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Production of Specific mRNA Transcripts, Usage of an Alternate Promoter, and Octamer-Binding Transcription Factors Influence the Surface Expression Levels of the HIV Coreceptor CCR5 on Primary T Cells\textsuperscript{1,2}

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Surface levels of CCR5 on memory CD4\textsuperscript{+} T cells influence HIV-1/AIDS susceptibility. Alternative promoter usage results in the generation of CCR5 mRNA isoforms that differ based on whether they contain or lack the untranslated exon 1. The impact of exon 1-containing transcripts on CCR5 surface expression is unknown. In this study, we show that the increased cell surface expression of CCR5 on primary T cells is associated with selective enrichment of exon 1-containing transcripts. The promoter that drives exon 1-containing transcripts is highly active in primary human T cells but not in transformed T cell lines. The transcription factors Oct-1 and -2 inhibit and enhance, respectively, the expression of exon 1-containing transcripts and CCR5 surface levels. However, polymorphisms at homologous octamer-binding sites in the CCR5 promoter of nonhuman primates abrogate the binding of these transcription factors. These results identify exon 1-containing transcripts, and the cis-trans factors that regulate the expression levels of these mRNA isoforms as key parameters that affect CCR5 surface expression levels, and by extension, susceptibility to HIV/AIDS among humans, and possibly, the observed interspecies differences in susceptibility to lentiviral infection. The Journal of Immunology, 2007, 178: 5668–5681.

Because CCR5 serves as the major coreceptor for the cell entry of HIV-1 (1), its expression levels are a critical determinant of HIV/AIDS susceptibility. For example, homozygosity for a 32-bp deletion in the open reading frame (ORF)\textsuperscript{5} of CCR5 results in loss of surface expression and is asso-

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\textsuperscript{5} Abbreviations used in this paper: ORF, open reading frame; UTR, untranslated region; Pr, promoter; CHART-PCR, chromatin accessibility real-time PCR; TF, transcription factor; qPCR, quantitative PCR; CNS, conserved noncoding sequence; NCNS, nonconserved noncoding sequence; Cl, cycle threshold; ChIP, chromatin immunoprecipitation; IVTT, in vitro transcription and translation; AGM, African Green Monkey; HDV, HIV-derived vector; siRNA, small interfering RNA.

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transcripts (10, 11, 13). We therefore tested the hypothesis that 1) Pr2-driven exon 1-containing transcripts designated as CCR5A and CCR5B are important determinants of CCR5 expression in primary T cells; and 2) the prevailing viewpoint that Pr2 is not relevant to CCR5 gene expression is an artifact of conducting analyses in transformed cell lines. Our results affirm these two hypotheses and we demonstrate that the interactions between CCR5 Pr2 and the transcription factors Oct-1 and Oct-2 play an important role in CCR5 gene regulation and, consequently, in HIV/AIDS pathogenesis.

Materials and Methods

Plasmids

CCR5 Pr1 reporter plasmids pl1, p1b, and plc are as described previously (10). Pr2 reporter plasmids p2a, p2b, p2c, p2d, and p2e were cloned into the KpnI and Smal restriction enzyme sites of the promoterless PGL3-Basic vector (Promega) and the extent and location of the constructs are shown in Fig. 4a. The plasmid p2dm was created by site-directed mutagenesis of the octamer (Oct) binding site in the plasmid p2d (Stratagene). Oligonucleotides used for all experiments except for CHART-PCR are shown in Table I. The ORFs of transcription factors (TF) Oct-1 and Oct-2 (gift from Dr. W. Herr, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were cloned into pEF6/V5-HisB (Invitrogen Life Technologies). Bicistronic HIV-derived vectors (HDV) that expressed either Oct-1 or Oct-2 and murine CD24 (HSA) were also created using approaches as described previously (25, 26).

RT-PCR and real-time quantitative RT-PCR

RNA from primary cells was isolated, treated with DNase, and reverse transcribed with random hexamers using a commercially available kit (Invitrogen Life Technologies). The cDNA was then amplified by PCR using forward primer Reverse primer Probe Deletion constructs (Fig. 4a) p2a (Forward) p2b (Forward) p2c (Forward) p2d (Forward) p2e (Forward) Reverse primer Quantitative PCR for ChIP assay (Fig. 5g) Forward primer; sequence in italics corresponds to CCR5 sequence Reverse primer EMSA (Fig. 6; sense oligonucleotides shown) NF-κB (~3420 to ~3406) AP1 (~3198 to ~3178) Oct-1/2 (~3005 to ~2991) Mutagenesis of the Pr2 octamer-binding site (Fig. 7d; mutated nucleotide shown in bold) Sense primer RT-PCR (Fig. 2, b and f; Fig. 3c; Fig. 7, b, f, and h) Primer x (exon 1, forward) Primer y (exon 3, forward) Primer z (exon 3, reverse) Quantitative RT-PCR (Fig. 2, d and e) Forward primer Reverse primer Probe

Table I. Oligonucleotides used in all experiments except for CHART-PCR

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<th>Oligonucleotides</th>
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<td>5'-tagtcctcagatgatatatc-3'</td>
<td>Primer y (exon 3, forward)</td>
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<td>5'-gattatcaagtgtcaagtc-3'</td>
<td>Primer z (exon 3, reverse)</td>
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<tr>
<td>Probe</td>
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<td>5g)</td>
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<tr>
<td>AP1 (~3198 to ~3178)</td>
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<tr>
<td>Oct-1/2 (~3005 to ~2991)</td>
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<td>Mutagenesis of the Pr2 octamer-binding</td>
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</tr>
<tr>
<td>site (Fig. 7d; mutated nucleotide</td>
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<td>shown in bold)</td>
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</tr>
<tr>
<td>Sense primer</td>
<td>SmaI-gcagagagactctccgca-3'</td>
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</tbody>
</table>

Cell lines, transfections, luciferase assays

The Monomac.1 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH) and Jurkat, COS, and human embryonic kidney (HEK) cell lines were obtained from the American Type Culture Collection. Cells growing in logarithmic phase were transfected with 1 µg of endonuclease-free plasmid DNA (Qiagen) using the Fugene (Roche) transfection reagent. Transfection efficiency was normalized by cotransfection of endonuclease-free plasmid DNA (Qiagen) using the Sequence Detection System software. Highly purified CCR5-positive and -negative memory T cells (CD3+CD45RA CD45RO+) and CCR5-negative naive T cells (CD3+CD45RA CD45RO) were used for determining exon 1 expression were obtained by sequential purification through AutoMACS (Miltenyi Biotec) and the FACSaria cell-sorter system (BD Biosciences). Cell purity was confirmed to be >96% by FACS.

Determination of CCR5 cell surface expression

Cell surface staining was performed using standard protocols. Samples were analyzed with a FACSCalibur four-color cytometer using CellQuest Pro software (BD Pharmingen). Live lymphocytes were gated based on forward- and side-scatter properties. At least 10,000 gated events were collected for each sample. The following Abs were used for staining: anti-human CD3, CD4, CD45RA, CD45RO, CCR5 (clone 2D7), and an anti-mouse mAb against CD24 (HSA) cell surface Ag (BD Pharmingen).
Transfection of PBMC and T cell subsets

All studies were approved by the Institutional Review Boards at University of Texas Health Science Center at San Antonio (UTHSCSA) and Vanderbilt University. Four to 5 million PBMCs obtained from normal volunteers were nucleofected by following the manufacturer’s protocol (Amaxa). Briefly, PBMCs were stimulated with immobilized CD3/CD28 Abs (BD Pharmingen) for 3 days using protocols similar to those described previously (28, 29). The cell culture and TCR activation protocol is as shown in Fig. 1a. Of note, in experiments designed to delineate the CCR5 transcriptional activity following TCR activation, the growth medium was not supplemented with additional cytokines.

Retaining CD44+ T cells were isolated to a purity of >99% using Dynabead technology (Invitrogen Life Technologies) as described previously (30). To purify memory T cells, purified CD44+ T cells were incubated with an anti-CD45RA Ab and negatively sorted with anti-mouse Dynabeads. Naive CD44+ T cells were purified from cord blood using a similar protocol. The purity of memory and naive T cells was typically >95%. Purified CD44+ total resting T cells, CD44-CD45RO- memory T cells, and CD44-CD45RO+ CD45RA+ naive T cells were stimulated through the TCR using CD3 and CD28 Abs (BD Biosciences) for 48 h and were then nucleofected with CCR5 reporter constructs. Oct-2-specific siRNA and control siRNA were obtained from Santa Cruz Biotechnology. A decrease in Oct-2 expression following nucleofection of the siRNA was confirmed using TaqMan Gene Expression Assays (Applied Biosystems).

Comparative genomics

Comparative analysis of the human, dog, and mouse CCR5 loci to identify conserved noncoding sequences (CNS) and nonconserved noncoding sequences (NCNS) was performed using Vista tools (available at http://genome.lbl.gov/vista/index.shtml).

CHART-PCR

Naive and memory T cells were obtained from freshly isolated PBMC using the AutoMACS system. The purity of the memory T cell population was >90% as assessed by flow cytometry. Chromatin accessibility at the CCR5 locus in naive and memory cells was determined by using CHART-PCR according to previously described protocols (31). The nuclei that were obtained following Nonidet P-40 lysis were pelleted and resuspended in Mnase digestion buffer. The nuclei were then treated with 5 U of Mnase for 5 min at room temperature and the digestions were terminated with Mnase stop solution. The samples were then incubated overnight in the presence of SDS and proteinase K and genomic DNA was extracted from these samples using a QIAamp blood DNA minikit (Qiagen). The digestion (or accessibility) of the genomic DNA was quantified using SYBR Green real-time PCR. Optimal primer pairs were selected using the CHART-PCR program (www.cbs.dtu.dk/ SCRIPT/chartscheme.html) and their specificity was confirmed by using Megablast against the human genome; the nucleotide sequences of these primers used to amplify the CNS and NCNS regions are shown in Table II. The genomic DNA was quantified using SYBR Green real-time PCR. Optimal primer concentrations against Ct values. The DNA concentration obtained from each primer set was calculated for the efficiency of the primer, which was calculated from the slope of its standard curve. The final formula used was: DNA (ng) = (1/[(efficiency of primer × (Ct – constant) – 1 × slope)], where

![FIGURE 1. Kinetics of CCR5 surface expression levels on primary T cells following TCR stimulation.](http://www.jimmunol.org/)

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**Table II. Primers used for CHART-PCR**

| CNS/NCNS | Nucleotide Position | Primer Sequences
|----------|---------------------|-------------------|
| NCNS1    | −20,247 to −20,146  | (F)-cccctagatcagccccagtctt  
| NCNS2    | −8,590 to −8,389   | (R)-agacaccgagggagagcatctt  
| CNS1     | −7,043 to −6,834   | (F)-actcttgatctctgacgctccat  
| CNS2     | −6,766 to −6,565   | (R)-tgagagtctgtatggtggtgtct  
| CNS3     | −6,478 to −6,360   | (F)-atcgtactacatgtcagctacgc  
| CNS4     | −5,467 to −5,324   | (R)-agatgtgctaaccttgctggtg  
| CNS5     | −4,880 to −4,679   | (F)-ccctagtgtgctcctgctgca  
| CNS6     | −4,527 to −4,380   | (R)-tgctcgttgctgctgcatccct  
| CNS7     | −4,248 to −4,084   | (F)-actcttgatccttgcagcctca  
| CNS8     | −3,207 to −3,006   | (R)-tgatgtgctaaccttgctggtg  
| CNS9     | −2,274 to −2,073   | (F)-gagttgatgtgatgggtgtggat  
| NCNS3    | −1,707 to −1,707   | (R)-gctctgcacataacactcaccac  
| NCNS4    | −1,046 to −959     | (F)-agctgtacctacatggcactagc  
| NCNS5    | −7,043 to −6,766   | (R)-agatgtgctaaccttgctggtg  
| NCNS6    | −6,766 to −6,478   | (F)-ctcatgtagttggcccttgca  
| NCNS7    | −6,478 to −6,171   | (R)-ggtgtttcgtgccatccct  
| NCNS8    | −5,467 to −5,170   | (F)-tcacagcatgataagctgctagc  
| NCNS9    | −4,880 to −4,527   | (R)-gtgtgttctagtaaccactcagg  
| NCNS10   | −4,248 to −3,980   | (F)-tgtgggcttttgactagatgaatg  
| NCNS11   | −3,207 to −2,948   | (R)-cagttcttccttttaagttgctt  
| NCNS12   | −2,274 to −2,016   | (F)-tttacaggaaacccatagaaaccttt  
| NCNS13   | −1,046 to −889     | (R)-tcacctcaaatcatttgtgatcctg  

* Nucleotide positions are relative to the start of the CCR5 coding sequence on a human genomic contig (GenBank accession no. U95626).

β F and R indicate forward and reverse primers, respectively.

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stage 2.95°C for 10 min (1 cycle), stage 3, 9.5°C for 15 s, and 60°C for 1 min (40 cycles). To convert the cycle threshold (Ct) values from the CHART-PCR amplification plots to percent accessibility, a standard curve was generated using serial dilutions of human genomic DNA and the appropriate primer sets. The Ct values generated were used to calculate the DNA amount using the standard curve by plotting log DNA concentrations against Ct values. The DNA concentration obtained from each primer set was calculated for the efficiency of the primer, which was calculated from the slope of its standard curve. The final formula used was: DNA (ng) = (1/[(efficiency of primer × (Ct – constant) – 1 × slope)], where
the constant and the slope were determined by regressing the log-transformed sample DNA concentration over Ct. For normalization of data within a single donor, we first calculated the average amount of DNA obtained following amplification of all the regions in the Mnase-treated naive T cells (reference value) and this value was used to divide the amount of DNA amplified from each of the CNS or NCNS regions in Mnase-treated memory cells. Relative accessibility was calculated as the ratio of the amount of DNA amplified from unstimulated and TCR-activated purified CD4+ T cells. IL-2 indicates activated CD4+ T cells. IL-2 indicates activated CD4+ T cells that were maintained in IL-2 after TCR activation.

**Chromatin immunoprecipitation assay (ChIP)**

ChIP to detect in vivo bound acetylated Histone H3 to CCR5 Pr2 was performed with a commercially available kit (Upstate Biotechnology). Immunoprecipitated DNA was subjected to real-time quantitative PCR using Lux fluorogenic primers (Invitrogen Life Technologies; primer sequences described previously (25)).

**Virus production and infections**

VSV-G pseudotyped, replication-incompetent HDVs were generated as described previously (25).

**EMSA**

The methods for nuclear extract preparation, EMSAs and supershift assays were as described previously (11), and the oligonucleotides used are in Table I. In some EMSAs, in vitro-transcribed and translated TFs were used.

**Results**

**Relationship between CCR5 expression levels and CCR5 exon 1-containing transcripts**

We first validated a cell culture and TCR stimulation protocol (Fig. 1a) that allowed us to determine whether T cells that express higher amounts of CCR5 on the cell surface also express higher amounts of a specific kind of CCR5 mRNA isoform. Consistent with previous reports (28, 29), the experimental protocol shown in Fig. 1a resulted in a significant up-regulation in CCR5 surface expression on PBMCs (Fig. 1b) and most of these leukocytes were T cells of the memory phenotype, the primary target of HIV cell entry (Fig. 1, c and d). Of note, the increase in CCR5 expression was donor dependent.

**Cloning of orthologous CCR5 Pr2 sequences from nonhuman primates**

Pr2 sequences from chimpanzee and African Green Monkeys (AGM; sabaeus, grivet, and vervet) were PCR amplified and cloned into Topo 2.1 vector (Invitrogen Life Technologies) and were sequenced.
To determine whether the observed increase in CCR5 surface expression levels detected after TCR stimulation was associated with a concomitant increase in the production of specific CCR5 mRNA isoforms, we developed radioactive and real-time PCR-based assays to distinguish and quantify the different transcripts produced. A PCR assay to distinguish between exon 1-containing CCR5 transcripts vs those isoforms whose 5' most ends originate within exon 2A or 2B (truncated isoforms; Fig. 2a) cannot be designed. This is because exon 1-containing CCR5 transcripts also contain exon 2B (CCR5B mRNA) or both exons 2A and 2B (CCR5A mRNA) (Fig. 2a). We therefore designed two PCR assays that amplify either the total pool of all CCR5 mRNA isoforms produced, regardless of whether they originated in exons 1 or 2 (using primers y and z; Fig. 2a), or exon 1-containing CCR5A or CCR5B isoforms (primers x and z; Fig. 2a). Using the radioactive-based PCR assay, we detected abundant expression of total CCR5 mRNA transcripts in unstimulated PBMCs (Fig. 2b). However, among the total pool of CCR5 transcripts in unstimulated PBMCs, there was minimal expression of CCR5B mRNA isoforms, and no expression of CCR5A transcripts (Fig. 2b). By contrast, in TCR-activated PBMCs, there was a significant enrichment of exon 1-containing transcripts (Fig. 2b and c); results of the real-time PCR assays supported these findings obtained by the radioactive-based PCR assay (Fig. 2, d and e).

To confirm these results obtained from whole PBMCs, we analyzed the nature of CCR5 transcripts present in purified CD4+ T cells. Consistent with the CCR5 mRNA expression pattern detected in unstimulated vs TCR-stimulated nonpurified PBMCs (Fig. 2, b–e), we found that exon 1-containing transcripts were detected in only TCR-stimulated CD4+ T cells (Fig. 2f). Notably, the amount and pattern of expression of exon 1-containing transcripts varied among donors, suggesting that the amount of these transcripts produced could be a determinant of interindividual variations in CCR5 surface expression (Fig. 2, b–f).

To conclusively demonstrate that CCR5 expression on the cell surface of HIV target cells is strongly associated with expression of exon 1-containing CCR5 transcripts, we conducted the analyses shown in Fig. 3. We determined the expression of CCR5 mRNA isoforms in three highly purified T cell populations: naive T cells, and CD4+CCR5− and CD4+CCR5+ memory T cells (Fig. 3). Predictably, CCR5 surface expression levels were negligible in naive compared with memory T cells (Fig. 3a and b). Consistent with the differential pattern of CCR5 surface expression on these T cell subsets, there was also a clear gradient in the amount of total CCR5 mRNA present in these cells which was: memory CD4+CCR5− > memory CD4+CCR5+ > naive T cells (Fig. 3c). However, exon 1-containing transcripts were detected only in those memory T cells that also expressed CCR5 on the cell surface (Fig. 3c). Taken together, we interpreted the results presented thus far to indicate that expression of exon 1-containing mRNA isoforms is an important determinant of CCR5 surface expression (Fig. 3c).

Transcriptional activity of CCR5 Pr1 and Pr2 in primary T cells

We next sought to understand the molecular factors that regulate expression of exon 1-containing transcripts. We determined whether the sequences upstream of exon 1 that we have previously designated as CCR5 Pr2 were transcriptionally active. In a previous report, we found that compared with Pr1-derived reporter constructs, Pr2-derived reporter constructs had weak transcriptional
activity in a surrogate T cell (Jurkat) and monocyctic (THP-1) cell line, as well as an erythroleukemia cell line (K562) (10). To resolve this discordance, we compared the transcriptional activity of CCR5 Pr1 and Pr2 luciferase-based reporter constructs in transformed and primary cells. Consistent with results of our previous study (10), we found that compared with reporter constructs that contained sequences derived from Pr1, the transcriptional strength of reporter constructs derived from Pr2 were comparable in the three purified T cell subsets that we examined (Fig. 4, a and b). We extended these observations to the monomac.1 cell line, a surrogate for a myeloid cell environment (Fig. 4, a and b).

We next compared the transcription strength of the same Pr1- and Pr2-derived reporter constructs in unstimulated PBMCs obtained from four normal donors. We first confirmed that primary PBMCs can be transfected efficiently using the nucleofection procedure (Fig. 4c). To account for the donor-to-donor variability in both the transfection efficiency as well as the activity of the reporter constructs, we normalized the luciferase values of the reporter constructs to those value obtained from a Pr1 construct designated as p1a (Fig. 4a). The results showed that contrary to the findings in transformed cell lines, in primary PBMCs, the reporter constructs derived from Pr2 had nearly equivalent or higher transcriptional activities than those constructs derived from Pr1 (Fig. 4d).

We next compared the transcriptional strength of Pr1 and Pr2 constructs in autologous PBMCs from four normal donors, before and after TCR activation (Fig. 4e). In unstimulated and TCR-activated PBMCs, the transcriptional activity of the Pr2-derived reporter constructs was consistently similar to or greater than those of Pr1-derived constructs (Fig. 4e). Furthermore, we found that Pr2 constructs had greater transcriptional activity in TCR-activated PBMCs than in autologous unstimulated PBMCs (Fig. 4e). In contrast, the transcriptional strength of each of the Pr1 reporter constructs examined in unstimulated and TCR-activated PBMCs were similar (Fig. 4e and data not shown).

For further validation that Pr2 is transcriptionally active in primary HIV target cells, we conducted similar analyses in purified T cell populations. With the single exception of the p1a Pr1 construct that was highly active in CD4+ T cells derived from cord blood, the transcriptional strength of the constructs derived from Pr1 and Pr2 were comparable in the three purified T cell subsets that we examined (Fig. 4f). Collectively, these findings demonstrated that 1) sequences designated as the CCR5 Pr2 are functionally active in primary T cells but not in transformed cell lines; 2) the transcriptional strength of the Pr1 and Pr2 constructs in resting PBMCs is similar; and 3) the transcriptional activity of Pr2 constructs increases after T cell activation; notably, this increase in transcriptional activity mirrors the increased expression of Pr2-driven exon 1-containing transcripts.

**CCR5 Pr2 is associated with a relaxed chromatin and acetylated histone H3 in memory T cells**

The aforementioned results provided in vitro evidence that Pr2 is functionally active in primary T cells. To accrue in vivo evidence that Pr2 is functionally active in primary T cells, we used two approaches. The first experimental approach used CHART-PCR, a newly developed technique that determines the accessibility of chromatin to nucleases (31). CHART-PCR relies on the principle that an open chromatin conformation in a chromosomal region confers greater sensitivity to nuclease digestion and that this sensitivity (i.e., chromatin accessibility) can be quantified by real-time PCR. Thus, in CHART-PCR, a primer pair that is designed to

![Diagram](http://www.jimmunol.org)
amplify across a genomic region will yield lower amounts of PCR products when it encompasses a nuclease-sensitive site than one that lacks such a site. Hence, compared with the Southern blot approach that is traditionally used to examine chromatin remodeling, the CHART-PCR is more sensitive, is amenable to high throughput methodologies, and can be used to map nuclease-sensitive sites in small quantities of purified primary cell populations (31).

To determine the nuclease accessibility of the CCR5 locus by CHART-PCR, we designed a series of primer pairs that spanned ~20 kb upstream of the CCR5 ORF and included genomic sequences upstream of the CCR5 Pr2 as well as the CCR2 ORF (Fig. 5a). For optimal placement of the primers for CHART-PCR, we used comparative genomics to identify genomic regions that are highly conserved between humans and dogs and/or mice. This approach has been used successfully to identify critical regions involved in gene regulation as well as chromatin remodeling (32).

Alignment of the human and dog CCR5 loci revealed nine CNS, designated as CNS 1–9 (Fig. 5a). The extent of the regions amplified is shown in Table II. The percentage homology between humans and dogs in the CNS and NCNS regions identified by Vista analysis is shown in parentheses. The data are representative of one of three experiments. The data were corrected for the differences in efficiency of the primer sets and were normalized as described under Materials and Methods. Dissociation curve analysis revealed that after PCR amplification all the primer sets yielded a single peak, suggesting that the PCR products were specific. This was confirmed by resolving the products by agarose gel electrophoresis, which also yielded a single band (data not shown). The authenticity of the PCR products was also established by...
cloning and sequencing the PCR-amplified products (data not shown).

Fig. 5 outlines the experimental design for the CHART-PCR analysis. We compared the degree of nuclease accessibility at each of the CNS and NCNS regions, and determined whether highly purified populations of naive and memory T cells differed in the degree of accessibility in these regions. Representative qPCR plots obtained from a NCNS (NCNS3) and a CNS (CNS7) are shown in Fig. 5, and respectively. The data showed that: 1) the amplification curves obtained after PCR of genomic DNA derived from naive and memory cells that were not treated with Mnase overlapped (curves with closed symbols in Fig. 5, and ); 2) compared with untreated cells, the amplification curves were shifted to the right when PCR was conducted on genomic DNA extracted from cells that were treated with Mnase (compare curves with closed and open symbols in Fig. 5, and ); 3) the amplification curves for PCR on genomic DNA from Mnase-treated T cells showed that compared with curves obtained in naive cells, those obtained in memory cells were shifted further to the right (compare open circle and triangles in Fig. 5, and ); and 4) a qualitative comparison of the amplification curves for the CNS7 and NCNS3 regions in Mnase-treated memory cells showed that there was a greater shift to right for the curve from the CNS7 region compared with that from the NCNS3 region (compare open circles in Fig. 5, and ).

To quantify the differences in chromatin accessibility among the different CNS and NCNS regions in naive and memory T cells, we conducted the analyses shown in Fig. 5, and . The plot in Fig. 5e was generated as described in Materials and Methods and was corrected for various confounders such as differences in primer
efficiency. The normalized DNA quantity is a surrogate measure of the chromatin accessibility with a higher number reflecting lower accessibility (Fig. 5e). The degree of accessibility was similar in both memory and naive T cells at the CNS 1–5 and NCNS 1–3 regions, i.e., until ~4.5 kb upstream of the start of the CCR5 ORF. By contrast, the four CNS regions that are within this 4.5-kb region (CNS 6–9) were more accessible to Mnase digestion in memory than naive T cells (Fig. 5e). To normalize CHART-PCR data among different donors, we determined accessibility in different CNS regions using NCNS1 as a baseline in two independent donors and the plots are shown in Fig. 5f. Thus, taken together, the findings of CHART-PCR showed that the degree of accessibility in the CNS regions in both Pr1 (CNS8) and Pr2 (CNS7) of CCR5 were comparable and additional regions that are upstream of Pr2 and intron 2 might also be involved in regulating CCR5 gene expression in vivo.

The second in vivo approach that we used to determine whether the CCR5 Pr2 was functionally active in primary cells is based on the notion that chromatin in a relaxed state is coupled to active transcription and is often associated with covalently modified histones (33). To examine the association of modified histones with Pr2, we performed ChIP assays in TCR-activated PBMCs using an Ab that specifically recognizes acetylated histone H3. By real-time qPCR using CCR5 Pr2-specific primers or primers that amplify the control gene ex-globin, we compared the amount of the precipitated DNA obtained with an Ab directed against acetylated histone H3 vs a control Ab. The normalized DNA quantity is a surrogate measure of the accessibility with a higher number reflecting lower efficiency. The normalized DNA quantity is a surrogate measure of the chromatin accessibility with a higher number reflecting lower accessibility (Fig. 5e). The degree of accessibility was similar in both memory and naive T cells at the CNS 1–5 and NCNS 1–3 regions, i.e., until ~4.5 kb upstream of the start of the CCR5 ORF. By contrast, the four CNS regions that are within this 4.5-kb region (CNS 6–9) were more accessible to Mnase digestion in memory than naive T cells (Fig. 5e). To normalize CHART-PCR data among different donors, we determined accessibility in different CNS regions using NCNS1 as a baseline in two independent donors and the plots are shown in Fig. 5f. Thus, taken together, the findings of CHART-PCR showed that the degree of accessibility in the CNS regions in both Pr1 (CNS8) and Pr2 (CNS7) of CCR5 were comparable and additional regions that are upstream of Pr2 and intron 2 might also be involved in regulating CCR5 gene expression in vivo.

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### Table III. Predicted TF-binding sites on CCR5 Pr2

<table>
<thead>
<tr>
<th>Family/Matrix</th>
<th>TF</th>
<th>Nucleotide Position</th>
<th>Anchor</th>
<th>Strand</th>
<th>Nucleotide Sequence</th>
<th>TF-Binding Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB/CREB</td>
<td>NF-κB</td>
<td>-3420 to -3406</td>
<td>-3413</td>
<td>(+)</td>
<td>tctggcTTCCaaa</td>
<td>sggmrvTTCC</td>
</tr>
<tr>
<td>TCF7F</td>
<td>TCF11 homodimers</td>
<td>-3402 to -3396</td>
<td>-3499</td>
<td>(+)</td>
<td>GTCAatt</td>
<td>GTCAatt</td>
</tr>
<tr>
<td>ECAT</td>
<td>Nuclear factor Y</td>
<td>-3309 to -3295</td>
<td>-3302</td>
<td>(+)</td>
<td>gtggaaCCAAattttat</td>
<td>trrrCCAAattmrnn</td>
</tr>
<tr>
<td>AP-1F</td>
<td>c-fos/c-jun</td>
<td>-3198 to -3178</td>
<td>-3188</td>
<td>(-)</td>
<td>ctaatgaGCAatccac</td>
<td>nntgaSTCAann</td>
</tr>
<tr>
<td>TCF7F</td>
<td>TCF11 homodimers</td>
<td>-3139 to -3153</td>
<td>-3156</td>
<td>(-)</td>
<td>GTCAatt</td>
<td>GTCAatt</td>
</tr>
<tr>
<td>SMAD3</td>
<td>SMAD3</td>
<td>-3134 to -3126</td>
<td>-3130</td>
<td>(-)</td>
<td>GTCTGtga</td>
<td>GTCTGtga</td>
</tr>
<tr>
<td>NF1F</td>
<td>Oct factor 1</td>
<td>-3096 to -3078</td>
<td>-3087</td>
<td>(+)</td>
<td>gctTGGGactctgactaca</td>
<td>nntTGGGnn</td>
</tr>
<tr>
<td>OCT1</td>
<td>Oct-1, -2 consensus</td>
<td>-3005 to -2991</td>
<td>-2998</td>
<td>(-)</td>
<td>agAAGCcaatgaga</td>
<td>nrtATGCaatahagn</td>
</tr>
<tr>
<td>ETSF</td>
<td>Erm/Etk1</td>
<td>-2952 to -2936</td>
<td>-2944</td>
<td>(+)</td>
<td>ggtcaccGGAaggccaga</td>
<td>ccGGAArynn</td>
</tr>
</tbody>
</table>

* a—adenine, g—guanine, c—cytosine, t—thymidine, r—G or A, s—G or C, m—A or C, n—A or G or C or T, W—A or T.

* Bases marked in bold show high degree of conservation (consensus index value > 60) in the matrix at that position.

* Bases indicated in capital letters denote the core sequence used by MatInspector Professional for predicting the binding sites.

FIGURE 7. Oct TFs influence CCR5 surface expression and Pr2-driven expression of CCR5A and CCR5B. a, Human naive cord blood CD4+ T cells were activated and transduced with HDV or HDV-expressing Oct-1 and Oct-2 TFs. The transduced cells also express mouse cell surface molecule HSA that was used to sort Oct-1- or -2-expressing cells. Sorted cells were reactivated by TCR stimulation as described in Materials and Methods, stained with PE-conjugated anti-CCR5 Ab, and analyzed by flow cytometry. Histograms show the relative difference in CCR5 expression levels compared with control Materials and Methods.

b, Oct-1 overexpressing primary cord blood CD4+ T cells contain lower amounts of exon 1-containing transcripts than Oct-2-overexpressing cells. c, Oct-2 competitively inhibits Oct-1 binding to an oligomer that contains the octamer-binding consensus sequence. EMSA showing that increasing amounts of IVTT Oct-2 displaced IVTT generated Oct-1 that was bound to the octamer binding site. d, Mutation in the octamer binding site in p2d. e, Oct-2 reporter construct, resulted in decreased transcriptional activity in activated PBMCs. The transduced cells are representative of two independent experiments. f, Oct-2 mRNA expression following nucleofection of PBMCs with Oct-2 siRNA or control siRNA. mRNA levels were as determined by quantitative RT-PCR. 18S RNA was used as an internal control. g, Autoradiogram showing the specificity of Oct-2 siRNA in reducing Oct-2 protein expression as assessed by a supershift assay. HEK 293 cells were transfected with an Oct-2 expression vector and Oct-2 specific siRNA. No reduction was observed in the expression of Oct-1. h, Oct-2-specific siRNA decreases the expression of exon 1-containing CCR5 transcripts in two independent donors. Error bars show SD from mean.
control β-globin sequences, were selectively enriched in chromatin complexes precipitated with acetylated histone H3 (Fig. 5g and data not shown). Additionally, no enrichment was detected with the control Ab (Fig. 5g). These results suggested that CCR5 Pr2 is associated with posttranscriptionally modified histones that serve as a strong signature for its transcriptional activity in vivo. This inference is in accord with the results obtained by CHART-PCR which also demonstrated that Pr2 is likely to be functionally active in vivo.

**TF-binding profile of CCR5 Pr2**

Relative to Pr1, very little is known about the cis-trans interactions that regulate the expression of the CCR5 Pr2 transcriptional unit in any cellular context. In silico analysis of the Pr2 sequences revealed the presence of consensus binding sites for NF-κB, AP-1, Oct-1, and Oct-2, TF that are thought to be up-regulated upon T cell activation (Fig. 6a and Table III). We therefore focused on these TFs because they might provide a molecular basis for the increased activity of Pr2 in activated T cells. Consistent with this possibility, by EMSA, we found that the Pr2 cis elements bound NF-κB, AP-1, Oct-1, and Oct-2 in TCR-stimulated PBMCs (Fig. 6, b–e, and data not shown for AP-1). Among these TFs, Oct-2 has been implicated previously in the regulation of CCR5 gene expression (16). However, Oct-1 and Oct-2 have identical binding sites (34) and the role of Oct-1 in CCR5 gene expression remains unknown. Thus, in our study design, we placed a special emphasis on elucidating whether Oct-1 or Oct-2 TFs and their interactions with cis-binding elements in CCR5 Pr2 played a role in regulating CCR5 protein and mRNA expression (Figs. 7 and 8).
Effects of Oct-1 and Oct-2 on CCR5 mRNA and protein expression

To determine the effect of Oct-1 and Oct-2 binding to cis-binding sites in CCR5 on its surface and mRNA expression levels, we first engineered activated T cells to overexpress Oct-1 and Oct-2 TFs, and compared the CCR5 protein and transcript levels in these cells. Compared with T cells transduced with the control vector, cells overexpressing Oct-1 had lower CCR5 surface expression levels (Fig. 7a, left). In contrast, CCR5 surface expression was up-regulated in T cells that overexpressed Oct-2 (Fig. 7a, right). Concordant with these findings, relative to CD4+ T cells overexpressing Oct-2, those overexpressing Oct-1 had reduced expression levels of exon 1-containing mRNA (Fig. 7b). We also determined the expression of “total” CCR5 RNA following Oct-1 and Oct-2 overexpression in primary cells (data not shown). There was a substantial decrease in “total” CCR5 transcript levels when Oct-1 was overexpressed whereas there were no changes detected upon Oct-2 overexpression (data not shown).

The aforementioned findings suggested that despite binding to identical cis motifs, Oct-1 and Oct-2 had opposing effects on CCR5 surface expression. We therefore hypothesized that a competitive interaction between these two TFs for a common cis motif might provide a mechanistic basis for their contrasting effects on CCR5 protein/mRNA expression. Consistent with this thesis, we found that increasing concentrations of in vitro-synthesized Oct-2 oligomer (Fig. 7c) found that increasing concentrations of in vitro-synthesized Oct-2 might provide a mechanistic basis for their contrasting effects on CCR5 expression are mediated through the Oct-binding motifs, Oct-1 and Oct-2 had opposing effects on

Factors affecting CCR5 expression in the context of T cell activation include transcriptional and post-transcriptional events. We next determined whether some of the aforementioned effects of Oct-1/2 on CCR5 expression are mediated through the Oct-binding cis-site in Pr2 at position −2998 (Fig. 6a). Underscoring the importance of the Oct motif for CCR5 Pr2 activity in T cells, mutation of the octamer binding site in the Pr2 reporter construct p2D was associated with a marked decrease (~70%) in its transcriptional activity in TCR-activated PBMC (Fig. 7d).

To define a direct association between Oct-2- and Pr2-mediated transcription, we determined whether siRNA-mediated reduction of Oct-2 expression in T cells will inhibit the expression of exon 1-containing transcripts (CCRS5 and CCR5B). In primary T cells, the siRNA for Oct-2 reduced by over 60% expression of Oct-2 mRNA (Fig. 7e). The Oct-2 siRNA was also highly specific for depletion of Oct-2 protein as assessed by mobility shift assays (Fig. 7f). In cells from three independent donors, nucleofection of Oct-2 siRNA but not control siRNA into TCR-activated PBMCs led to a decrease in the expression levels of the exon 1-containing CCR5A and CCR5B transcripts (Fig. 7g) and, in some instances, complete abrogation of CCR5A mRNA expression (e.g., donor 2 in Fig. 7g). The summary data from the three donors is shown in Fig. 7h.

Evolutionary importance of octamer binding site in Pr2

We used an evolutionary approach to gain further insights into the relative importance of octamer binding sites that we identified in human Pr2. We cloned and sequenced the Pr2 regions from humans, chimpanzees, and AGM (sabaeus, grivet, and vervet; Fig. 8a). Alignment of the region corresponding to the octamer sequences was notable for two findings (Fig. 8b). First, relative to the human sequence, there was a single nucleotide difference that is common to both chimpanzee and AGM in the core sequence of the consensus octamer binding site (Fig. 8b). Second, AGM has a species-specific nucleotide difference relative to human and chimpanzee CCR5 sequences (Fig. 8b). From a functional perspective, these species-specific differences result in loss of binding of Oct-1 (Fig. 8c) and Oct-2 (Fig. 8d).

Discussion

The current studies provide new insights into the molecular determinants that regulate CCR5 cell surface expression. Based on the results of previous studies, we and others had previously concluded that cis-trans interactions mediated via Pr1 were more important than those in Pr2 in regulating CCR5 gene expression. Consequently, the prevailing viewpoint is that the physiological relevance of Pr2-driven, exon 1-containing transcripts (CCRS5A and CCR5B mRNA isoforms; Fig. 2a) to CCR5 surface expression levels is minimal. We revisited these assumptions.

Our results highlight seven findings. First, T cell activation results in the enrichment of exon 1-containing transcripts. Second, there is a clear association between production of these mRNA isoforms and robust CCR5 surface levels in T cells that are targets of HIV infection (Figs. 1–3). Third, Pr2 is highly active in primary T cells but not in transformed T cell lines (Fig. 4). Fourth, two different approaches indicate that the region encompassing Pr2 is functionally active in vivo (Fig. 5). Fifth, Pr2 binds TFs that are expressed in TCR-activated cells (Fig. 6). Sixth, Oct-1 and Oct-2 are key negative and positive modulators of CCR5 surface expression, respectively, and can mediate part of their effects by binding to cis-binding sites in Pr2 (Figs. 7 and 8). Seventh, expression of exon-1 containing transcripts appears to be a target for selection (Fig. 8). Based on these findings derived from analyses in primary T cells that are targets for HIV-1 infection, we infer that Pr2-dependent cis-trans interactions and the consequent production of exon 1-containing transcripts are an important determinant of CCR5 surface expression. Below, we discuss the relevance of each of these seven major findings of this study.

To the best of our knowledge, we provide the first example among the chemokine receptor family where usage of an alternative promoter and the consequent expression of novel transcripts is linked to the cell activation state. Our current findings indicate that in unstimulated T cells, CCR5 expression is driven primarily by Pr1 and results in the production of mRNA isoforms that originate in exon 2A or 2B. By contrast, stimulation of the TCR induces signals that result in the induction of Pr2 and consequently the expression of exon 1-containing transcripts. We speculate that usage of an alternate promoter might allow T cells to respond appropriately to antigenic stimuli. This might be physiologically relevant because we found that the gain in expression of exon 1-containing CCR5 transcripts in memory T cells was found only in those cells that also expressed CCR5 on the surface. Thus, these Pr2-driven gene transcription events might be important for normal homeostasis as CCR5 and its ligands have been shown to play a role in T cell costimulation (35–37), migration (38), and polarization (39–41). Additionally, a TCR-induced gain in expression of exon 1-containing transcripts might also have relevance for HIV-1 pathogenesis as immune activation of latently infected resting CD4+ T cells triggers viral replication.

There are some striking similarities in the expression profile of total CCR5 mRNA in T cells across species. In a nonhuman pri-
expression (Fig. 3c). At this juncture, we do not completely understand why there is a discordance between levels of exon 1-lacking CCR5 mRNA transcripts and CCR5 surface expression. One possibility is that there are differences in translatability between transcripts that contain or lack exon 1 sequences. Nevertheless, our study closely links Pr2 transcriptional activity and expression of specific mRNA isoforms to CCR5 surface expression and consequently to HIV-1 pathogenesis.

We observed that there was a striking discordance between the transcriptional strength of Pr2 in a surrogate T cell line (Fig. 4b) vs primary T cells. This finding underscores the importance of accounting for the physiological cellular milieu while analyzing gene transcription in vitro. There are very few studies that have conducted comparative analyses of identical cis regions in primary vs transformed cellular environments, or in highly purified T cell subsets such as those studied herein. The limited studies that have conducted such comparative analyses provide a similar cautionary note regarding the full translation of findings from transformed cell lines to primary cells (43, 44).

In addition to the in vitro data supporting a role of Pr2 in CCR5 expression in primary cells, we also provide in vivo support for this. We used a comparative genomics approach to identify conserved regions between humans and dogs and then used CHART-PCR to identify regions that are more accessible to nuclease digestion, a surrogate for an open chromatin conformation. We found that a genomic region 4.5 kb upstream of the CCR5 ORF that encompassed the Pr1 and Pr2, and the 5′ end of intron 2 contained conserved regions that had a more open chromatin conformation in memory than naive T cells. Validating our findings, one of the conserved regions, CNS6 was more accessible in memory cells overlapped with that identified by a completely different method. Roh et al. (45) conducted a high-resolution genome-wide mapping of diacetylation of histone H3 at Lys9 and Lys14 in resting and anti-CD3/CD28-activated human T cells. These authors found that chromatin accessibility and gene expression of a genetic domain correlated with hyperacetylation of promoters and other regulatory elements but not with generally elevated acetylation of the entire domain. A total of 46,813 islands of acetylation were identified and these regions were significantly correlated with CNS expression in vitro. There are very few studies that have conducted comparative analyses of identical cis-binding site for Oct-1/2 that we suggest are important cis-binding motif, the functional differences in their regulatory role may now be extended to transcriptional regulation of CCR5 in T cells wherein the relative balance in the levels of Oct-1 and Oct-2 may affect CCR5 transcriptional activity and, potentially, surface expression levels. This mode of control of gene expression through cross-competition for a common binding motif is a frequent regulatory theme and has been described for several TF families (47−50). However, there are only a limited number of T cell-specific genes that are known to be regulated by octamer-binding proteins (51−55).

In our previous studies, we found that exon 1-containing transcripts are also expressed in non-T cell environments such as dendritic cells and monocytes (10). It is therefore possible that cis elements other than the octamer binding site regulate the expression of exon 1 in these cell types. This is supported by studies of Giri et al. (56) who demonstrated the presence of an Egr-1 binding site in Pr2 that may mediate CCR5 up-regulation in TH-1 monocytes following treatment with human amyloid peptides.

We exploited the “natural mutagenesis” that exists between human and nonhuman primates with respect to CCR5 sequences and found that nucleotide differences in the orthologous Pr2 cis-regions of chimpanzees and AGM disrupts binding of Oct-1/2 at position −2998 in Pr2. This might provide a possible species-specific mechanism by which primate populations modulate CCR5 gene expression. It is conceivable that loss of the Oct site and other polymorphisms may be responsible for lower levels of CCR5 expression observed in natural primate hosts of SIV infection (e.g., Sooty mangabey) that do not progress to AIDS compared with hosts in which SIV or HIV infection leads to AIDS (57). Extending this notion, the results of our current and previous studies (11) suggest that sequences that influence the production of exon 1-containing transcripts are targets for selection. In this study, we found that the cis-binding site for Oct-1/2 that we suggest are important for expression of exon 1-containing transcripts and surface expression in humans is not functional in nonhuman primates. Previously, we found that the intron-exon splice sites that influence the production of exon 1-containing isoforms CCR5A and CCR5B were polymorphic (11). These polymorphisms result in the inability to produce CCR5A in chimpanzees and CCR5B in AGM (11). The relevance of these polymorphic sequences and their potential impact on reduced expression of exon 1-containing transcripts and CCR5 surface expression is illustrated by the recent findings of Pandrea et al. (57) who showed that natural hosts for SIV infection express remarkably low levels of CCR5 on CD4+ T cells from gut. Thus, taken together, we propose that in some natural hosts of SIV, polymorphic sequences that affect the Oct-1/2 cis-binding site in Pr2 or splicing patterns of CCR5 mRNA might result in the reduced CCR5 expression (57).
In summary, our results have implications from two respects. First, the striking differences observed in the molecular determinants that regulate CCR5 gene expression in transformed surrogate T-cell lines vs primary cells brings into sharp focus the fact that the vast majority of our understanding regarding the cis-trans factors that regulate most genes comes from experiments conducted in transformed cell lines, which as our current findings suggest, might not fully mimic primary cell environments. Second, we propose that by fine tuning the T-cell surface expression of CCR5 at the transcriptional level, and in turn the availability of target cells for viral infection, CCR5 Pr2-dependent gene transcription may be a critical determinant of HIV/AIDS susceptibility.

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Disclosures
The authors have no financial conflict of interest.

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