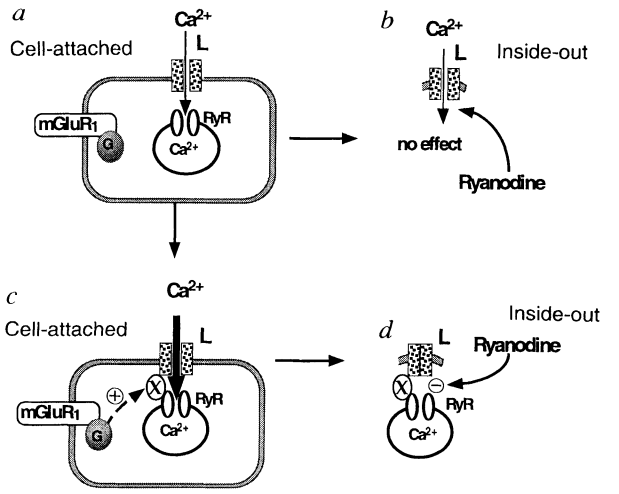

FIG. 3 Model for the coupling between the ryanodine receptor and L-type Ca^{2+} channel induced by mGluR1 stimulation. In unstimulated cells ryanodine has no effect on the Ca^{2+} channel recorded in both cell-attached (*a*) and inside-out (*b*) configurations, indicating a lack of interaction between RyR and the membrane Ca^{2+} channels in nonstimulated cells. Stimulation with mGluR1 activates a PTX-insensitive G protein to generate an unidentified second messenger, which in turn triggers a facilitatory interaction between the RyR and the L-type Ca^{2+} channel, leading to a larger transmembrane Ca^{2+} current (*c*). Although the mechanism by which mGluR1 induces this coupling is unknown, the involvement of a second messenger is likely as t-ACPD applied in the bath activated channels under the cell-attached recording pipette. The facilitatory coupling between the RyR and the L-type Ca^{2+} channel is sufficiently tight to remain in excised patches where ryanodine disrupts this interaction (*d*). The model does not exclude the possibility that an unidentified intermediate factor (X) links RyR with the L-type Ca^{2+} channel.

all of the single-channel activity (Fig. 2*b*). An unidentified soluble intracellular factor may participate in the recovery of Ca^{2+}-channel activity after wash-out of t-ACPD, as the mGluR-induced channel stimulation persisted in inside-out patches after washing out agonist (Fig. 2*a*, *b*) whereas it returned to control levels in whole-cell experiments (Fig. 1*a*).

Coupling of RyRs to Ca^{2+} entry through L-type Ca^{2+} channels by mGluR1 may be important in regulating neuronal electrical activity and synaptic plasticity. Indeed, induction of long-term depression in cerebellar Purkinje cells requires, in addition to Na^+ influx[15], coincident mGluR1 activation[16], Ca^{2+} entry through voltage-sensitive Ca^{2+} channels[17] and Ca^{2+} release from ryanodine-sensitive stores[18]. □

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Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene

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HIV-1 and related viruses require co-receptors, in addition to CD4, to infect target cells. The chemokine receptor CCR-5 (ref. 1) was recently demonstrated to be a co-receptor for macrophage-tropic (M-tropic) HIV-1 strains[2–6], and the orphan[7] receptor LESTR (also called fusin) allows infection by strains adapted for growth in transformed T-cell lines (T-tropic strains). Here we show that a mutant allele of CCR-5 is present at a high frequency in caucasian populations (allele frequency, 0.092), but is absent in black populations from Western and Central Africa and Japanese populations. A 32-base-pair deletion within the coding region results in a frame shift, and generates a non-functional receptor that does not support membrane fusion or infection by macrophage- and dual-tropic HIV-1 strains. In a cohort of HIV-1-infected caucasian subjects, no individual homozygous for the mutation was found, and the frequency of heterozygotes was 35% lower than in the general population. White blood cells from an individual homozygous for the null allele were found to be highly resistant to infection by M-tropic HIV-1 viruses, confirming that CCR-5 is the major co-receptor for primary HIV-1 strains. The lower frequency of heterozygotes in seropositive patients may indicate partial resistance.

Few molecules have attracted as much immediate attention as the recently cloned and characterized CC-chemokine receptor-5 (CCR-5)[1]. CCR-5 was shown to respond to macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES (for regulation-upon-activation, normal T expressed and secreted), the three chemokines identified as the major HIV-suppressive factors produced by $CD8^+$ T cells[8], and released in higher amounts by $CD4^+$ T lymphocytes from uninfected but multiply exposed individuals[9]. It has been shown that CCR-5 represents the major co-receptor for primary M-tropic HIV-1 strains[2–6]. M-tropic strains predominate during the asymptomatic phase of the disease in infected individuals, and are thought to cause HIV-1 transmission. In some individuals, T-tropic viruses capable of replicating

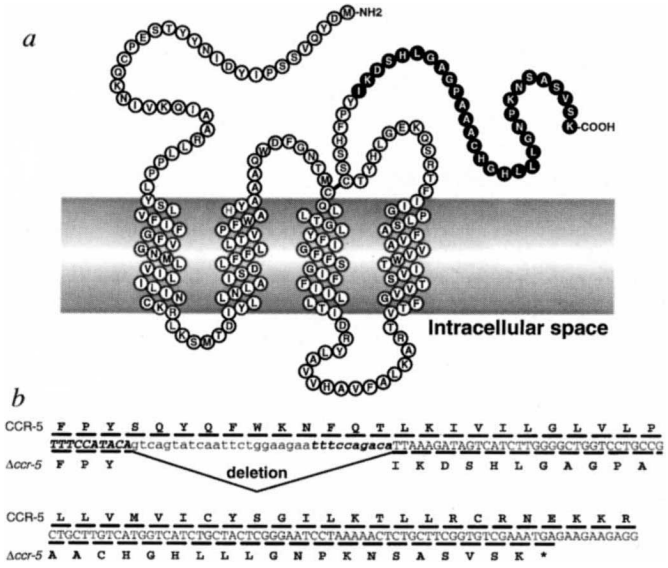

FIG. 1 Structure of the mutant form of human CCR-5. *a*, The amino-acid sequence of the non-functional Δ*ccr-5* protein. The transmembrane organization is given by analogy with the predicted transmembrane structure of the wild-type CCR-5, although the correct maturation of the mutant protein up to the plasma membrane has not been demonstrated. Amino acids represented in black correspond to unnatural residues resulting from the frame shift caused by the deletion. The mutant protein lacks the last three transmembrane segments of CCR-5, as well as the regions involved in G-protein-coupling. *b*, Nucleotide sequence of the *CCR-5* gene surrounding the deleted region, and translation into the normal receptor (top) or the truncated mutant (Δ*ccr-5*, bottom). The 10-bp direct repeat is represented in bold italics.

in transformed T-cell lines emerge with time. These virus strains use LESTR (or fusin) as a co-receptor[7], an orphan receptor also belonging to the chemokine receptor family, but not yet characterized functionally[10–12]. Dual-tropic viruses, which may represent transitional forms of the virus in late stages of infection[13], were shown to use both CCR-5 and LESTR as co-receptors, as well as the CC-chemokine receptors CCR-2b and CCR-3 (ref. 4). The broad spectrum of co-receptor usage of dual-tropic viruses suggests that, within infected individuals, the virus may evolve at least in part from selection by a variety of co-receptors expressed on different cell types.

Some individuals remain uninfected despite repeated exposure to HIV-1 (refs 9, 14, 15). In some of these exposed-uninfected individuals, the reason is the relatively low risk of contamination after a single contact with the virus, but it has been postulated that truly resistant individuals do exist. Indeed, $CD4^+$ lymphocytes isolated from exposed-uninfected individuals are highly resistant to infection by primary M-tropic, but not T-tropic, HIV-1 strains. Also, peripheral blood mononuclear cells (PBMCs) from different donors are not infected equally with various HIV-1 strains[16–18]. Given the importance of CCR-5 in the fusion event that mediates infection by M-tropic viruses, we postulated that variants of CCR-5 could be responsible for the relative or absolute resistance to HIV-1 infection exhibited by some individuals, and possibly also for the variability of disease progression in infected patients[19]. To test this hypothesis, we selected three HIV-1 infected patients in which disease progression is slow, and four seronegative individuals as controls; the full coding region of their CCR-5 gene was amplified by the polymerase chain reaction (PCR) and sequenced. To our surprise, one of the slow progressors, but also two of the uninfected controls, exhibited heterozygosity at the CCR-5 locus for a biallelic polymorphism. The frequent allele corresponded to the published CCR-5 sequence[1], and the minor one displayed a 32-bp deletion within the coding sequence, in a region corresponding to the second extracellular loop of the receptor (Fig. 1). Cloning of the PCR product and sequencing of several clones confirmed the deletion. The deletion causes a frame shift, which is expected to result in premature termination of translation. The protein encoded by this mutant allele (Δ*ccr-5*) therefore lacks the last three transmembrane segments of the receptor. A 10-bp direct repeat flanking the deleted region (Fig. 1*b*) on both sides is expected to have promoted the recombination event leading to the deletion. Numerous mutagenesis studies performed on various classes of G-protein-coupled receptors, including chemokine receptors, show that such a truncated protein is not functional in terms of chemokine-induced signal transduction: it lacks the third intracellular loop and carboxy-terminal cytoplasmic domains, the two regions involved primarily in G-protein coupling[20]. To test whether the truncated protein was able to function as an HIV-1 co-receptor, we tested its ability to support membrane fusion by both primary M-tropic and dual-tropic virus env proteins. The recombinant protein was expressed in quail QT6 cells together with human CD4. The QT6 cells were then mixed with HeLa cells expressing the indicated viral env protein, and the extent of cell–cell fusion was measured using a sensitive and quantitative gene-reporter assay. In contrast to wild-type CCR-5, the truncated receptor did not allow fusion with cells expressing the env protein from either M-tropic or dual-tropic viruses (Fig. 2*a*). Coexpression of Δ*ccr-5* with wild-type CCR-5 reduced the efficiency of fusion for both JR-FL and 89.6 envelopes in three independent experiments. In comparison, expression of the Duffy chemokine receptor, which does not function as an HIV co-receptor, with wild-type CCR-5 had no effect on fusion efficiency. Whether this *in vitro* inhibitory effect also occurs *in vivo* is not yet known. We also found that Δ*ccr-5* failed to support infection by either JR-FL or 89.6 (Fig. 2*b*). Thus Δ*ccr-5* fails to support either membrane fusion or virus infection.

Based on the 14 chromosomes tested in the first experiment, the

TABLE 1 Genotype and allele frequencies of CCR-5 and Δ*ccr-5* in cohorts of Caucasians

	Seronegative			Seropositive			
	Number	Frequency	s.e.	Number	Frequency	s.e.	χ^2
Genotypes							
CCR-5/CCR-5	582	0.827	0.014	645	0.892	0.012	1 degree of freedom
CCR-5/Δ*ccr-5*	114	0.162	0.014	78	0.108	0.012	12.7
Δ*ccr-5*/Δ*ccr-5*	8	0.011	0.004	0	0.000	<0.001	$P < 0.0005$
Total	704	1.000		723	1.000		
Alleles							1 degree of freedom
CCR-5	1278	0.908	0.008	1368	0.946	0.006	15.1
Δ*ccr-5*	130	0.092	0.008	78	0.054	0.006	$P < 0.0005$
Total	1408	1.000		1446	1.000		

Genotype and allele frequencies for *CCR-5* and Δ*ccr-5* in seronegative and seropositive cohorts of Caucasians. Standard errors of the genotype and allelic frequency estimates were calculated as $\sqrt{P(1-P)/n}$. The standard error for the Δ*ccr-5*/Δ*ccr-5* genotype in the seropositive cohort (no homozygotes found) was assumed to be smaller than if a single individual with this genotype was observed. Genotype and allelic frequencies in seronegative and seropositive samples were compared by 2×2 contingency tables yielding χ^2 with 1 degree of freedom. *CCR5*/Δ*ccr5* heterozygous and Δ*ccr5*/Δ*ccr5* homozygous classes were poled when comparing genotype frequencies to account for the expected values <5 in the homozygous classes. For the origin of samples, see text.

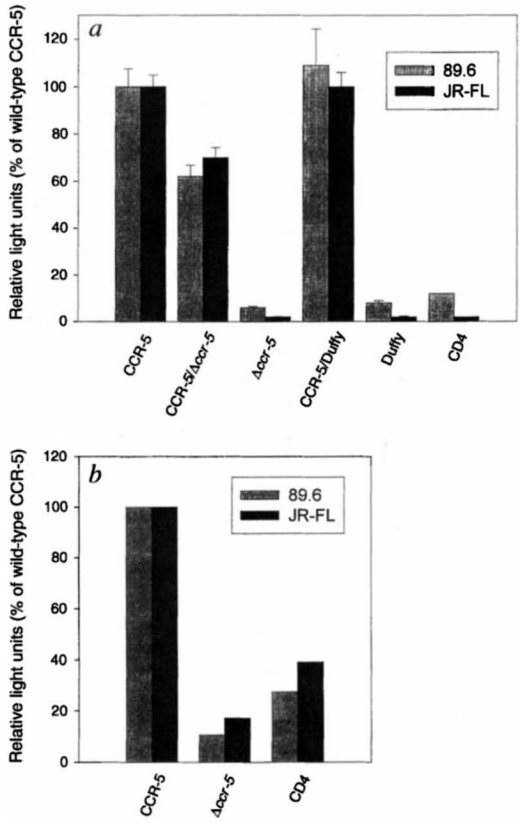

FIG. 2 *a*, Quantification of env protein-mediated fusion by Luciferase assay. To quantify cell-cell fusion events, a modified version of the gene reporter fusion assay[23] was used. Japanese quail QT6 fibrosarcoma cells were transfected or cotransfected as indicated with the pcDNA3 vector (Invitrogen) containing the coding sequence for wild-type CCR-5, the truncated *Δccr-5* mutant or the Duffy chemokine receptors, or with the pCDNA3 vector alone. The target cells were also transfected with human CD4 expressed form the CMV promoter and the luciferase gene under the control fo the T7 promoter. HeLa effector cells were infected (multiplicity of infection = 10) with vaccinia vectors expressing T7-polymerase (vTF1.1) and either the JR-FL (vCB28) or 89.6 (vBD3) envelope proteins. The Luciferase activity resulting from cell fusion is expressed as the percentage of the activity (in relative light units) obtained for wild-type CCR-5. Error bars indicate s.d. within a single experiment (each point was performed in triplicate). Similar results were obtained in two additional experiments. *b*, Quail QT6 cells were transfected with plasmids encoding human CD4, wild-type CCR-5 or *Δccr-5*, and LTR-Luciferase[24]. Two days later, cells were infected with equivalent amounts of JR-FL and 89.6 (as judged by viral p24), and three days after infectin the cells were lysed and Luciferase activity assayed as above. Similar results have been obtained in three independent experiments.

deleted *Δccr-5* allele was rather frequent in the caucasian population. A more accurate frequency was estimated by testing (Fig. 3) a large cohort of caucasian individuals, including unrelated members of the CEPH (Centre d'Etude des Polymorphismes Humains) families, some members of the IRIBHN staff, and a bank of anonymous DNA samples from healthy individuals collected by the Genetics Department of the Erasme Hospital in Brussels. From a total of more than 700 healthy individuals, the allele frequencies were found to be 0.908 for the wild-type allele, and 0.092 for the mutant allele (Table 1). The genotype frequencies observed in the population were not significantly different from the expected Hardy–Weinberg distribution (*CCR-5/CCR-5*, 0.827 versus 0.824; *CCR-5/Δccr-5*, 0.162 versus 0.167; *Δccr-5/Δccr-5*, 0.011 versus 0.008; ($\chi^2_2 = 0.81$; $P < 0.5$), suggesting that the null allele has no drastic effect on fitness. It was confirmed using two CEPH families that the wild-type *CCR-5* gene and its *Δccr-5* variant were allelic, and segregated in a normal mendelian fashion (data not shown). Cohorts of 124 DNA samples originating from Western and Central Africa (collected from Zaire, Burkina Fasso, Cameroun, Senegal and Benin) and 248 samples of Japanes origin did not reveal a single *Δccr-5* mutant allele, suggesting that this allele is either absent or extremely rare in Africa and Japan.

The consequences of the existence of a null allele of *CCR-5* in the normal caucasian population were then considered in terms of susceptibility to infection by HIV-1. If, as predicted, CCR-5 plays a major (not redundant) role in the entry of most primary virus strains into cells, then *Δccr-5/Δccr-5* individuals should be particularly resistant to HIV-1 challenge, both *in vitro* and *in vivo*. The frequency of the *Δccr-5/Δccr-5* genotype should therefore be significantly lower in HIV-1 infected patients, and increased in exposed-uninfected individuals. Also, if heterozygotes have a statistical advantage owing to either the lower number of functional receptors on their white blood cells or to possible dominant-negative properties of the mutant allele, the frequency of heterozygotes (and mutant alleles) should be decreased in HIV-infected populations. These hypotheses were tested by genotyping a large number of seropositive caucasian individuals ($n = 723$) belonging to cohorts originating from various hospitals of major Belgian cities and Paris (Table 1). Within this large series, the frequency of the null *Δccr-5* allele was significantly reduced from 0.092 to 0.054 ($\chi^2_1 = 15.1$; $P < 0.0005$; see Table 1). This reduction in the *Δccr5* allele frequency in the seropositive cohort was due both to a reduction in the frequency of *CCR5/Δccr5* heterozygotes (0.108 versus 0.162) and *Δccr5/Δccr5* homozygotes (0 versus 0.01) ($\chi^2_1 = 12.7$; $P < 0.0005$; Table 1). Great care was taken to include in both the HIV+ and HIV− caucasian cohorts only individuals with a similar geographic origin and with European patronymes. This makes it extremely unlikely that the observed differences in genotype frequencies could result form a different genetic background rather than a true difference in susceptibility to infection by HIV.

TABLE 2 Infection of PBMC from *Δccr5/Δccr5*, *CCR5/Δccr5* and *CCR5/CCR5* individuals with HIV-1 strains

	BAL	JR-FL	SF162	89.6	IIIB
Δccr5/Δccr5	0.112	0	0	>22.3	>22.3
	0.104	0	0	>22.3	>22.3
CCR5/CCR5, *CR5/Δccr5*	>22.3	>22.3	>22.3	>22.3	>22.3
	>22.3	>22.3	>22.3	>22.3	>22.3

M-tropic (BAL, JR-FL, SF162), dual-tropic (89.6) and T-tropic (IIIB) HIV-1 strains were used to infect PBMCs prepared from 1 *Δccr5/Δccr5*, 3 CR5/*Δccr5* and 2 CCR5/CCR5 individuals. p24 antigen (ng ml^{-1}) was assayed 7 days after infection. Most of the measurements were off-scale for the investigated range (>22.3 ng ml^{-1}). The values represent duplicate infections for each condition. All samples derived from *CR5/Δccr5* and *CCR5/CCR5* individuals gave similar off-scale results with all strains, and are therefore represented together.

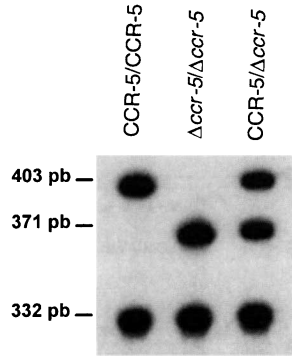

FIG. 3 Genotyping of individuals by PCR. Autoradiography illustrating the pattern resulting from PCR amplification and *Eco*RI cleavage for individuals homozygous for the wild-type *CCR-5* allele (*CCR-5/CCR-5*), the null *Δccr-5* allele (*Δccr-5/Δccr-5*), and for heterozygotes (*CCR-5/Δccr-5*). A 735-bp PCR product is cleaved into a common band of 332 bp for both alleles, and into 403 and 371 bp bands for the wild-type and mutant alleles, respectively.

Taken together, the functional and statistical data suggest that CCR-5 is indeed the major co-receptor responsible for natural infection by M-tropic HIV-1 strains. However, the severely truncated *Δccr-5* protein cannot function as an HIV-1 co-receptor (Fig. 2*a*, *b*, Table 2). This finding, coupled with the epidemiological data (Table 1), strongly suggests that individuals homozygous for the null *Δccr-5* allele (about 1% of the caucasian population) will be highly resistant to HIV-1 infection. It is unclear at this point whether resistance to HIV-1 is absolute or relative, and whether resistance will vary depending on the mode of viral transmission. Larger cohorts of seropositive individuals will have to be tested to clarify this point. Assuming that the genotypic frequencies in both cohorts are true estimates (Table 1), the relative risk of *CCR-5/Δccr-5* and *Δccr-5/Δccr-5* versus *CCR-5/CCR-5* individuals is 0.625 and 0, respectively. Both a decrease in functional CCR-5 number, and a dominant-negative effect of *Δccr-5 in vivo* comparable to that suggested from our *in vitro* experiments (Fig. 2*a*), are possible explanations for this relative protection of heterozygotes. The mutant allele, which can be regarded as a natural knock-out in humans, is not accompanied by an obvious phenotype in homozygous individuals. This may be due to the documented redundancy of chemokines and their receptors[21]. Future studies will reveal whether subtle correlations can be found with disease states, especially inflammatory, immune or infectious diseases, given the predominant role of chemokine receptors in the recruitment of white blood cells towards the sites of inflammation[21,22]. Nevertheless, the lack of overt phenotype of homozygotes, together with the relative protection that characterizes heterozygous subjects, suggests that pharmacological agents that selectively block the ability of HIV-1 to use CCR-5 as a cofactor could be effective in preventing HIV-1 infection, and would be predicted not to be associated with major side effects resulting from CCR-5 inactivation. Whether these agents will be effective in delaying the transition of seropositive patients towards AIDS is more questionable, considering the wide range of chemokine receptors used as co-receptors by some HIV-1 primary isolates[4].

The relatively high frequency of the null allele in the caucasian population could be the result of genetic drift or some undefined selective advantage it may have conferred to its carriers in the distant past. It will be important to search for additional polymorphisms in CCR-5 and other entry cofactors among diverse populations, as this may help explain different clinical outcomes, such as the exposed-uninfected phenotype, and long-term survivors □

Methods

CCR-5 gene amplification and sequencing. The full-size coding region of the CCR-5 gene was amplified by PCR, using 5′-TCGAGGATCCAAGATGGATTATCAAGT-3′ and 5′-CTGATCTAGAGCCATGTGCACAACTCT-3′ as forward and reverse primers, respectively. The PCR products were sequenced on both strands using the same oligonucleotides as primers, as well as internal primers, and fluorochrome-labelled dideoxynucleotides as terminators. The sequencing products were run on an Applied Biosystem sequencer, and ambiguous positions were searched along the coding sequence. When the presence of a deletion was suspected from direct sequencing, the PCR products were cloned after restriction with *Bam*HI and *Xba*I endonucleases into pcDNA3. Several clones were sequenced to confirm the deletion. The deletion was identical in three unrelated individuals investigated by sequencing.

Fusion and infection assays. Details concerning plasmid and viral vectors have been previously described[4]. All transfections were performed with an identical quantity of plasmid DNA using pcDNA3 as carrier when necessary. To initiate fusion, target and effector cells were mixed in 24-well plates at 37 °C in the presence of ara-C and rifampicin, and allowed to fuse for 8 h. Cells were lysed in 150 μl of reporter lysis buffer (Promega) and assayed for Luciferase activity according to the manufacturer's instructions (Promega). The LTR-Luciferase infection assay (Fig. 2*b*) was performed as described[24].

Genotyping of individuals by PCR. PCRs were performed on genomic DNA samples, using 5′-CCTGGCTGTCGTCCATGCTG-3′ and 5′-CTGATCTAGAGCCATGTGCACAACTCT-3′ as forward and reverse primers, respectively. Reaction mixtures consisted of 30 μl 10 mM Tris-HCl buffer, pH 8.0, containing 50 mM KCl, 0.75 mM $MgCl_2$, 0.2 mM dCTP, dGTP and dTTP, 0.1 mM dATP, 0.5 μCi [α-^{32}P]-dATP, 0.01% gelatin, 5% DMSO, 200 ng target DNA, 60 ng of each of the primers and 1.5 U *Taq* polymerase. PCR conditions were: 93 °C for 2 min 30 s, 93 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min; 30 cycles; 72 °C for 6 min. After the PCR reaction, samples were incubated for 60 min at 37 °C with 10 U *Eco*RI, and 2 μl of the denatured reaction mixture was applied onto a denaturing 5% polyacrylamide gel containing 35% formamide and 5.6 M urea. Bands were detected by autoradiography.

Statistical analysis. Assume that *p1*, *p2*, *p3* and *p′1*, *p′2*, *p′3* are the genotype frequencies of *CCR5/CCR5*, *CCR5/Δccr5*, *Δccr5/Δccr5* individuals in HIV− and HIV+ cohorts, respectively; that *x1*, *x2*, *x3* are the probabilities to become HIV+ conditional on *CCR5/CCR5*, *CCR5/Δccr5* and *Δccr5/Δccr5* genotypes, respectively. The relative risk for *CCR5/Δccr5* and *Δccr5/Δccr5* to contract the disease compared with *CCR5/CCR5* individuals was computed as *x2/x1* and *x3/x1*, respectively from $p'1 = (p1*x1)/(p1*x1 + p2*x2 + p3*x3)$; $p'2 = (p2*x2)/(p1*x1 + p2*x2 + p3*x3)$; $p'3 = (p3*x3)/(p1*x1 + p2*x2 + p3*x3)$.

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