Genotyping of the CCR5 Chemokine Receptor by Isothermal NASBA Amplification and Differential Probe Hybridization

JOSEPH W. ROMANO,1 SURYA TETALI,2 EUN MI LEE,1 ROXANNE N. SHURTLIFF,1 XUE PING WANG,2 SAVITA PAHWA,3 MARK H. KAPLAN,2 AND CHRISTINE C. GINOCCHIO2*

Advanced BioScience Laboratories, Inc., Kensington, Maryland 20895, 1 and Department of Infectious Disease and Department of Pediatrics and Immunology, 3 North Shore University Hospital, Manhasset, New York 11030

Received 9 April 1999/Returned for modification 24 June 1999/Accepted 7 September 1999

The human CCR5 chemokine receptor functions as a coreceptor with CD4 for infection by macrophage-tropic isolates of human immunodeficiency virus type 1 (HIV-1). A mutated CCR5 allele which encodes a protein that does not function as a coreceptor for HIV-1 has been identified. Thus, expression of the wild-type and/or mutation allele is relevant to determining the infectibility of patient peripheral blood mononuclear cells (PBMC) and affects disease progression in vivo. We developed a qualitative CCR5 genotyping assay using NASBA, an isothermal nucleic acid amplification technology. The method involves three enzymes and two oligonucleotides and targets the CCR5 mRNA, which is expressed in PBMC at a copy number higher than 2, the number of copies of DNA present encoding the gene. The single oligonucleotide set amplifies both alleles, and genotyping is achieved by separate hybridizations of wild-type- and mutation-specific probes directed to the single-stranded RNA amplification product. Assay sensitivity and specificity were demonstrated with RNAs produced in vitro from plasmid clones bearing the DNA encoding each allele. No detectable cross-reactivity between wild-type and mutation probes was found, and 50 copies of each allele were readily detectable. Analysis of patient samples found that 20% were heterozygous and 1% were homozygous for the CCR5 mutation. Thus, NASBA is a sensitive and specific means of rapidly determining CCR5 genotype and provides several technical advantages over alternative assay systems.

The CC-CKR5 (or CCR5) cell surface molecule serves as the natural receptor for the CC (or β)-chemokines (reviewed in reference 16). The CCR5 molecule has also been shown to serve as a coreceptor, along with the CD4 molecule, for the entry of macrophage-tropic, non-syncytium-inducing (i.e., primary) strains of human immunodeficiency virus type 1 (HIV-1) during infection (1, 7). A series of studies have demonstrated that the CCR5 genotype is important in determining host susceptibility to HIV-1 infection, as well as in determining disease progression (9, 15). For example, it was demonstrated that cells from two HIV-1-negative individuals with multiple exposures to HIV-1+ patients were resistant to infection by primary macrophage-tropic virus in vitro (12). Sequence analysis determined that both of these individuals encoded the same homozygous deletion mutation of 32 bp in the CCR5 gene. This deletion mutation in both alleles of the gene results in a truncated form of the receptor that is not detected on the cell surface. A more extensive survey of patients has revealed that approximately 3% of 612 HIV-1-exposed but antibody-negative individuals were homozygous for this deletion mutation (6). In the same study, it was shown that the deletion allele occurred at a frequency of about 0.1% in the U.S. Caucasian population. There were no homozygous deletion mutations detected among more than 1,300 HIV-1+ patients, and the frequency of heterozygosity for the mutation was elevated among patients surviving for more than 10 years. Interestingly, the deletion mutation appears to be of low prevalence among people of Asian or African ancestry (9). The relevance of the CCR5 receptor in HIV-1 infection is further established by the observation that the β-chemokines RANTES, MIP-1α, and MIP-1β (which naturally bind the CCR5 receptor) will block infection of macrophage-tropic isolates of HIV-1 in vitro (5).

The studies which addressed the CCR5 genotype and its role in HIV-1 pathogenesis involved the use of several molecular methods for the genotyping process. In this report, we describe the use of the isothermal nucleic acid sequence-based amplification (NASBA) method for CCR5 genotyping analysis. NASBA (10) is an isothermal process that is highly appropriate for the amplification of RNA. Amplification is achieved through the coordinated activities of three enzymes (avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase) and two DNA oligonucleotides that are specific for the target sequence of interest. Amplification in NASBA is transcription based and results in the production of large amounts of single-stranded RNA that is antisense to the original target sequence. The single-stranded RNA product can then be readily detected through the hybridization of an appropriately labeled oligonucleotide DNA probe. With CCR5, a single NASBA system which amplifies both the wild-type and the mutated alleles was designed. Discrimination between the two amplification products was achieved at the detection step by means of independent hybridizations with wild-type- and mutation-specific probes. It is the abundance of the CCR5 transcript in patient peripheral blood mononuclear cells (PBMC) and the appropriateness of NASBA for RNA amplification which enabled development of this genotyping assay. The isothermal nature of the amplification procedure along with an easily detected single-stranded RNA amplification product further justifies the use of NASBA technology in genotyping analyses.

MATERIALS AND METHODS

Obtaining and processing of biological samples. After obtaining informed consent, whole-blood samples were collected in VACUTAINER EDTA anticoagulant tubes (Becton Dickinson, Franklin Lakes, N.J.) from 89 randomly se-
lected HIV-1-seropositive patients attending the North Shore University Hospital Center for AIDS Research and Treatment (Manhasset, N.Y.). Additional samples were collected from four human T-cell leukemia virus type 1 (HTLV-1)-positive patients and four HIV-1-seronegative persons with a history of high-risk exposure to HIV-1. Whole bloods were centrifuged for 20 min at 10,000 × g in a swinging-bucket rotor (Sorvall R; Beckman Instruments, Fullerton, Calif.) within 4 h of specimen collection. PBMC were obtained from the whole blood either by centrifugation through Ficoll Hypaque or through the use of cell preparation tubes (Becton Dickinson). Resulting PBMC were either viably cryopreserved for use later or processed directly for nucleic acid extractions. Nucleic acids were isolated from approximately 10⁵ cells (NASBA assay only) or 2 × 10⁵ cells (PCR and NASBA assays) by the guanidinium thiocyanate-acetic acid procedure of Boom et al. (3). After isolation, 5 to 10% of the nucleic acid extract was used in the NASBA reactions and PCRs. Alternatively, to obtain total RNA, the method of Chomczynski and Sacchi (4) was used.

**Amplification by NASBA of CCR5 Transcripts**

RNA Amplification. The NASBA technique was achieved by a modified version of the procedure of Romano et al. (18). Briefly, the 20-μl amplification reaction mixture contained 5 μl of the nucleic acid extract material in a solution containing 40 mM Tris (pH 8.5); 5 mM dithiothreitol; 12 mM MgCl₂; 70 mM KCl; 2.0 mM (each) ATP, CTP, and UTP; 1.5 mM GTP; 0.5 mM ITP; 1.0 mM (each) dATP, dCTP, dGTP, and dTTP; 0.1 μg of bovine serum albumin per μl; 0.1 U of RNase H; 40 U of T7 RNA polymerase; 8 μl of avian myeloblastosis virus reverse transcriptase; 15 units dimethyl sulfoxide; and 0.2 μM (each) oligonucleotides (P1 and P2). Two sets of oligonucleotides, specific for CCR5 amplification, were synthesized. Design of these oligonucleotides was based on the reported sequence for the CCR5 gene (GenBank accession no. U57840), and the oligonucleotides were as follows: P1A, 5′-AACTCTACCAGCTTACATAGGGAGATAGCCAGGAAGCAGCGGCAGGACCAGCC AA CGAATACGATACGAGCTGTGCTACAGGAGAC15′; P1B, 5′-AATTCTATACGACTTACATAGGGAGATAGCCAGGAAGCAGCGGCAGGACCAGCC AA CGAATACGATACGAGCTGTGCTACAGGAGAC15′; P2A, 5′-GGCTGTGTTTGCGTCTCTCCCAATGAGATGACCATGA3′; P2B, 5′-TTTGGGGTGAGAC15′-labeled probe specific for the mutated allele (5′-TATTTCCTGCTC3′) corre-

**Hybridization Analysis of CCR5 NASBA Products.** The portion of the CCR5 gene targeted by the specified oligonucleotides spans positions 430 through 650 (the A of the ATG methionine is base 1). The probe for the wild-type amplification product (5′-AGTCAATCCTTGGAGAAGTCCCAAGTTAGATGACCATGA3′, complementary to the reverse strand) was designed in the electrochemical detection (ECL) or NASBA approach was conducted on biotinylated DNA PCR products (100 μm tris-HCl [pH 8.3], 500 μM MgCl₂, 0.1 μM (each) dNTPs, 0.2 U of T7 RNA polymerase, 40 U of RNase H, and 40 U of T7 DNA polymerase in a 20-μl reaction mixture). Amplification products were analyzed by gel electrophoresis on a 2% agarose gel (nucleic acid marker, 0.5 to 100 kilobase units), and the amplified products were visualized by ethidium bromide staining. The resulting NASBA products were analyzed by gel electrophoresis before and after hybridization analysis.

**RESULTS**

**Basic assay performance.** A schematic representation of the CCR5 genotyping NASBA assay is provided in Fig. 1. The approach was to conduct a single NASBA of RNA obtained from patient PBMC and subject the resulting amplicons to independent hybridization analyses with probes that are specific for either the wild-type or the mutated allele. Multiple oligonucleotides for CCR5 amplification by NASBA were designed such that there were two antisense P1 sequences downstream from the 32-base deletion mutation site and two sense P2 oligonucleotides (A and B) upstream from the deletion site. Map positions are provided in Materials and Methods. All four combinations were used in initial amplification reaction evaluations. Resulting amplicons were screened by hybridization analysis with either the wild-type probe (WT), which is specific for the sequence that is deleted from the mutated allele, or with the mutation-specific probe (Mut), which straddles the resulting junction region created by the deletion. The capture probe, used in ECL detection of amplification products, is located immediately downstream from P2A (not shown). The P1A-P2B primer combination was used in all subsequent experiments.

**FIG. 1.** Strategy for genotyping at the CCR5 locus by NASBA. The wild-type CCR5 allele contains a 32-base sequence (black bar) which is deleted from the mutated allele. The oligonucleotides used for amplification by NASBA of the CCR5 mRNA were designed as shown with two antisense P1 oligonucleotides (A and B) downstream from the deletion site and two sense P2 oligonucleotides (A and B) upstream from the deletion site. Map positions are provided in Materials and Methods. All four combinations were used in initial amplification reaction evaluations. Resulting amplicons were screened by hybridization analysis with either the wild-type probe (WT), which is specific for the sequence that is deleted from the mutated allele, or with the mutation-specific probe (Mut), which straddles the resulting junction region created by the deletion. The capture probe, used in ECL detection of amplification products, is located immediately downstream from P2A (not shown). The P1A-P2B primer combination was used in all subsequent experiments.
on the alternative RNAs. Mut-specific probes. Results show that the assay is capable of at least 50-copy sensitivity (WT and Mut) and that there is no cross-reactivity between the specific probes with the ECL system described in Materials and Methods. Each input RNA was amplified in duplicate, and each reaction mixture was tested with both the WT- and Mut-specific probes. Results show that the assay is capable of at least 50-copy sensitivity (WT and Mut) and that there is no cross-reactivity between the specific probes on the alternative RNAs.

Sensitivity of the NASBA assay. Several strategies were employed to evaluate the sensitivity of the NASBA assay for CCR5 mRNA. Initially, it was determined that the assay could detect the CCR5 target mRNA sequence in as little as 50 pg of total RNA obtained from PBMC. In the standard NASBA reaction conducted with PBMC extracts obtained by the method of Boom et al. (3), CCR5 mRNA was readily detected with input material equivalent to 5,000 copies. However, a more informative evaluation of sensitivity requires the use of RNA produced in vitro that corresponds to the CCR5 target sequence. To obtain this RNA, PCR was conducted with genomic DNA that had been extracted from pooled human PBMC with primers that spanned the entire coding region of the CCR5 gene. Since the CCR5 gene does not contain introns, the DNA sequence is contiguous with the transcribed RNA (19). Further, the use of DNA extracted from pooled PBMC enhances the likelihood of both wild-type and mutated forms of the gene being amplified in the PCR. The resulting PCR product was ligated directly into the TA cloning vector pCR2.1, and bacterial transformants were screened with a 32P-labeled capture probe (see Materials and Methods) which hybridizes to both wild-type and mutated clones. Through additional screening steps, independent clones harboring the wild-type and mutated CCR5 alleles were isolated and the identities of these clones were confirmed by means of DNA sequence analysis. The specific wild-type and mutated CCR5 plasmids were then used to produce separate batches of both forms of RNA by means of in vitro transcription.

NASBA reactions were performed with independent titration dilutions of the wild-type and mutated CCR5 in vitro-produced RNA products. Both forms of RNA were amplified with the same oligonucleotide set (i.e., P1A and P2B); however, amplification products were diagnosed separately by hybridization with ruthenium-labeled versions of either the wild-type or the mutation probe. Also included in this hybridization reaction mixture was a capture probe which has sequence common to both alleles and is immobilized on the surface of a magnetic bead. Thus, the hybridization reaction product consists of the NASBA amplicons annealed to the capture probe on the bead surface, with the allele-specific ruthenium-labeled detector probe being bound at an independent position of the amplicons. These hybridization products are then evaluated in an ECL reader that quantifies the amount of light induced during the ECL process (2). The amount of light is a function of the quantity of amplicon that is bound to both the magnetic bead and the detector probe. Thus, results of the hybridization analysis were determined by automated quantitation of the resulting ECL signal with the NASBA QR system.

It was found that the assay exhibited approximately equal sensitivities for both forms of the CCR5 RNA. The assay detected 50 copies of in vitro-produced wild-type and mutated RNAs in duplicate attempts, as presented in Fig. 2. Although the results presented in Fig. 2 indicate that 5-copy sensitivity is possible, the inability to detect this amount of template in both trials of mutated RNA establishes a 50-copy limit of sensitivity for the assay. Further, it was found that there was no cross-reactivity between the wild-type- and mutation-specific probes. Results of these sensitivity and specificity experiments are summarized in Fig. 2. Importantly, the CCR5 sequence is highly homologous to the sequence of an independent chemokine receptor, CCR2 (GenBank accession no. U80924). However, the CCR5 NASBA assay failed to detect $5 \times 10^5$ copies of in vitro-produced RNA corresponding
to the CCR2 transcript (data not shown). However, this CCR2 RNA was readily detected by an NASBA assay specific for CCR2 transcripts.

Analysis of patient PBMC samples. After the initial evaluation of the NASBA-based CCR5 assay, the system was applied to the analysis of patient PBMC samples. Nucleic acids were obtained by the silica-based extraction method of Boom et al. (3), which leads to the isolation of both RNA and DNA. However, the isothermal nature of NASBA renders the amplification method specific for the RNA versions of the targets; there is no opportunity for a double-stranded DNA molecule to denature. Therefore, there is no contribution to the NASBA product from the background DNA obtained from the cells. After amplification as described earlier, reaction products were subjected to both 32P-labeled probe detection and ECL-based hybridization analysis. For the 32P-labeled probe detection system, duplicate 5-μl aliquots of the NASBA reaction product were either resolved through independent agarose gels and then vacuum transferred to nylon membranes or vacuum transferred directly as slot blots. After independent hybridizations with the allele-specific probes, the genotypes of individual patients could be determined by scoring for the presence or absence of bands on the resulting autoradiograms. A homozygous wild-type genotype was indicated by the presence of a band only on the wild-type probe autoradiogram; the homozygous mutation genotype was indicated by the presence of a band only on the mutation probe autoradiogram. Heterozygotes had bands on both autoradiograms. Results from an analysis of eight patients are summarized in Fig. 3. Evaluation of these autoradiograms indicated that patients 2, 3, 4, and 8 were homozygous for the wild-type allele and that patients 1, 5, 6, and 7 were heterozygous at the CCR5 locus.

Patient samples were also analyzed by means of the ECL-based detection system. After the NASBA process was applied, resulting amplicons from each patient were subjected to independent liquid-phase hybridization analyses with the wild-type- and mutation-specific ruthenium-labeled probes. These hybridization products were then analyzed in the NASBA QR System ECL reader. After appropriate subtraction of background signals, results were scored as positive or negative for the wild-type and mutation probes based on the resulting ECL signal. Figure 4 summarizes the results obtained from the same eight patients represented in Fig. 3. Figure 4A depicts the ECL results for the control materials used during the patient analysis. Clearly, the ECL-based assay is specific, with no cross-reactivity between wild-type- and mutation-specific probes. Furthermore, all of the negative control reactions were scored at background ECL levels. In Fig. 4B, results obtained from the analysis of patient PBMC are provided. This ECL-based version of the assay again demonstrated that patients 2, 3, 4, and 8 had the homozygous wild-type CCR5 genotype and that patients 1, 5, 6, and 7 had the heterozygous genotype.

The specificity of the NASBA-ECL assay was further vali-
FIG. 5. CCR5 genotyping by NASBA and DNA PCR. (A) NASBA products generated from the PBMC of eight patients were subjected to ECL-based detection analysis by independent hybridization with wild-type (WT)- and mutation (Mut)-specific probes. Bars WT Con, wild-type control samples produced signals with only the WT probe; bars Mut Con, mutation control samples produced signals with only the Mut probe; bars Neg Con, negative-control samples had undetectable signals with both probes. The PBMC of patients 3, 6, 7, 8, and 10 (bars so numbered) produced positive signals with the WT probe only, indicating a homozygous wild-type genotype. The PBMC of patients 4 and 9 produced signals with both the WT and Mut probes, indicating a heterozygous (wild-type/32-bp-deletion) genotype. The PBMC of patient 5 produced a positive signal with the Mut probe only, indicating a homozygous 32-bp-deletion genotype. (B) CCR5 PCR amplification products generated from PBMC from the same eight patients whose results are shown in panel A were visualized by ethidium bromide staining after electrophoresis on a 2% agarose gel. Three different amplification patterns were observed. Lane 1, DNA size marker; lane 2, negative water PCR control (no bands visualized); lanes 3, 6, 7, 8, and 10, homozygous wild-type amplicons (547-bp band); lanes 4 and 9, heterozygous deletion amplicons (547- and 515-bp bands); lane 5, homozygous deletion amplicons (515-bp band).

dated by comparing the CCR5 genotype results obtained by the NASBA assay with results obtained from the analysis of identical samples by a DNA PCR assay (8). Initially, nucleic acids extracted from $10^6$ PBMC were used for both the NASBA and DNA PCR assays. Although the CCR5 genotype was readily determined by the NASBA assay for all samples, several samples were not amplified sufficiently by PCR to determine the genotype (data not shown). Subsequently, all comparative studies were performed with an input volume of $2 \times 10^5$ PBMC. The NASBA and DNA PCR genotype results of 61 patients tested were 100% concordant. An example of comparison results identifying all three genotype profiles is shown in Fig. 5, with panel A depicting NASBA results and panel B depicting DNA PCR results obtained for eight patients.

A summary of the CCR5 NASBA genotyping analysis of multiple patient PBMC extracts is provided in Table 1. The populations analyzed included 79 HIV-1$^1$ patients, 10 HIV-1$^1$ long-term survivors, 4 HTLV-1$^1$ patients, and 4 HIV-1-sero-

negative patients with a history of high-risk exposure to HIV-1 through intravenous drug use and/or heterosexual contact. In total, 97 patients were analyzed; 79% were homozygous for the wild-type gene, 20% were heterozygous, and 1% were homozygous for the 32-bp deletion.

The CCR5 gene encodes a cell surface chemokine receptor molecule that also functions as a coreceptor for macrophage-tropic isolates of HIV-1. Importantly, a naturally occurring mutated allele for the CCR5 gene which encodes a 32-base deletion has been identified. This mutated allele produces a truncated form of the protein that is not expressed on the cell surface. Consequently, PBMC from patients who are homozygous for the deletion mutation cannot be infected with macrophage-tropic virus and appear to be resistant to infection in vivo despite multiple exposures (12). Heterozygosity at this gene apparently correlates with a slower progression of HIV-1-related disease (6, 8, 9). Consequently, patient genotyping at this locus is important in assessing prognosis.

We have developed a rapid, sensitive, and specific genotyping assay for the human CCR5 gene. The assay utilizes isothermal amplification by NASBA of CCR5 RNA obtained from patient PBMC. Although detection and actual sequence typing of the resulting amplicons was achievable with radiolabeled probes in blot-based hybridization analysis, a technically more favorable ECL-based detection method was developed. This nonspecific, liquid hybridization detection method involves ruthenium-labeled probes that are specific for amplicons generated from the wild-type and mutated alleles of CCR5. Hybridization results are evaluated in this system with an automated ECL reader, allowing for rapid diagnosis of NASBA products. This assay system has several advantages over other amplification-based methods which can be applied in genotyping. First, it is isothermal and does not require specialized equipment for amplification. The nucleic acids are obtained from the clinical material by the extraction method of Boom et al., which can be applied to a wide range of sample types. Second, the product of the NASBA amplification process is single-stranded RNA, which is readily analyzed by probe hybridization without the need for a denaturing step. Genotyping assays directed at DNA typically depend on the presence of only two copies of target material per cell. The NASBA assay targets RNA and therefore exploits the presence of a more abundant target material in cells where the gene is expressed.

**TABLE 1. CCR5 genotyping analysis of patient PBMC by NASBA**

| Patient status | No. of patients with PBMC CCR5 genotype$^a$ |
|---------------|------------------------------------------|
|               | WT/WT | WT/Mut | Mut/Mut | Total |
| HIV-1$^1$     | 65     | 14     | 0       | 79    |
| HIV-1$^1$ LTS | 6      | 4      | 0       | 10    |
| HIV-1$^1$ HRE | 3      | 0      | 1       | 4     |
| HTLV-1$^1$    | 3      | 0      | 1       | 4     |

$^a$ LTS, long-term survivors; HRE, patients subjected to high-risk exposure.

$^b$ WT, wild-type allele; Mut, mutation allele. Seventy-nine percent of all patients were homozygous for the wild-type allele, 20% were heterozygous, and 1% were homozygous for the 32-bp deletion.
Importantly, when the NASBA system was applied to clinical specimens (i.e., patient PBMC), the frequency of the mutated allele was found to be similar to what had been previously reported (23). The frequency of heterozygotes among individuals of western European heritage is approximately 20%. The NASBA-measured frequency in this study was also 20%. Only one individual (with a history of intravenous drug abuse) who was homozygous for the mutated allele was identified in this study. This person is the spouse of an HIV-1-infected individual and has remained HIV-1 seronegative despite frequent high-risk exposure through the sharing of needles with other HIV-1-infected individuals and through spousal heterosexual contact. Similarly, several other investigators have identified persons with the homozygous 32-bp deletion genotype who are resistant to HIV-1 infection (6, 9, 12, 17, 20–23). The fact that only one individual with the homozygous mutated genotype was found in this study is consistent with the facts that nearly all of the patients tested were HIV-1 positive and that the frequency of homozygosity in the general population is only 1% (12). Interestingly, three persons with a history of multiple exposure to HIV-1 through intravenous drug abuse, but not infected with HIV-1, demonstrated homozygous wild-type CCR5 genotypes. Additional studies are in progress to determine the nature of HIV-1 infection resistance in these persons.

Interestingly, the CCR5 NASBA genotyping assay has been used in parallel with a PCR assay directed against CCR5 DNA, and the results obtained with the two assays were 100% concordant. An advantage of the NASBA assay was the ability to perform CCR5 genotyping on a significantly lower number of PBMC than was required for the DNA PCR assay (1 × 10^5 [NASBA] versus 2 × 10^6 [PCR]). Although it may be possible to reconfigure the DNA PCR assay for greater sensitivity by means of an alternative detection method, the results presented here indicate greater sensitivity by the NASBA assay targeting CCR5 mRNA. Furthermore, NASBA CCR5 genotyping can be successfully performed directly on whole-blood samples, eliminating the time-consuming process of PBMC isolation (21a). Thus, NASBA technology provides a rapid, simple, and specific means for accurate genotype determination at the CCR5 locus. The clinical significance of this marker in the prognosis of HIV-1-infected patients and the ease with which this genotyping assay can be applied suggest that application of the NASBA assay on a wide scale is appropriate. Further, the NASBA assay provides an effective means for conducting population demographic studies. Moreover, NASBA technology can be applied to the detection of other relevant polymorphisms identified in the CCR5 gene (11, 13, 14), perhaps in a multiplex format. In summary, targeting the CCR5 RNA transcript with the NASBA assay system is an effective method for determining the genotype at this locus.

ACKNOWLEDGMENT

This study was funded in part by the Jane and Dayton Brown and Dayton Brown Jr., Molecular Diagnostics Laboratory at North Shore–LIJ Health System Laboratory, Lake Success, N.Y.

REFERENCES

1. Alkhatib, G., C. Combiadire, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger. 1996. CC-CCR5: a RANTES, MIP-1a, MIP-1b receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272: 1955–1958.

2. Blackburn, G., H. Shah, J. Kenney, R. A. Ramin, J. Link, J. Peterman, M. J. Powell, A. Shah, D. B. Talley, S. K. Tyagi, E. Wilkins, T. G. Wu, and R. J. Massey. 1991. Electromembranescence detection for development of immunoassays and DNA probe assays for clinical diagnostics. Clin. Chem. 37:1534–1539.

3. Boom, R., C. J. Sol, M. M. Salisam, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28:495–503.

4. Czeczotkynski, P., and N. Saiki. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.

5. Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Letizia. 1995. Identification of RANTES, MIP-1a, and MIP-1b as the major HIV-suppressive factors produced by CD8+ T cells. Science 270:1811–1815.

6. Dean, M., M. Carrington, C. Winkler, G. A. Huttley, M. W. Smith, R. Allikmets, J. J. Goedert, S. P. Buchbinder, E. Vittinghoff, E. Gomperts, S. Donfeld, D. Vlahov, R. Kaslow, A. Saah, L. Rinaldo, R. Detels, and S. J. O’Brien. 1996. Genetic recombination of HIV-1 integration template for AIDS by a deletion allele of the CCR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science 271:1856–1862.

7. Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cavanaugh, P. J. Maddon, R. A. Kopf, J. P. Moore, and W. A. Paxton. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CCR5. Nature 381:667–673.

8. Eugen-Olsen, J., A. K. Iversen, P. Garred, U. Koppelhus, C. Pedersen, T. L. Benfield, A. M. Sorensen, T. Katzenstein, E. Dickm ess, J. Gersto 6f, P. Skinhoj, A. Sveigaard, J. O. Nielsen, and B. Hofmann. 1997. Heterozygosity for a deletion in the CCR5 gene leads to prolonged AIDS-free survival and slower CD4 T-cell decline in a cohort of HIV-seropositive individuals. AIDS 11:305–310.

9. Huang, Y., W. A. Paxton, S. M. Wolinsky, A. U. Neumann, L. Zhang, T. He, S. Kang, D. Ceradini, Z. Jun, K. Yazdanbakhsh, K. Kunzman, D. Erickson, E. Drago, N. R. Landau, J. Phair, D. D. Ho, and R. A. Kopf. 1996. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat. Med. 2:1240–1243.

10. Kiebits, T., B. van Gemen, D. van Strijp, R. Schukkin, M. Dirckx, H. Adriaanse, L. Malek, R. Sooknanan, and P. Lens. 1991. NASBA iso thermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J. Virol. Methods 35:273–286.

11. Kostrikis, L. G., Y. Huang, J. P. Moore, S. Wolinsky, L. Zhang, Y. Guo, J. Phaie, A. U. Neumann, and D. D. Ho. 1998. A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promoter mutation. Nat. Med. 4:350–353.

12. Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Kopf, and N. R. Landau. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 86:367–377.

13. Martin, M. P., M. Dean, M. W. Smith, C. Winkler, B. Gerrard, N. L. Micheal, B. Lee, R. W. Doms, J. Margolick, S. Buchbinder, J. J. Goedert, T. R. O’Brien, M. W. Hilgartner, D. Vlahov, S. J. Rucker, and M. J. Lomb, J. J. Goedert, T. R. O’Brien, M. W. Hilgartner, D. Vlahov, S. J. Rucker, and M. J. Lomb. 1996. Identification of HIV-1-infected patients and the ease with which this genotyping assay can be applied suggest that application of the NASBA assay on a wide scale is appropriate. Further, the NASBA assay provides an effective means for conducting population demographic studies. Moreover, NASBA technology can be applied to the detection of other relevant polymorphisms identified in the CCR5 gene (11, 13, 14), perhaps in a multiplex format. In summary, targeting the CCR5 RNA transcript with the NASBA assay system is an effective method for determining the genotype at this locus.

ACKNOWLEDGMENT

This study was funded in part by the Jane and Dayton Brown and Dayton Brown Jr., Molecular Diagnostics Laboratory at North Shore–LIJ Health System Laboratory, Lake Success, N.Y.
Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study. Science 277:959–965.

21a. Tetali, S. Unpublished data.

22. Wilkinson, D. A., E. A. Operskalski, M. P. Busch, J. W. Mosley, and R. A. Koup. 1998. A 32-bp deletion within the CCR5 locus protects against transmission of parenterally acquired human immunodeficiency virus but does not affect progression to AIDS defining illness. J. Infect. Dis. 178:1163–1166.

23. Zimmerman, P. A., A. Buckler-White, G. Alkhatib, T. Spalding, J. Kubofcik, C. Combadiere, D. Weissman, O. Cohen, A. Rubbert, G. Lam, M. Vaccarezza, P. E. Kennedy, V. Kumaraswami, J. V. Giorgi, R. Detels, J. Hunter, M. Chopek, E. A. Berger, A. S. Fauci, T. B. Nutman, and P. M. Murphy. 1997. Inherited resistance to HIV-1 conferred by an inactivating mutation in CC chemokine receptor 5: studies in populations with contrasting clinical phenotypes, defined racial background, and quantified risk. Mol. Med. 3:23–36.