrophage-tropic HIV-1 in the context of specific chimera structures (4, 8, 34, 38). The finding of sites in CCR5 that are critical for infections by all tested isolates of macrophage-tropic HIV-1 has the important corollary that targeting these sites with monoclonal antibodies or drugs might reduce transmission or be clinically beneficial to infected individuals.

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