CCR5Δ32 59537-G/A Promoter Polymorphism Is Associated with Low Translational Efficiency and the Loss of CCR5Δ32 Protective Effects[†]

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We have recently demonstrated that the CCR5Δ32 protein interacts with CCR5 and CXCR4 and down-modulates their cell surface expression. We have also reported the absence of detectable expression of the truncated CCR5Δ32 protein in four out of six human immunodeficiency virus-infected (HIV⁺) CCR5^{-/-} individuals. To explain the defect in protein expression in these samples, we cloned and sequenced the promoter regions of the six HIV⁺ individuals. We have identified several polymorphisms in the CCR5Δ32 promoter region, but these polymorphisms were not associated with significant differences in mRNA levels. Coupled *in vitro* transcription/translation and polyribosome analysis demonstrated a strong association between a variant genotype designated CCR5Δ32 59537-A/A and a low translation efficiency. Protein analysis indicated that the peripheral blood mononuclear cells from two of the HIV⁺ CCR5^{-/-} individuals carrying the CCR5Δ32 59537-A/A variant expressed trace amounts of CCR5Δ32 protein compared to the individuals carrying the CCR5Δ32 59537-G/G genotype. The results imply that the absence of CCR5Δ32 protein in two HIV⁺ individuals is due to a genetic defect in the translation of the protein. Together, these results highlight the importance of the CCR5Δ32 protein as an HIV suppressive factor and provide further insight into the mechanism of the protective effect of the CCR5Δ32 mutation.

The CC chemokine receptor 5 (CCR5) is the coreceptor utilized by human immunodeficiency virus type 1 (HIV-1) to infect human CD4⁺ macrophages and T lymphocytes (4, 10, 12–14). A naturally occurring 32-base pair deletion in the human CCR5 gene is highly associated with resistance to HIV-1 infection (8, 11, 13, 14, 38). CCR5Δ32 encodes a truncated protein that is not detected on the cell surface and therefore is not functional as a coreceptor (8, 11, 13, 14, 38). The majority of individuals carrying the two alleles of the CCR5Δ32 mutation (CCR5^{-/-}) are highly protected against HIV-1 infection (reviewed in reference 32). Individuals who are heterozygous for the mutant allele (CCR5^{+/-}) are not protected against infection, but once infected, their progression to AIDS is delayed (8, 11, 13, 27, 36, 38), indicating that partial resistance can occur in the presence of a single copy of CCR5Δ32.

In rare cases, CCR5Δ32 homozygosity was associated with HIV-1 infection (5, 7, 15, 22, 28, 33, 34, 36), but in these cases, the mechanism of infection has not been defined. In most cases, the exclusive use of CXCR4 by virus isolates or the presence of the *env* gene sequences typical of X4 viruses was observed. Isolation of the dual-tropic (R5X4) HIV-1 from infected individuals homozygous for the CCR5Δ32 allele has also been reported (15, 16, 31).

Our previous work suggested that the HIV resistance in CCR5Δ32 homozygotes may result from both the genetic loss

of CCR5 from the cell surface as well as the active down-regulation of CXCR4 expression by the mutant CCR5Δ32 protein. We and others have demonstrated that the CCR5Δ32 protein may form heterodimers with the wild-type CCR5 and CXCR4 proteins, which are retained in the endoplasmic reticulum and result in reduced cell surface expression of the coreceptors (2, 6, 9, 37). We have previously demonstrated the absence of detectable CCR5Δ32 protein in four infected CCR5^{-/-} individuals (1, 2). Here, we have examined the effect of polymorphisms in the promoter region and the 5' untranslated region (UTR) on CCR5Δ32 expression.

To explain the absence or low abundance of the CCR5Δ32 protein in infected CCR5^{-/-} individuals, we cloned the promoter regions of the HIV-infected (HIV⁺) CCR5Δ32 homozygous individuals. We identified a 59537-G/A polymorphism in exon 3 (E3) that is a part of the CCR5Δ32 promoter region and the 5' UTR. Promoter analysis of luciferase reporter constructs did not reveal significant effects on mRNA transcription; however, the 59537-A/A genotype was strongly associated with low translational efficiency of the CCR5Δ32 protein. The results provide important insight into the mechanism of resistance to HIV-1 infection and pathogenesis.

MATERIALS AND METHODS

Cell lines and CCR5^{-/-} PBMC samples. Human embryonic kidney (HEK293) and HeLa cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The peripheral blood mononuclear cell (PBMC) samples (six HIV⁺ and some HIV-negative [HIV⁻] CCR5^{-/-} cells) have been described previously (1). The clinical profile of patient VH has been described previously (7, 31). VM and VH are two CCR5^{-/-} individuals; VM is uninfected, while VH is infected, and the mode of infection appears to be via homosexual intercourse. The three HIV⁺ SEROCO samples are all from CCR5^{-/-} individuals who are homosexuals. The SEROCO sample 2 has been reported previously (36). The Shep sample 164 has been documented

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previously (34) and examined in our previous studies (1, 2). The MACS infected *CCR5*^{-/-} sample, ID 20705, has been reported previously (15). The other HIV⁻ *CCR5*^{-/-} samples and the samples from individuals homozygous for the wild-type *CCR5* allele (*CCR5*^{+/+}) were obtained from the MACS cohort. PBMCs were stimulated with phytohemagglutinin (PHA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and recombinant interleukin 2 (IL-2; 100 units/ml).

Reverse transcription (RT)-PCR analysis of mRNA transcription in *CCR5*^{-/-} PBMCs. Total RNA of transfected cells was isolated from PBMCs using an RNeasy mini-kit (Qiagen, MD) reagent. The RNA (1 μg) was reverse transcribed using oligo(dT)₂₀ primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting first-strand DNA (2 μl) was amplified to a final volume of 25 μl containing 10 pmol of each primer and 1 unit of *Taq* polymerase (Promega, Madison, WI). The primers used to amplify the E2-Δ32 transcript (183 bp) were forward primer (FP) 5'-ATTCTGTGTAGTGGGATG-3' and reverse primer (RP) 5'-TGAAGGAGGGTGGAGTTAA-3'. The primers used to amplify the E3-Δ32 transcript (54 bp) were FP 5'-CTCATCTG GCCAGAAGAG-3' and RP 5'-CGGGGAGAGTTTCTTGTA-3'. Amplification conditions were denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s for 35 cycles. The amplified products (5 μl) were subjected to electrophoresis on 2% agarose and stained with ethidium bromide.

PCR cloning of the *CCR5*Δ32 promoter regions. Genomic DNA was isolated by using a DNeasy tissue kit (Qiagen, MD) reagent. The primers were designed according to GenBank sequence U95626. The upstream promoter (*P*_U) was amplified using the FP 5'-AAGCTAGCAGGAAATGGAAGCTTGGGCA-3' and the RP 5'-CGAAGCTTCGTGACCTTGGCTCTAGAAT-3'. The downstream promoter (*P*_D) was amplified using the FP 5'-GCAAGCTTCGCTTCTTAGAGATCACAA-3' and the RP 5'-CGAAGCTTCCAACTGTGACCCTTTCC-3'. The amplified *P*_U fragment maps to sequences between 57236 and 58761, while the amplified *P*_D fragment represents sequences from 58491 to 59728. Briefly, DNA samples and primers were first denatured at 94°C for 2 min and then subjected to 35 cycles of PCR amplification (95°C for 15 s, 55°C for 30 s, and 68°C for 1 min per kb). PCRs were performed using Platinum Pfx DNA polymerase (Invitrogen). PCR products were purified by using a Quick Gel Extraction kit (Invitrogen) through an agarose gel and cloned into a pGEM T Easy vector (Promega) after A tailing. The resulting positive promoter clones were sequenced to confirm their identities. The *P*_D and *P*_U fragments cloned into the pGEM T-Easy vector were digested with HindIII and then ligated to make *P*_U+*P*_D, which was joined at an overlapping HindIII site at a position from 58525 to 58530 (Fig. 1C).

The *P*_U, *P*_D, and *P*_U+*P*_D promoter region fragments in the pGEM T-Easy vector were digested with NotI, filled in with Klenow fragment, ligated into the pRF vector (Promega) that had been digested with NheI/XbaI to remove the T7 promoter and the *Renilla* luciferase gene, and blunt ended. Promoter activity assays were done using a dual-luciferase reporter assay kit according to the manufacturer's instructions (Promega). Briefly, 0.1 million HeLa cells per well were seeded into a 24-well plate for overnight growth. Cotransfection of 0.5 μg DNA of the *P*_U, *P*_D, or *P*_U+*P*_D promoter construct (Fig. 1C) along with 0.2 μg of the pRL-CMV vector (*Renilla* luciferase under cytomegalovirus [CMV] promoter control) was accomplished by using Lipofectamine 2000 (Invitrogen). The empty pGL3B vector was used as a blank control. Transfected cells were incubated for 72 h and then washed twice in cold phosphate-buffered saline (PBS) buffer and lysed with 100 μl passive lysis buffer (Promega) by shaking for 15 min at room temperature. A 20-μl sample of cell lysate was taken to measure luciferase activity by using an automated AutoLumat LB 953 Berthold luminometer.

PCR cloning of *CCR5*Δ32 5' UTR fragments and construction of 5' UTR reporter genes. The *CCR5*Δ32 5' UTR E1 was amplified using the FP 5'-CTT CAGATAGATTAT-3' and the RP 5'-CGAGTACTATGCCAGATACGTAGG-3'. E2/3 was amplified using the FP 5'-CGAGTACTATTCTGTAGTGGGATG-3' and the RP 5'-CGAGTACTCGGGGAGAGTTTCTTGTA-3'. E3 was amplified using the FP 5'-CGAGTACTCTCATCTGGCCAGAAGAG-3' and the RP 5'-CGAGTACTCGGGGAGAGTTTCTTGTA-3'. The E1, E2/3, and E3 5' UTR fragments were subcloned into the pGEM T Easy vector, digested with Scal, and ligated to make E1/2/3 or E1/3 with an overlapping Scal site. For constructing the T7 promoter luciferase reporter system, the pRF vector (Promega) was digested with EcoRV/XbaI to remove the *Renilla* luciferase gene and then self-ligated to make a new pT7FL vector. The *CCR5*Δ32 5' UTR E3, E2/3, E1/3, and E1/2/3 fragments in the pGEM T Easy vector were digested with SpeI/EcoRI and subcloned into the same SpeI/EcoRI digestion site of the pT7FL vector. The structure of the different 5' UTR fragments was designed as reported by Mummidi et al. (30).

To study the in vivo translation efficiency of the constructs, 0.5 μg of each expression vector was transfected into HEK293 cells, using Lipofectamine 2000

reagent (Invitrogen), together with 0.2 μg of P Tracer-LacZ (Invitrogen), which contains the LacZ gene under the control of the T7 promoter. After 4 h of transfection, the expression was activated by infection with the vaccinia virus vTF7-3. On the following day, the cells were washed twice in cold PBS buffer and then lysed with passive lysis buffer. The dual-luciferase enzymatic activity assay was carried out according to the manufacturer's protocol (Promega) and measured by luminometer. The β-galactosidase activity was measured according to standard procedure (3). The translation efficiency was calculated by dividing the luciferase units by the β-galactosidase units.

For in vitro-coupled transcription/translation, we utilized the linked in vitro SP6/T7 transcription/translation nonradioactive kit (catalog no. 1 814 346; Roche). The system is based on in vitro transcription of DNA sequences inserted downstream of the T7 promoter into capped RNA, using linearized or supercoiled DNA as the template. Without any further purification, the transcribed mRNA is subsequently translated in rabbit reticulocyte lysates. All the components required for the in vitro transcription reaction, as well as for the nonradioactive in vitro translation reaction, are provided in the kit, premixed and ready to use. Briefly, 1 μg of linear DNA of each construct (E1/2/3, E3G, or E3A) was incubated in T7 transcription buffer at 30°C for 15 min and frozen at -70°C, after a 10-μl aliquot was taken for Northern blotting analysis. The samples were then thawed, and a 10-μl aliquot from each construct was mixed with 40 μl of the translation mix. The mixtures were incubated for 1 h at 30°C, and then 5 μl of the mixtures was diluted in 45 μl of luciferase reaction buffer, and the luciferase produced was measured using a luminometer.

Construction of a CMV-5' UTR E3G or E3A luciferase reporter system and polyribosome analysis. For the CMV promoter luciferase reporter system, we used the vector pFL-CMV as described in the previous section. E3 containing the G or A polymorphism fragment in the pGEM TEasy vector was digested with SpeI/EcoRI and subcloned into the same SpeI/EcoRI digestion site as that of the pFL-CMV vector. To analyze the translational efficiency of the E3 region, HEK293 cells were cotransfected with 0.5 μg of the recombinant E3-pFL-CMV vector together with 0.2 μg of the pRL-CMV vector (which contains the *Renilla* luciferase gene under the control of the CMV promoter). The *Renilla* luciferase served as an internal control for transfection efficiency normalization, considering the pGL3B vector (Promega) only as a blank control. The transfected cells were lysed at 3, 6, 12, and 48 h and diluted in 45 μl luciferase reaction buffer, and the luciferase produced was measured using a luminometer.

For polyribosome analysis, approximately 5×10^6 cells (HEK293 cells) were transfected with 10 μg of pFL-CMV-5' UTR G or pFL-CMV-5' UTR A and incubated at 37°C for 48 h. The cells were treated with 0.35 mM cycloheximide (Sigma, St. Louis, MO) for 10 min, washed with PBS, and lysed with lysis buffer (20 mM HEPES-KOH [pH 7.4], 10 mM KCl, 5 mM MgCl₂, 0.3% NP-40, 0.5 mM dithiothreitol, 200 U/ml RNasin [Roche, Indianapolis, IN], and 15 μl/ml protease inhibitor mixture [Sigma], with 50 mM EDTA [pH 8.0]). The cell lysates were scraped into a 1.5-ml microcentrifuge tube and passed five times or more through a 27-gauge needle. Nuclei were pelleted by centrifugation at 12,000 rpm for 20 min at 4°C, and the supernatant was layered on top of a 20% to 60% linear sucrose gradient in gradient buffer (10 mM HEPES-KOH [pH 7.4], 150 mM KCl, 0.5 mM MgCl₂, 1 mM dithiothreitol, 50 U/ml RNasin). The gradient was made with Gradient Maker (catalog no. GM-100; CBS Scientific, Del Mar, CA). Gradients were centrifuged at 35,000 rpm for 2 h in a Beckman SW41 rotor and recovered in 18 equal fractions. The optical density was measured at 254 nm. Fractions were treated with DNase (GIBCO-BRL, Rockville, MD) and then extracted twice with phenol, and the RNA was precipitated with ethanol, washed twice with 70% ethanol, and stored at -80°C.

For Northern blotting analyses, the entire RNA sample from each fraction was used. Samples were separated on 1.2% agarose gels containing 15% formaldehyde, transferred to Zeta-Probe membranes (Bio-Rad Laboratories, Hercules, CA), and incubated with a luciferase-specific DNA probe. The luciferase probe was prepared by using a random primer DNA-labeling system (Gibson-BRL) with a gel-purified Luc PCR fragment and [³²P]dCTP (GE Healthcare, Piscataway, NJ). The membranes were cross-linked using a UV cross-linker (Stratagene, La Jolla, CA) and hybridized to the ³²P-labeled probe. After undergoing hybridization and washing, the membrane was exposed to X rays at -70°C and developed.

Metabolic cell labeling and immunoprecipitation. The *CCR5*^{-/-} PBMCs were activated with PHA plus IL-2 for 3 days and metabolically labeled with 200 μCi of [³⁵S]methionine and 200 μCi of [³⁵S]cysteine (Amersham Corp., Pittsburgh, PA) for 3 h, as described previously (2). The cell lysates were immunoprecipitated by using a previously described polyclonal antibody raised against the last 31 frame-shift amino acids in the protein, the *CCR5*Δ32-specific antibody (2). The resulting immune complexes were reacted with protein A-Sepharose beads (GE Healthcare, Piscataway, NJ), washed three times, and analyzed by sodium

FIG. 1. (A) Schematic diagram of the *CCR5Δ32* gene promoter. The promoter regions and potential transcripts resulting from *CCR5Δ32* are as originally described for *CCR5* by Mummidi et al. (30). E1, E2, E3, and E4 represent the four exons, P_U refers to the upstream promoter, and P_D refers to the downstream promoter, as described by Mummidi et al. (30). The position of the 59537 G/A-polymorphism in the E3 region is indicated. This polymorphism corresponds to -1951, according to the numbering system that designates the first nucleotide of the translation start site of *CCR5Δ32* as position 1 and the nucleotide immediately upstream of this as position -1. (B) RT-PCR analysis of endogenous *CCR5Δ32* mRNA isolated from PBMCs with the indicated *CCR5* genotypes. Labeling on the right indicates the identity of the primers used. Equivalent amounts (5 μg) of poly(A)⁺ mRNA were used in this analysis. The primers to amplify β-actin were used to control for gel loading. The amplified products were analyzed with a 1% agarose gel.

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then dried and exposed to X-ray film.

RESULTS

RT-PCR analysis of the *CCR5Δ32* transcripts in PBMCs from six HIV⁺ patients. To analyze *CCR5Δ32* RNA transcription, we first compared endogenous mRNA expression by RT-PCR analysis using poly(A) mRNA isolated from the different *CCR5*^{-/-} PBMCs. Previous studies demonstrated multiple transcripts from the *CCR5* promoter (30). Figure 1A shows the *CCR5* promoter organization and the predicted mRNA transcripts as originally described (30). Mummidi et al. identified two *CCR5* promoter regions: the P_U, a weak promoter which resides proximal to E1, and the P_D, a stronger promoter which is located upstream of E3 (30). Both *CCR5* promoter regions have been demonstrated to be active in CD4⁺ T lymphocytes (23). Figure 1A shows a schematic structure of the predicted *CCR5Δ32* mRNA transcripts as previously reported (30) and the location of the 59537-G/A polymorphism. This polymorphism corresponds to -1951, according to the numbering system that designates the first nucleotide of the translation start site of *CCR5Δ32* as position 1 and the nucleotide immediately upstream of this site as position -1.

RT-PCR analysis using primers that amplify the large transcript (*CCR5A* in reference 30) indicated the absence of this mRNA species in four HIV⁺ *CCR5*^{-/-} samples (Fig. 1B, E2 to E4). However, RT-PCR analysis using primers that map to the E3 region (Fig. 1B, E3 to E4) indicated no significant differ-

ences in the abundance of this RNA species among the tested *CCR5*^{-/-} samples (Fig. 1B, E3 to E4). The quality of samples loaded was verified by amplification of β-actin mRNA from the same RNA quantity (Fig. 1B, bottom gel). The absence of a detectable large transcript in the HIV⁺ *CCR5*^{-/-} PBMCs and the fact that it is unknown which transcribed mRNA species are translated in vivo prompted us to examine the promoter regions for potential polymorphisms.

Polymorphisms in *CCR5Δ32* promoter regions and their effect on reporter gene expression. We sequenced both promoter regions in the *CCR5*^{-/-} samples and identified a number of sequence polymorphisms in all *CCR5*^{-/-} PBMC samples, as indicated in Table 1. To determine whether the promoter polymorphism had any effect on the transcriptional activity of *CCR5Δ32* promoter DNA, fragments corresponding to the P_U and P_D regions cloned from the *CCR5*^{-/-} samples were cloned upstream of the firefly luciferase reporter gene (Fig. 2A).

In order to determine whether these polymorphisms impact *CCR5* promoter activity, we utilized a reporter assay system that has been used previously to analyze the transcriptional activity of *CCR5* promoter (17, 30). We generated promoter constructs that contained either P_U, P_D, or both promoter regions joined in one fragment upstream of the firefly luciferase (Fig. 2A). The different promoter plasmid constructs were cotransfected into HEK293 cells along with plasmid DNA encoding the *Renilla* luciferase under the CMV promoter. The results demonstrate no significant differences in *CCR5Δ32* pro-

TABLE 1. Sequence polymorphisms of the *CCR5* promoter region^a

Nucleotide site	GenBank nucleotide	Wild type (UN ^{+/+})	MACS sample		SEROCO sample			Shep sample 164 (IN ^{-/-})	Clinical sample	
			UN ^{-/-}	IN ^{-/-}	1 (IN ^{-/-})	2 (IN ^{-/-})	3 (IN ^{-/-})		VM (UN ^{-/-})	VH (IN ^{-/-})
-4123	57365	C	C	C	C	C	T	C	C	ND
-4011	57477	A	A	A	G	A	A	A	A/G	ND
-4004	57484	G	G	G	G	G	G	G	A	ND
-3911	57577	C	T	T	T	T	T	T	T	ND
-3852	57636	C	T	T	T	T	T	T	T	ND
-3589	57899	C	C	C	C	C	C	C/T	C	ND
-3399	58089	A	G/A	A	A	A	A	A	A	ND
-3387	58101	C	T	T	T	T	T	T	T	ND
-3287	58201	T	T	T	T	T	T/G	T	T	ND
-3181	58307	A	A	A	A	A	A	A	G/A	ND
-3159	58329	A	A	G	A	A	A	A	A	ND
-3071	58417	A	A	A	G/A	A	A	A	A	ND
-2933	58555	A	G	G	G	G	G	G	G	ND
-2892	58596	A	G	G	G	G	G	G	G	G
-2773	58715	A	G	G	G	G	G	G	G	G
-2554	58934	T/G	G	G	G	G	G	G	G	G
-2459	59029	G	A	A	A	A	A	A	A	A
-2150	59338	A	A	A	A	A	A	A	A	A
-2136	59352	C	C	C	C	C	C	C	C	C
-2135	59353	T	C	C	C	C	C	C	C	C
-2132	59356	C	C	C	C	C	C	C	C	C
-2115	59373	C	C	C	C	C	C	C	C	C
-2086	59402	G	A	A	A	A	A	A	A	A
-2078	59410	T	T	T	T	T	T	T	T	T
-2048	59440	C	C	C	C	C	C	C	C	C
-1951	59537	G	G	G	A	A	G	G	G	G
-1835	59653	C	C	C	C	C	C	C	C	C

^a Cellular DNA from nine different individuals (six HIV⁺ *CCR5*^{-/-} patients, two HIV⁻ *CCR5*^{-/-} patients, and one HIV⁻ *CCR5*^{+/+} patient) was used to clone the *CCR5*Δ32 promoter fragments. Six DNA clones of each sample containing each promoter fragment were subjected to DNA sequence analysis in both strands. When the sequencing data showed the same nucleotide in all six clones, it was reported as a single A, G, C, or T. The T/G sequence is reported when the sequence data of the first six clones returned three clones with a T and three with a G. In all cases, we sent six more clones for sequence analysis to confirm the potential heterozygosity or homozygosity. ND, not determined; UN, uninfected; IN, infected.

motor activities associated with these polymorphisms (Fig. 2B). Northern blotting analysis of the mRNA produced in HEK293 cells transfected with the different promoter constructs confirmed the relative abundance of the expressed RNA species driven from each promoter construct (Fig. 2C). These results demonstrate that the observed promoter polymorphisms had no significant impact on *CCR5*Δ32 transcriptional activity.

Translational analysis of the 5' UTRs of *CCR5*Δ32. Since the *CCR5*Δ32 59537-G/A polymorphism is located in E3 of the *CCR5* gene, it is reasonable to assume an effect on translational efficiency. Since it is located in E3, this polymorphism (Fig. 3A) is present in the 5' UTR of all transcripts that are transcribed from the *CCR5* promoter (30). It has been demonstrated previously that complex splicing and multiple transcriptional start sites result in several *CCR5* transcripts that differ in their 5' UTR (30). To analyze the translational efficiency of the different 5' UTRs, the sequences of the different 5' UTRs were constructed as previously described (30) and were cloned upstream of the firefly luciferase under the control of the T7 promoter (Fig. 3A). Production of the mRNA was activated by infection with a vaccinia virus recombinant encoding T7 RNA polymerase. The 5' UTR constructs were individually cotransfected with the pT7-LacZ vector (containing LacZ under T7 promoter control) into HEK293 cells, and the translational efficiency of the UTR was measured as the ratio of luciferase:β-galactosidase. The results indicate that the short-

est 5' UTR (E3) was the most efficient at translation and produced the highest luciferase:β-galactosidase ratio (Fig. 3B). Low translation efficiency was observed consistently with the longer 5' UTR that had E2 and E3 (E2/3) and consistently with the background levels obtained with the longest UTR (i.e., E1/2/3) (Fig. 3B). To determine the translational efficiency of the E3 5' UTR containing the A polymorphism (E3A), we compared its efficiency with that of its counterpart that has a G at that position (E3G). We consistently found that E3G has a significantly higher translational efficiency than E3A (Fig. 3B).

To verify the results obtained with the T7 polymerase system, we used an in vitro-coupled transcription/translation system (Promega). The translational efficiency of the E3G and E3A UTR constructs was examined by measuring the luciferase activity produced with rabbit reticulocyte lysate supplemented with T7 RNA polymerase. The results of these experiments consistently produced translational efficiency of E3G that was higher than E3A (Fig. 3C). The construct containing the E1/2/3 UTR had a very low translational efficiency in these experiments (Fig. 3C). Northern blotting analysis confirmed that the observed results were not due to higher mRNA produced from the E3G-based constructs (Fig. 3D) or the E3G RNA produced in the in vitro-coupled transcription/translation system (Fig. 3E). Taken together, these results suggest that the mRNA containing the E3 UTR is the RNA species

FIG. 2. Transcriptional activities of the *CCR5*Δ32 promoter variants. (A) Schematic diagram illustrating the different promoter constructs used in subsequent experiments to measure the transcriptional activities of the promoter variants described in this study. The promoter regions identified by the indicated GenBank nucleotide numbers were cloned upstream of the firefly luciferase, using the simian virus 40 polyadenylation signal (SV40 PA) for the 3' end processing and the termination signal. The 59537 polymorphism corresponds to the reported -1951 position relative to that of the translation initiation codon. (B) The *CCR5* Pr-Luciferase plasmids were individually cotransfected with a plasmid containing the *Renilla* luciferase into HEK293 cells and incubated for 48 h at 37°C. Transcriptional activity of the cloned promoters was measured by calculating the ratio of firefly:*Renilla* luciferase production. (C) Northern blotting analysis of the luciferase mRNA produced in transfected HEK293 cells. Blots were probed with a fragment corresponding to the firefly luciferase. The broken line represents the background levels measured in empty vector-transfected HEK293 cells with no luciferase production. The blots were also probed with a DNA fragment corresponding to β-actin to control for gel loading. Rel., relative levels.

most efficiently translated and that the G/A polymorphism in E3 significantly affects translational efficiency.

The 59537-G 5' UTR but not the 59537-A 5' UTR augments ribosomal association. To determine the in vivo effect of the G/A polymorphism, we transfected 293 cells with the constructs containing the polymorphic 5' UTR under the control of the CMV promoter (Fig. 4A). The different 5' UTR constructs were individually transfected along with the plasmid DNA encoding *Renilla* luciferase under the CMV promoter. Transfected cells were lysed after incubation for 3, 6, 12, and 48 h, and luciferase activity was measured. The results indicate that the E3A 5' UTR construct produced significantly lower luciferase activity than the E3G 5' UTR, even after 48 h of incubation (Fig. 4B).

To directly examine the effect of the 5' UTR polymorphism on translational efficiency in vivo, we isolated poly(A) mRNA from cells transfected with the chimeric 5' UTR constructs. Transcriptionally active ribosomes (polysomes) were separated from the nonactive (free ribosomal), using a sucrose gradient. Cytoplasmic extracts were prepared from the transfected cells and analyzed. Eighteen fractions were collected, and absor-

bance was measured (Fig. 4C). The RNA extracted from the gradient fractions was analyzed by Northern blotting hybridization using a probe corresponding to the luciferase-coding region (Fig. 4D). To analyze the cytoplasmic distribution of luciferase RNA, all 18 fractions of the ribosomal profile were subjected to Northern blotting analysis. The results demonstrate that the chimeric RNA with the E3G 5' UTR was completely associated with actively translating polysomes, while the chimeric RNA carrying E3A 5' UTR was partially associated with polysomes, indicating a recruitment defect. Figure 4C shows the absorbance profiles of the 18 fractions.

Figure 4D shows that the chimeric mRNA with the 5' UTR (E3G) is almost completely associated with actively translating polysomes, while the mRNA with the polymorphism (E3A) is only partially associated with the polysome fractions. Polysome disruption by EDTA treatment results in a dramatic shift of the sedimentation pattern from the heavy polysomes to the lighter and free fractions of the gradient (Fig. 4D, bottom two blots). Treatment with EDTA resulted in the disappearance of the mRNA from the polysome fraction, confirming the specificity of this assay. These results suggest a translational control

FIG. 3. Translational analysis of the 5' UTRs of *CCR5Δ32*. (A) Schematic diagrams of the maps of the pGL3-based plasmid construct containing the different 5' UTRs. The location of the 59537-G/A polymorphism in the *CCR5Δ32* promoter is indicated. The 5' UTR regions of *CCR5Δ32* (as originally described by Mummidi et al. [30]) were individually cloned under the T7 promoter in the pGL3 vector (Promega) and upstream of the firefly luciferase gene. The plasmids containing the different T7-UTR-luciferase constructs were cotransfected along with pT7-*lacZ* into HeLa cells, and expression of the constructs was activated by infection with vTF7-3 encoding the T7 RNA polymerase. (B) Expression efficiency of the constructs transcribing the luciferase gene linked with the different 5' UTRs. Transfection efficiency was determined by cotransfecting pT7-*lacZ* that contains β-galactosidase under T7 promoter (translation efficiency was calculated as the luciferase/β-gal ratio). The experiment was reproduced at least three times. (C) In vitro transcription/translation of the indicated 5' UTR constructs. The indicated 5' UTR plasmid construct (1.0 μg) was incubated in rabbit reticulocyte lysate (Promega) supplemented with T7 polymerase. Results are shown for the luciferase produced in one experiment performed in duplicate and reproduced three times. (D and E) Northern blotting analysis of the firefly luciferase mRNA produced in transfected HeLa cells (D) and as a result of in vitro transcription (E). Rel., relative levels.

mechanism in two HIV⁺ *CCR5*^{-/-} individuals with a 59537-A polymorphism in the 5' UTR.

Endogenous *CCR5Δ32* protein expression in the HIV⁺ PBMC samples correlate with the in vitro translation data. We have previously reported that the *CCR5Δ32* protein was not detected by Western blotting in four of the six HIV⁺ samples (1). Since the *CCR5Δ32* protein was not detected in the two samples (sample 1 and sample 2) that have a 59537-A/A genotype, we examined the endogenous mRNA expression by RT-PCR and compared that to the three uninfected *CCR5*^{-/-} samples with a 59537-G/G genotype. The results demonstrated abundant mRNA expression in the two samples with the 59537-A/A genotype that was similar in intensity to that obtained with the samples with the 59537-G/G genotype (Fig. 5A). We then examined the *CCR5Δ32* protein expression in PBMC samples radiolabeled with [³⁵S]methionine and [³⁵S]cysteine. The radiolabeled proteins were immunoprecipitated with antibodies that specifically detect the *CCR5Δ32* protein. The immunoprecipitated proteins were analyzed with SDS-PAGE and detected by autoradiography. The results show high *CCR5Δ32* protein expression in the *CCR5*^{-/-}

PBMC samples with a 59537-G/G genotype and relatively low levels in the samples with a 59537-A/A genotype (Fig. 5B). Immunoprecipitating equivalent amounts of cell lysates with antibodies specific to β-actin revealed equivalent gel loading. These results strongly support our findings of the translational efficiency of the 5' UTR constructs and indicate that the expression of the *CCR5Δ32* protein is critical for the resistance phenotype.

DISCUSSION

Polymorphisms in *CCR5* have been implicated in modulating AIDS disease progression and resistance to HIV-1 infection (32). Several studies have suggested that polymorphisms in the *CCR5* promoter region may result in different levels of mRNA transcription that might explain some of the observed variability in the progression to AIDS (19, 25, 26, 29). The literature reported different data concerning *CCR5* promoter polymorphisms. While some studies reported an association between the *CCR5* promoter polymorphisms and the transcription efficiency (26), others have found that promoter polymor-

FIG. 4. Expression efficiency and distribution of transcripts containing the *CCR5*Δ32 5' UTR regulatory elements in polysome gradients. (A) The structural map of the reporter constructs containing either E3G or E3A 5' UTR upstream of the firefly luciferase reporter gene is shown. (B) The protein expression efficiency of the two E3G and E3A 5' UTR constructs in transfected HEK293 cells is shown. To normalize for the transfection efficiency, the cells were transfected with another plasmid containing *Renilla* luciferase under the control of the CMV promoter. The cells were lysed and analyzed for luciferase expression at the indicated times posttransfection. (C and D) Polysome analysis of luciferase mRNA extracted from transfected HEK293 cells that were transiently transfected with 2.5 μg of E3G or E3A 5' UTR reporter constructs and incubated for 48 h. Cell lysis and sucrose gradient sedimentation analyses of polysomes were performed as described in Materials and Methods. Fractions (0.5 ml each) were collected, and their optical density was measured at a wavelength of 254 nm (C). The fractions were labeled from top to bottom to indicate their positions in the gradient. RNA was extracted from each fraction and subjected to Northern blotting analysis (D). The blots were hybridized with a probe specific to the firefly luciferase gene. The two blots at the bottom show the polysomal profiles as a result of EDTA treatment. The addition of 50 nM EDTA results in the dissociation of ribosomes from mRNA and a shift of luciferase mRNA to the top of the gradient.

phisms had no significant effects on the *CCR5* promoter activity in vitro (24). Here, we report a number of promoter polymorphisms in a number of HIV⁺ *CCR5*^{-/-} individuals, none of which had a significant impact on the transcriptional activity of the promoter in vitro. We report for the first time that one of these polymorphisms that are located in E3 of the *CCR5*Δ32 promoter (and is a part of the 5' UTR) had a significant effect on the translation efficiency of the *CCR5*Δ32 protein. Our results do not absolutely prove that the 59537 variant is solely responsible for HIV infection of these *CCR5*Δ32 individuals. Other unknown host factors might also be involved in the loss of the *CCR5*Δ32 protective effect in vivo.

The 59537-A polymorphism in the 5' UTR was associated with weak translational activity of the *CCR5*Δ32 protein in two different unrelated HIV⁺ *CCR5*^{-/-} individuals. This finding is supported by immunoprecipitation experiments showing very low levels of the endogenous *CCR5*Δ32 protein in the two samples of HIV⁺ PBMCs. The two samples with the 59537-A genotype had significantly lower levels of the *CCR5*Δ32 protein than the three other *CCR5*^{-/-} PBMC samples with the 59537-G genotype. Mutations that cause disease through increased or decreased efficiency of mRNA translation have been extensively documented, defining translational patho-

physiology as a novel mechanism of human disease (reviewed in references 20 and 21). For example, it has been demonstrated that a G-to-C transversion in the 5' UTR in the second exon of the *BRCA1* gene is able to affect mRNA translation in vitro and in vivo (35), possibly affecting tumor development and/or progression.

The low translational efficiency of the 59537-A 5' UTR may explain the low abundance of the *CCR5*Δ32 protein in two infected *CCR5*^{-/-} individuals. The reporter assays implied that 59537-G in the 5' UTR is associated with the higher efficiency of *CCR5*Δ32 protein expression that correlated with resistance to HIV-1 infection. We have previously reported that the protective effect of the *CCR5*Δ32 protein in the two HIV⁺ samples with a 59537-A genotype could be rescued by the expression of recombinant *CCR5*Δ32 protein (1), implying that the absence of the protective effect is strongly associated with the low levels of *CCR5*Δ32 protein expression. The low expression levels of the *CCR5*Δ32 protein in the two HIV⁺ *CCR5*^{-/-} subjects could not be due to a change the *CCR5*Δ32 protein amino acid sequence, since no polymorphisms in their open reading frames were detected (data not shown).

Previous studies described an association between accelerated AIDS progression and homozygosity for the *CCR5*P1/P1

FIG. 5. Endogenous expression of CCR5Δ32 mRNA and protein in PBMCs isolated from *CCR5*^{-/-} individuals with 59537G/A polymorphism. (A) RT-PCR analysis of endogenous mRNA expression. Poly(A)⁺ mRNA isolated from PHA plus IL-2-activated PBMCs was used as a template to amplify E3 to E4 transcripts in the indicated samples. The amplified products were analyzed with a 1% agarose gel. Amplification of β-actin was performed as a control. (B) The PHA plus IL-2-activated PBMC samples were radiolabeled with [³⁵S]methionine and [³⁵S]cysteine for 6 h. Lysates of the radiolabeled cells were prepared, and equivalent amounts were immunoprecipitated with either anti-CCR5Δ32 antibodies or antibodies to β-actin. The positions of the CCR5Δ32 and β-actin proteins are indicated. Uninfected (UN)^{-/-} refers to PBMCs from HIV⁻ *CCR5*^{-/-} individuals. Infected (IN)^{-/-} refers to PBMCs from HIV⁺ *CCR5*^{-/-} patients.

genotype (25). Martin et al. described a polymorphic region in the *CCR5* promoter, from E1 to E3 (GenBank no. 58934 to 59537). Analysis of that region revealed 10 polymorphic nucleotide residues that specify 10 *CCR5* promoter region haplotype alleles designated *CCR5P1* through *CCR5P10*. The *CCR5P1/P1* genotype contains a G at position 59537 (59537-G/G genotype) and has been found to be more frequent than the 59537-A/A genotype (25). We found that the presence of a G in the 5' UTR of a reporter gene results in more efficient protein translation. Martin et al. reported that the 59537-G/G genotype in *CCR5*^{+/+} individuals is associated with accelerated disease progression (25). It is possible that the increased CCR5 expression in vivo might result in a larger number of cells that initially get infected with HIV-1. It is also possible that other factors may be involved in the accelerated disease progression of these individuals and that the 59537-G/G genotype is only one of the factors involved.

It is not clear why four of the HIV⁺ *CCR5*^{-/-} samples did not express the E2-to-E4-related transcript. This transcript is similar to the previously described large CCR5A transcript (30). The E2-to-E4-related transcript could also represent one of the truncated mRNA species that has the E2 and E3 regions fused, as previously reported (30). Analysis of the effect of the promoter polymorphisms in the reporter gene activation assay did not reveal significant differences for the observed polymorphic promoter regions. The protein translation experiments consistently demonstrated that the CCR5Δ32 transcripts with

the shortest 5' UTR (E3 fused to E4) results in the most efficiently translated protein. We were unable to detect a significant translational efficiency for the longest 5' UTR region that has the three exons fused (E1/2/3), suggesting that it is not a major translation product in vivo.

The observation that the 59537-G/A variant was not associated with a transcriptional defect does not exclude a possible role for other polymorphisms in promoter activity. Previous studies suggested that a 59029-G/A polymorphism in the *CCR5* promoter was associated with a differential regulation of CCR5 transcription (26). Other studies demonstrated that some individuals who practice high-risk sexual behavior may resist infection because they possess one CCR5Δ32 allele plus a 59029-A/G genotype combination, which is apparently associated with relatively weak CCR5 expression (18). This new-found complexity in the vagaries of CCR5Δ32 expression and function might increase the clarity of the genomic analysis of risk factors for HIV infection and disease progression.

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