Expression of CCR5, a major coreceptor for human immunodeficiency virus type 1 (HIV-1), is regulated by a number of transcription factors. Here we report that the YY1 transcription factor down-regulates CCR5 promoter activity and that overexpression of YY1 reduces cell surface CCR5 expression and infectibility by R5-HIV-1. Because YY1 also down-regulates promoter activities of CXCR4, another major coreceptor for HIV-1 and HIV-1 long terminal repeat, this transcription factor may play a critical role in the pathogenesis of HIV-1 disease.

YY1 is ubiquitous transcription factor that regulates a number of cellular and viral promoters, depending on the gene as well as the cell type in question (reviewed in Ref. 1). This transcription factor down-regulates expression from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (2) as well as the promoter for CXCR4, a major coreceptor for X4-HIV-1 (3). Furthermore, YY1 may influence maturation and uncoating of the HIV-1 virions through its interaction with cyclophilin A (4). Thus, the YY1 transcription factor may play a critical role in the pathogenesis of HIV-1 disease.

CCR5, a receptor for the CC chemokines regulated upon activation, normal T cell-expressed and -secreted, macrophage inflammatory protein-1α (14) or the Human T Cell Nucleofector™ kit (Amaxa Biosystems). In brief, 5 × 10⁶ PBMC were resuspended in 100 μl of Human T Cell Nucleofector solution, mixed with a total of 5 μg of plasmid DNA, and pulsed using the Nucleofector program U-14. Luciferase assays for transient expression assays were performed as described previously (13). 293 cells were transfected as described previously (13).

Expression of CCR5, a receptor for the CC chemokines regulated upon activation, normal T cell-expressed and -secreted, macrophage inflammatory protein-1α and -1β is essential for cellular entry of R5-HIV-1 (reviewed in Ref. 5). Levels of its expression appear to be critical for infectibility by R5 HIV-1 (6) or rate of disease progression in infected individuals (7). We have cloned and characterized the promoter region of CCR5 and identified several transcription factors that transactivate it (8–10).

In this study, we demonstrate that YY1 down-regulates CCR5 expression, and overexpression of YY1 reduces cell surface CCR5 expression and infectibility by R5-HIV-1, further emphasizing its importance in the pathogenesis of HIV-1 disease.

YY1 Transcription Factor Down-regulates Expression of CCR5, a Major Coreceptor for HIV-1*

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EXPERIMENTAL PROCEDURES

Plasmids—Plasmids pGL-CCR5 and pGL-CCR5/YY1 were described previously (8). Plasmid pGL-CCR5 (YY1-WT) contains the CCR5 promoter sequence spanning −607 to +61 relative to the major transcription start site (TSS) (8), followed by the luciferase reporter gene in plasmid pGL3-basic (Promega Corp., Madison, WI). Plasmid pGL-CCR5 (YY1-MT) has mutations (shown in bold letters below) at −594 and −593 relative to the TSS on the YY1 motif (indicated by underline) (AAAAAGATGGGGAA—AAAAAGATCCGAAA). Plasmids pCMV-YY1 and pPhES2−327/+59, a luciferase reporter driven by human cyclooxygenase-2 gene promoter, are generous gifts from T. Shenk (Princeton University, Princeton, NJ) (11) and H. Inoue (National Cardiovascular Research Center Institute, Osaka, Japan), respectively (12). To construct plasmid pcDNA/GFP-YY1, the YY1 coding region was amplified by polymerase chain reaction using forward primer 5′-ATGGCCTCGGGACACCCCTCTAC-3′ and reverse primer 5′-TCACGGGATGGTTGGTTGGGCTAGC-3′, and cloned into pcDNA3.1/NT-GFP-TOPO (Invitrogen). The resultant plasmid expresses green fluorescent protein-GFP/YY1 fusion protein under the control of the human cytomegalovirus major immediate early promoter. To construct pcDNA/GFP-YY1, pcDNA3.1/NT-GFP-TOPO was digested with XbaI, treated with T4 DNA polymerase, and re-ligated with T4 DNA ligase.

Cells—Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers (Red Cross Blood Center in Nagasaki Prefecture, Japan), and CD4+ T cell-enriched PBMC were propagated as described previously (13). Where indicated, CD4+ T cell-enriched PBMC were stimulated with recombinant human interleukin-2 (IL-2) (100 units/ml) for 7 days.

Transfection and Transient Expression Assays—Transfections of PBMC were performed using electroporation as described previously (14) or the Human T Cell Nucleofector™ kit (Amaza Biosystems). In brief, 5 × 10⁶ PBMC were resuspended in 100 μl of Human T Cell Nucleofector solution, mixed with a total of 5 μg of plasmid DNA, and pulsed using the Nucleofector program U-14. Luciferase assays for transient expression assays were performed as described previously (14). 293 cells were transfected as described previously (13).

Gel Mobility Shift Assays—Nuclear extracts were prepared from PBMC or 293 cells as described previously (15), and gel mobility shift assays were performed as described previously (15). Anti-YY1 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides used for competition studies are listed in Table I.

Flow Cytometric Analysis—Expression of cell surface CCR5 or CD4 was determined by staining cells with monoclonal anti-CCR5 Ab 2D7 or phycoerythrin (PE)-conjugate or monoclonal anti-CD4 Ab RPA-T4 PE-conjugate (BD PharMingen), respectively, and analyzing in FACSscan (Becton-Dickinson Immunocytometry Systems, San Jose, CA). GFP expression from pcDNA/GFP or pcDNA/GFP-YY1 was also analyzed in FACScan.

Single-round Viral Infection and Flow Cytometric Analysis for Intracellular Expression of p24 Ag—The recombinant, replication-deficient virus NL4–3lucR–E − that had been complemented with R5 JR-FL Env was designated NL4–3lucJR-FL here and propagated as described previously (13). Approximately 5 × 10⁶ PBMC were stimulated with hIL-2 (100 units/ml) for 7 days, transfected with 5 μg of pcDNA/GFP or pcDNA/GFP-YY1, and mock-infected or infected with NL4–3lucJR-FL at 10 h after transfection.

For intracellular p24 Ag staining, the transfected and (mock-)infected cells were washed once in phosphate-buffered saline containing 5%
CD4 T cell-enriched PBMC were transfected with 2.5 g of pGL-CCR5, pGL-CCR5 (−417), or phPES2 (−327/+59) along with 2.5 g of pCMV-YY1 or pcDNA3.1. Luciferase assays in the transfected cell lysates were performed 24 h after transfection. Reporter activity was shown as luciferase activity relative to that without pCMV-YY1 cotransfection. Results are reported as means ± S.E. from three independent experiments.

RESULTS

Overexpression of YY1 Down-regulates CCR5 Promoter—Because we and others have demonstrated that the YY1 transcription factor mediates a variety of effects on HIV-1 infection, we wanted to investigate whether this transcription factor plays a role in CCR5 expression on CD4+ T cells, a target for HIV-1. First, we transfected CD4+ T cell-enriched PBMC or 293 cells with pGL-CCR5 along with pCMV-YY1 or control plasmid. Gel mobility shift assay using YY1-C oligonucleotide (Table I) containing the YY1 consensus binding site demonstrated that the endogenous level of YY1 expression in PBMC or 293 cells was increased by transfection with pCMV-YY1 (Fig. 1A). Overexpression of YY1 down-regulated CCR5 promoter activity in a dose-dependent manner (Fig. 1B); however, truncation of the CCR5 promoter region down to −417 relative to the TSS resulted in much reduced responsiveness to YY1 (Fig. 1C), suggesting that a region upstream from −417 relative to the TSS contains a YY1-responsive element(s). In contrast, YY1 had little, if any, effect on Cox-2 promoter activity (Fig. 1C).

Identification of a YY1 Binding Element on the CCR5 Promoter—By scanning the DNA sequence, we found two candidate sites for YY1 binding on the CCR5 promoter region upstream from −417 relative to the TSS. Gel mobility shift assays demonstrated that one of the two, spanning from −607 to −584 relative to the TSS, can form a DNA-protein complex that was disrupted by the YY1 consensus oligonucleotide (Fig. 2A, lanes 7 and 8). Unlabelled R5 (−607/−584) oligonucleotide, but not R5 (−607/−584)-MT in which the putative YY1 site was mutated (Table I), also disrupted the DNA-protein complex forma-

### Table I

| Oligonucleotide | Sequence |
|-----------------|---------|
| R5 (−607/−584)  | 5’-TCCAGAAAAAGATGCGAAGACCTGT-3’ |
| R5 (−607/−584)-MT | 5’-TCCAGAAAAAGATGCGAAGACCTGT-3’ |
| R5 (−786/−763)  | 5’-CGCTCCGCCGCACTCTGGGCTGCTG-3’ |
| YY1-C           | 5’-GTATTTCGCCAGAAGAAAGAC-3’ |

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Fig. 2. YY1 binds to CCR5 promoter. A, gel mobility shift assays and competition studies. R5(−607/−584) probe was incubated with PBMC nuclear extracts in the presence of a 50- (lanes 5, 7, and 9) or 500-fold (lanes 4, 6, 8, and 10) molar excess of non-labeled oligonucleotides (see Table I), indicated above the figure as competitors. Lane 1 represents probe alone. Lanes 2–10 represent reactions in the presence of PBMC nuclear extracts. FP indicates free probe. An arrow indicates the YY1 complex. B, gel-shift interference analysis with anti-YY1 antibody. The reaction mixture for binding between R5(−607/−584) oligonucleotide and PBMC extracts was incubated with either normal rabbit serum (lane 2) or anti-YY1 rabbit polyclonal antibody (lane 3). The solid arrow indicates the YY1 complex, which was disrupted by anti-YY1 antibody (lane 3), but not control serum (lane 2).

Fig. 3. YY1 down-regulates CCR5 promoter. A, effect of YY1 site mutation on CCR5 promoter activity. CD4+ T cell-enriched PBMC were unstimulated or stimulated with IL-2 (100 units/ml) for 7 days and transfected with 2.5 μg of pGL-CCR5(YY1-WT) or pGL-CCR5(YY1-MT). Reporter activities are shown as arbitrary light units. Results are reported as means ± S.E. from three independent experiments. B, the YY1 motif on the CCR5 promoter mediates YY1-mediated transrepression. Five million CD4+ T cell-enriched PBMC were transfected with 2.5 μg of pGL-CCR5(YY1-WT) or pGL-CCR5(YY1-MT) along with 2.5 μg of pCMV-YY1 or pcDNA3.1. Fold reduction indicates luciferase activity relative to basal promoter activity (pcDNA3.1 co-transfection). Results are reported as means ± S.E. from three independent experiments.

Fig. 4. Overexpression of YY1 reduces cell surface expression of CCR5. Five million CD4+ T cell-enriched PBMC were transfected with 5 μg of pcDNA/GFP (left panels) or pcDNA/GFP-YY1 (right panels), stained with IgG2a PE (upper panels) or anti-CCR5 PE (lower panels), and analyzed in FACScan. The dot blot shows green fluorescence intensity (GFP or GFP-YY1 expression) in x-axis and PE color intensity (CCR5 expression) in y-axis. Results are representative of three independent experiments.
10-fold; however, mutations on the YY1 site markedly reduced YY1 suppression of reporter activity (less than 3-fold reduction) (Fig. 3B). These results suggest that the YY1 binding site is functional and plays a critical role in YY1-mediated down-regulation of the CCR5 promoter. Although residual responsiveness of pGL-CCR5 (YY1-MT) to YY1 may imply the presence of other YY1 binding site(s) on the promoter, we could not find any sequence resembling the YY1 motif.

Overexpression of YY1 Down-regulates Cell Surface Expression of CCR5—As shown above, the YY1 transcription factor can down-regulate the CCR5 promoter; however, it is well known that YY1 can mediate totally different effects, depending upon promoter constructs and cell types to be tested. To demonstrate whether cell surface expression of CCR5 is actually down-regulated by YY1, we overexpressed YY1 by transfecting CD4⁺ T cell-enriched PBMC with pcDNA/GFP-YY1 and determined cell surface expression of CCR5 or CD4 by flow cytometry. Like pCMV-YY1, pcDNA/GFP-YY1 down-regulated reporter activity from pGL-CCR5 when co-transfected (data not shown). After a 7-day stimulation with IL-2, more than 30% of CD4⁺ T cell-enriched PBMC expressed CCR5 (Fig. 4, lower panels; data not shown). GFP-positive cells expressed CCR5 at levels comparable with GFP-negative cells after pcDNA/GFP transfection (Fig. 4, left panels). However, significantly fewer GFP-positive cells expressed CCR5 after pcDNA/GFP-YY1 transfection (Fig. 4, right panels). Thus, overexpression of YY1 appears to reduce cell surface expression of CCR5. On the contrary, CD4 expression on GFP-positive cells after pcDNA/GFP transfection (94%; mean fluorescence intensity 36.9) was comparable with that on GFP-positive cells after pcDNA/GFP-YY1 transfection (93%; mean fluorescence intensity 34.0).

Overexpression of YY1 Decreased Infectibility by R5-HIV-1—Having had the aforementioned results, we wanted to determine whether overexpression of YY1 could inhibit HIV-1 infection. Because YY1 has variable effects depending upon cell type and because continuous and strong expression of YY1 is toxic to cells (data not shown), YY1 was transiently overexpressed in IL-2-stimulated, CD4⁺ T cell-enriched PBMC by transfection with pcDNA/GFP-YY1. The transfected cells were then mock-infected or infected with NL4-3Luc/JR-FL. The cells were stained with anti-HIV-1 p24 Ab-PE or IgG1 PE (data not shown). The dot blot shows green fluorescence intensity (GFP-YY1 expression) in x-axis and PE color intensity (p24 expression) in y-axis. Results are representative of three independent experiments.

**Possible Anti-HIV-1 Activities Mediated by YY1**

| HIV-1 Replicative Cycle | YY1-Mediated effects |
|-------------------------|----------------------|
| Entry                   | : Down-regulation of CCR5/CXCR4 expression |
| Uncoating               | : Inefficient uncoating due to lack of cyclophilin A in virion |
| Reverse Transcription/Nuclear Translocation/Integration | |
| Transcription           | : Down regulation of LTR activity |
| Translation             | |
| Assembly/Maturation      | : Block to incorporation of cyclophilin A into virion, leading to inefficient uncoating during the next cycle |
| Budding                 | |

**Fig. 6. Possible anti-HIV-1 activities mediated by YY1.**

In this study we have demonstrated that the YY1 transcription factor can down-regulate expression of CCR5 at the promoter level. Levels of CCR5 expression appear to correlate well with infectibility of CD4⁺ T cells by R5 HIV-1 (6) and rate of disease progression (7). Expression of CCR5 appears to be highly regulated by a number of cytokines, cell activation, or differentiation (16–20). At the promoter level, several transcription factors have been demonstrated to up-regulate CCR5 expression, including p65 (RelA) (21), C/EBP-β (22), GATA-1 (9), and Octamer (10). The present study has extended our understanding of the molecular mechanism of regulation of CCR5 promoter activity by adding YY1 as the first transcription factor.
ttional repressor of the promoter. We further demonstrated that overexpression of YY1 actually down-regulates cell surface CCR5 expression and infectibility by R5-HIV-1.

We have previously demonstrated that YY1 down-regulates the promoter for CXCR4, another major co-receptor for HIV-1 (3). YY1 is also known to down-regulate the HIV-1 long terminal repeat promoter (2). Furthermore, YY1 associates with cyclophilin A, which may be critical for maturation and uncoating of HIV-1 virions through its interaction with Gag (4). Taken together with the present study, it is reasonable to consider that the YY1 transcription factor plays important roles in the pathogenesis of HIV-1 disease (Fig. 6). Further investigations to delineate the molecular and cellular mechanisms that regulate expression of HIV coreceptors and to develop therapeutic interventions using anti-HIV-1 host factor(s) such as YY1 are warranted.

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