

## Simple Determination of Human Immunodeficiency Virus Type 1 Syncytium-Inducing V3 Genotype by PCR

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**Human immunodeficiency virus type 1 (HIV-1) phenotype variability plays an important role in the pathogenesis of AIDS. The presence of syncytium-inducing (SI) HIV-1 isolates in infected individuals is associated with a rapid decline of CD4<sup>+</sup> T cells, rapid disease progression, and reduced survival time after AIDS diagnosis. The strong association between the SI capacity of HIV-1 and the presence of positively charged amino acid residues at positions 306 and/or 320 in the third variable domain (V3) of gp120 could here be confirmed in 97% of 402 primary HIV-1 isolates, indicating that the V3 genotype may be useful for prediction of the viral phenotype. The V3 DNA sequences revealed a remarkably limited codon usage for the amino acid residues that are responsible for virus phenotype. On the basis of this limited SI-specific DNA sequence variation, four SI-specific oligonucleotides were designed for selective amplification of V3 from SI but not non-SI HIV-1 isolates. This PCR analysis allowed the prediction of the biological phenotype of HIV-1 isolates on the basis of the V3 genotype and may prove to be useful for monitoring SI capacity of HIV-1 isolates in infected individuals.**

Human immunodeficiency virus type 1 (HIV-1) phenotype variability plays an important role in the pathogenesis of AIDS. In early asymptomatic HIV-1 infection, slowly replicating macrophage-tropic non-syncytium-inducing (NSI) isolates are predominant (19, 23). In the course of infection, HIV-1 isolates display increased replication rates and reduced macrophage tropism (4, 19, 22). In 50% of infected individuals, SI isolates emerge in the course of infection, coinciding with an accelerated loss of CD4<sup>+</sup> T lymphocytes, rapid disease progression, and reduced survival time following AIDS diagnosis (1, 14). The SI capacity of virus isolates, as monitored in seropositive individuals, is now used as a prognostic marker for the rate of CD4 cell decline and rapid progression to AIDS. The relative risk of disease progression for persons with SI variants is higher than the relative risk for those with, e.g., low CD4 cell numbers or p24 antigenemia and is independent of the latter two markers (12).

Next to the role of HIV-1 biological variability in the natural course of infection, efficacy of zidovudine treatment appeared to be associated with the biological phenotype of HIV-1. In asymptomatic individuals at high risk of progression to AIDS, only transient improvement in CD4 cell numbers and responses to CD3 monoclonal antibodies were observed during zidovudine treatment. However, zidovudine treatment delayed clinical progression significantly in persons who carried only NSI variants at the start and who did not develop SI variants during the study (13). Therefore, it may be important to analyze the HIV-1 phenotypes of participants in antiviral drug trials for proper randomization to improve the prescription of antiviral therapy.

Initially, SI capacity of HIV-1 isolates was determined by scoring syncytium formation upon inoculation of peripheral blood mononuclear cells (PBMC) with virus or cocultivation of

PBMC with virus-infected cells. Alternatively, the SI capacity of HIV-1 was determined by using MT2 cells as an indicator, since MT2 cells selectively support replication of SI variants only (14).

The V3 domain of HIV-1 gp120 has been demonstrated to be a major determinant of the SI capacities of virus isolates (5, 9). Construction of chimeric proviruses from SI and NSI molecular clones of HIV-1 has demonstrated that the V1-V2 and V4 regions may also be involved in SI capacity (7, 10, 11). Comparison of V3 domains from a panel of SI and NSI HIV-1 variants resulted in a clear correlation between viral phenotype and V3 conformation (8). In SI isolates, positively charged amino acid residues were found at position 306 and/or position 320 in V3, whereas in NSI isolates both residues were either negatively charged or uncharged (Fig. 1). In these naturally occurring HIV-1 isolates, the correlation between viral phenotype and V3 genotype seems absolute, independent of the V1-V2 and V4 regions. By replacement of V3 domains with substitutions at positions 306 and 320 in the proviral clone HXB2, De Jong et al. demonstrated that positively charged residues at either position were indeed causally involved in determining SI capacity (5).

We have here analyzed whether the observed correlation between SI capacity and V3 genotype allowed an alternative approach to monitoring of the SI phenotype of HIV-1. We emphasized that phenotype-specific DNA sequence variation responsible for amino acid substitutions at positions 306 and 320 could enable prediction of HIV-1 phenotype by, e.g., discriminative PCR or selective oligonucleotide hybridization.

### MATERIALS AND METHODS

**Viruses.** All virus isolates included in this study have been described previously (11, 20, 22, 23). Biologically cloned HIV-1 isolates were obtained by multiple cocultures of limiting dilutions of patient PBMC with phytohemagglutinin-stimulated PBMC from seronegative individuals as described previously (19). Both cloned and bulk isolates were propagated in phytohemagglutinin stimulated PBMC in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 20 U of recombinant interleukin 2 per ml, 100 U of penicillin per ml,

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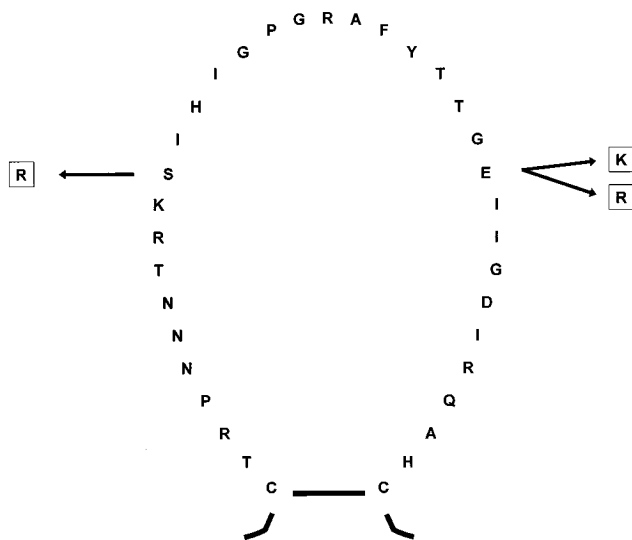


FIG. 1. Schematic presentation of amino acid sequence variation in V3 domains from SI and NSI HIV-1 isolates. Isolates with arginine (R) at position 306 in V3 and/or arginine or lysine (K) at position 320 are SI (8).

and 100  $\mu$ g of streptomycin per ml and tested for SI capacity in either PBMC or MT2 cells as described elsewhere (14).

**DNA isolation.** Total DNA was isolated by lysis of HIV-infected PBMC in lysis buffer L6 (0.08 M guanidium isothiocyanate [GIBCO/BRL], 0.08 M Tris-HCl [pH 6.4], 0.035 M EDTA, 2% [wt/vol] Triton X-100) (2). DNA was precipitated from the lysate by addition of 2 volumes of 2-propanol and 15 min of centrifugation at 13,000  $\times$  g, after which the pellet was washed with 70% ethanol and resuspended in water.

**PCR analysis.** For sequence analysis, gp120 V3 domains were amplified by PCR with primers PS A (5'-TACAATGTACACATGGAATT-3', nucleotide positions 6957 to 6976 relative to the HXB2 sequence, sense orientation) and PS D (5'-ATTACAGTAGAAAATCC-3', positions 7362 to 7381, antisense) in the first reaction and PS B (5'-TGGCAGTCTAGCAGAAGAAG-3', positions 7009 to 7028, sense) and PS C (5'-TCTGGGTCCCTCCTGAGGA-3', positions 7313 to 7332, antisense) in the nested reaction. For both reactions, DNA was denatured for a 5-min period at 95°C followed by 25 cycles of 1.5 min of denaturation at 95°C, 1.5 min of annealing at 50°C, and 1.5 min of extension at 72°C, a subsequent extra 5-min extension at 72°C, and soaking at 4°C. DNA was amplified in 50- $\mu$ l reaction mixtures containing 1 $\times$  *Taq* buffer (Promega), 0.2 mM (each) deoxynucleoside triphosphate (dNTP), 100 ng of each primer, 2.5 mM MgCl<sub>2</sub>, and 1 U of *Taq* DNA polymerase (Promega) overlaid with 50  $\mu$ l of mineral oil in a Perkin-Elmer Cetus thermocycler model 480. Genomic DNA (100 ng) was used as input in the initial PCR, and 5  $\mu$ l of this first reaction mixture was used as input for the nested reaction. Similar PCR conditions were used for selective amplification of SI V3 sequences, except that three primers (each 100 ng) were included in the nested reactions and annealing temperatures were optimized for specificity. Primer PS B was used in combination with primers SI 1 and SI 2 (5'-CTTATATCTCCTATTATTTT-3' and 5'-CTTATATCTCCTATTATTCT-3', respectively, positions 7187 to 7206, antisense) at an annealing temperature of 48°C, and primer PS C was used in combination with SI 3 and SI 4 (5'-ACAACAATACAAGAIAAGG-3' and 5'-ACAACAATACAAGAAI AAGA-3', respectively, positions 7122 to 7141, sense) at an annealing temperature of 54°C. PCR products were analyzed on 3% NuSieve agarose gels (FMC Bioproducts) stained with ethidium bromide.

**Sequence analysis.** Free dNTPs and primers were removed from nested PCR products with GeneClean (Bio 101) according to instructions from the manufacturer. DNA (250 ng) was used for direct sequencing with primers PS B and PS C, with Sequenase DNA polymerase (U.S. Biochemicals) at 50°C as described previously (21).

## RESULTS

**Sequence analysis of gp120 V3 domains from primary HIV-1 isolates.** To test whether the presence of positively charged amino acid residues at positions 306 and 320 in V3 would be applicable to phenotype prediction of HIV-1 isolates, we analyzed V3 sequences from 402 primary HIV-1 isolates obtained from 89 Dutch seropositive individuals. From 26 individuals,

both SI and NSI clones were obtained; from 63 individuals, exclusively NSI clones were obtained. In Fig. 2, amino acid sequences are shown aligned with the type B HIV-1 consensus sequence (16). If multiple clones with identical V3 sequences were obtained from an individual, only one sequence was used in further analysis, resulting in 249 unique V3 sequences from 58 SI and 191 NSI isolates. Within this set of V3 sequences, the correlation between the presence of positively charged amino acid residues at positions 306 and 320 in V3 and the SI capacity of the virus could be confirmed (8). The V3 domains of 55 of 58 SI isolates showed positively charged residues at either position 306 or position 320. The presence of a positively charged amino acid residue at position 320 coincided with a serine-to-glycine substitution at position 306 in all SI isolates. Of 191 NSI isolates, 186 contained negatively charged or uncharged residues at both positions 306 and 320 in V3. For 8 of 249 isolates, the phenotype did not correlate with the charge of amino acid residues at the fixed positions. Five NSI isolates (obtained from two infected individuals, S206 and S212) had a positively charged residue at position 320. Interestingly, the serine-to-glycine substitution at position 306 as seen in all SI isolates with basic residues at position 320 had not occurred in these NSI isolates, suggestive of an important contribution of the glycine residue to the SI phenotype of naturally occurring SI isolates. Three SI isolates (Ams-32.S, Ams-165.S, and Ams 165.27.S) had uncharged residues at both positions 306 and 320. For these isolates, insertion of positively charged amino acid residues between position 306 and the GPCR sequence at the tip of the V3 loop had occurred; this insertion may account for the SI capacity of these isolates. In conclusion, for 241 of 249 HIV-1 isolates, the charges of the amino acid residues at positions 306 and 320 in V3 correlated with the SI capacity ( $P < 0.0001$ , two-sided Fisher's exact test). In retrospect, the use of an assay based on discrimination of these two positively charged amino acid residues in V3 would have allowed accurate detection of SI HIV-1 variants in 85 of 89 seropositive individuals ( $P < 0.0001$ , two-sided Fisher's exact test).

**Analysis of codon usage at positions 306 and 320 in V3 of HIV-1 gp120.** Next, the possible use of PCR for discriminating SI from NSI HIV-1 on the basis of variability at amino acid positions 306 and 320 in V3 was investigated (Table 1). Given the relatively restricted amino acid sequence variation at positions 306 and 320, limited DNA sequence variation could account for differences in SI capacity. Although arginine residues may be coded for by six different codons, the codon use for the arginine residue present at position 306 of V3 in 50% of the SI isolates but absent in all NSI isolates was restricted to either AGG or AGA for all SI isolates. For all NSI isolates, the last nucleotide of the codon at position 306 was either a T or a C (i.e., AGT and AGC encoding serine and GGT encoding glycine). Thus, the arginine residue at position 306 in V3 may be detected by discrimination of A and G from T and C as the last nucleotide in codon 306. Arginine and lysine residues present at position 320 in 44.8% of the SI isolates but absent in 97.4% of the NSI isolates were encoded by codons AAA (lysine) and AGA (arginine) in all SI isolates. For 182 of 191 NSI sequences, either G or C was the first nucleotide in codon 320 (i.e., GAA and GAG encoding glutamic acid, GAT and GAC encoding aspartic acid, and CAA encoding glutamine). Thus, the arginine and lysine residues at position 320 in V3 may be detected by discrimination of A from G, C, and T as the first nucleotide in codon 320 (Table 1).

**Selective amplification of SI V3 sequences by PCR.** We took advantage of this limited phenotype-specific DNA sequence variation at positions 306 and 320 to design oligonucleotides for selective amplification of SI V3 sequences by PCR. Two

	306	320
CONSENSUS	CTFRPNNTKSI.HI.GPGRAFYTTGRIIGDIRQAHC	
H72-1.N		
M102.N		
SCE2.N		
SCW44.N		
H1140G8.N	H	
H1186.N		D
H138G10.N	G	
H139A8.N		A
H139H9.N		P
H157.N		N
H169-13.N	G	
H172PBL.N		A
H373.N		N
H455B4.N	H	
H455.N		N
H500B2.N	P	
H500.N		N
H505.N		N
H571-H10.N	T	
H638.N	G	
IIBAL.N		L
M102.N		
M102.N		V
M13750.N	G	
M137.N	M	
S101.N		V
S105.N		Q
S163.N	G	
S182.N		N
SCE5.N		S
SCE7.N		I
SCE8.N	I	
H1171.N	I	
H1186A6.N	P	D
H1186C2.N		S D
H1186C5.N		K D
H1186E3.N		N G
H1186H4.N		L D
H1234A9.N		L D
H138C10.N		G D
H138D5.N		D D
H138F12.N		G A
H139A6.N	I	N
H139F4.N		Y A
H157.N		T A N
H1682.N		A D
H169-16.N		G D
H172BAL.N		AP
H39-14.N		S N
H39-2.N		S I
H437.N		G D
H455A2.N	H	N
H500B3.N		P A
H500B4.N		P D
H500.N	I	P
H571.N		T D
H617-18.N		A D
H617-4.N		N F
H702.N		A A
H704-6.N		N W
H704-8.N		N W
H821.N		G Q
K114.N		D D
K13396.N		K D
M10218.N		V Y
M10229.N		D V
M13746.N		QM
M13751.N		NM
M13757.N		M K
S105BAL2.N		S Q
S150SP.S		M K
S1693.N		G A
S176BM.N		N A
S178.N		S D
S179.N		G T
S180.N		S V
SCE1.N		A D
H1186A1.N		L L D
H1186A5.N		ST D
H1186B2.N		Q P
H1186D4.N		P L
H138C1.N		G D R
H138F8.N		G A D
H139H12.N		Y A I
H157-17.N		R T N
H168.S		R Q N
H2321.N		S A D
H39.N		S A D
H53.N		P A D
H569.N		A A D
H6311.N		N A A
H704-4.N		M W V N
K114112.N		S W D
K114.N		S W D N
K114.N		P W D
K12769.N		S W D
K13388.N		K A D
K13394.N		K D
M110.N		S G T Y
M114.N		P W D
M1381.N		RG D
M13747.N		G M K
M13749.N		OM K
S151PBL.N		P I D
S168.N		N A D
S16.S		G V R
S199F11.N		G A D
S200.N		G A D
S205PBL.N		P A D
S24.N		PM A
S44.N		L K D
SCE10.N		S L A A
SCE9.N		S L A V
H1186A3.N		L L I D
H138H5.N		G A D
H139B9.N		G N A
H1681.N		G M A D
H169-12.N		P LA A
H1826.N		N A A R
H199BAL.N		G AA A
H199PBL.N		G AA A
H3202A21.N		G K A Q
H354.N		N WA D
H479.S		QG RR
H571-143.N		R T A T

	306	320
CONSENSUS	CTFRPNNTKSI.HI.GPGRAFYTTGRIIGDIRQAHC	
K11491.N		SK W D
K11493.N		N W D N
K114.N		P W D N
K13392.N		RG A D
M133.N		RG N D
M13768.N		G QM K
M13771.N		QM K E
S206.N		R P A Q
S150PBL.S		G I SK
S183.N		GL A D
S199C11.N		G S A D
S199D9.N		G PA D
S199.N		G K A D
SCB4.N		S S A V
H1186.N		QL K L D
H138H6.N		G G A D I
H159.N		NM I Q V
H186.N		I G P A D
H224.N		G L WF K
H3202A3.N		G M K A Q
H420.S		G AARK
H449.N		SM K A N
H4791.N		G N A Q N
H4798.S		QG Y RR
H6002A11.NV		S A D N
H6002H9.NV		P A D N
H6002.NV		Y A D N
H6042C6.NV		P A D N
H617-17.N		R N FA D
H7041.S		RV TM L
M11477.N		S V P W D
S206.N		KR S A K
S119MDM.N		R A A K
S119.N		A S A A K
S201PBL.S		G AA RV K
S32.S		S R HR N
S96MDM.N		H RG S T
SCB3.N		I S A DV
H138E2.S		D L R R
H224-40.N		G L WFA K
H373385.S		I KRMI N
H39-19.S		R SL S V R
H39.S		R SL S V R
H491A9.N		G R P K F
H6002D9.NV		Y AI D N
H6042.NV		Y A D KR
M114.N		S R V W D N
S212-120.N		Q ANSR N
S206.N		IKR S A K
S206.N		R P G A ER
S206.N		IKR S Q
S148PBL.N		G L GT A D
S164-40.N		G D V N Y
S175.S		RG Y V K R
S200G8.S		G R V A BK
S961.N		R FG D N
S962.N		RG G D N
H138.S		RD L R RK
H39-60.S		V R SL S V K
H39-9.S		F R SL S V R
H39.S		I R SL S V K
H486C10.S		G KR Y H DR
H486E11.N		G R P K F Q
H486F11.N		G KR P K F
H486F2.N		G R P KV F
H704.S		RV TM VM N
H72-25.S		RR R R I Q N
H72.S		Y RR S R M Q
H72.S		RR S R I Q N
K127.N		I TF Q A SN
S212.N		Q ANSP N Y
S213-101.NI		Q ANSP
S213-102.NI		Q AHSP
S206.N		IKR SM A Q
S206.N		IKR S A Q V
S127.S		RV TM VL K
S165-28.N		LS RGV TV
S165.N		LS RGV AV
S181.N		PM K A D K Y
SCA-8.N		V R QT A D N
H138E2.S		RD L R R K I
H224-42.S		Y G L WL RK K
H486A3.S		G KR Y H DR R
H486C5.S		G KR Y Q DR R
H486H3.S		G KR Y H DR K
H486.N		G KR P KV F
H704-7.S		RV TM VM V N
H704.S		RV TM VM N K
H72-28.S		I RR S R I Q N
H72-2.S		K RR I R I Q N
K127.N		I TF Q A SN Y
S206.N		IKR SM A Q N E
S127-17.S		RV TM VM N K
S164.N		K Q FG D N Y
S165-37.N		LS RGV AV G
S1691.S		G RIGHT V K
S1692.S		G RIGHT V K
S199A9.S		Y G R V A EK
S199D7.S		G R S VIA EK
S200F3.S		Y G R VIA EK
S200G9.S		Y G R V A EK
S37.S		RV TL VM K Y
SCA-4A11.N		G V R QT A A N
H138E3.S		RD L R R K V
H72-15.S		Y RRL S R I Q N
H72-8.S		RRV S R I Q NT
K12761.N		I TF Q A SN N Y
M127.N		I TF Q A SN K Y
M127.N		I TF Q A SN N Y
S164-23.S		TR KRR P G A ER E
S42.S		IKRR I HA G
SCA-4F9.N		G V R QT A A T N
H39-65.S		I R SL S VY R K Y
H39-67.S		V R SL S VY R K Y
M12737.N		I K TF Q A SN N Y
H18269.S		LSA KIRRM A K K
IIB.S		R R QR V IGK NM
S55.S		K G AV DI ADK LK
S164-18.S		R KIRR P G D E P Y
S164-26.S		R RIRR P G D E T Y
S164-28.S		R KIRR P G D E P Y
S164-23.S		TR KRR P G D T P Y
S165.S		LS RGVHIKHI V AV R
S165-27.S		LG K RGVHIKHI V AV R

FIG. 2. Alignment of 249 V3 loop sequences from 89 individuals with the B-type consensus sequence. Dashes represent similarity to the consensus, and points represent gaps introduced in the sequences for optimal alignment. The first character in each sequence name points to the source of the isolates: H indicates an isolate derived from a participant in the Amsterdam cohort studies on the natural course of HIV-1 infection in homosexual men, S indicates an Amsterdam isolate derived from an individual not participating in this cohort, SC indicates an isolate obtained during a primary infection, and M and K indicate isolates obtained from cases of vertical transmission (mothers and children, respectively). S212 and S213 are a donor-recipient pair, and S206 is a long-term-asymptomatic individual. The last character defines the SI capacity of the virus isolate (S for an SI isolate and N for an NSI isolate).

oligonucleotide primers spanning position 306 in V3 that had either a G or an A at the 3' position were synthesized (SI 3 and SI 4). Since all NSI isolates had either T or C at this position and mismatches at the extreme 3' end of PCR primers obstruct PCR amplification, use of these primers would selectively support amplification of SI V3 sequences by PCR. Similarly, two oligonucleotide primers spanning position 320 in V3 (SI 1 and SI 2) were designed. Oligonucleotides for selective amplification of SI isolates that had an arginine or lysine residue at position 320 should anneal to either AA or GA at the 3' position. Next to the SI-specific substitutions at the 3' ends, all four 20-mer oligonucleotides were homologous to the type B consensus V3 DNA sequence with the exception of an inosine residue in primers SI 3 and SI 4, to ensure amplification of V3 from all SI isolates (18). The four SI-specific primers were used in combination with the previously described oligonucleotides PS A to PS D that recognize relatively conserved sequences outside V3 to ensure annealing to both SI and NSI V3 sequences (21) (primers are schematically presented in Fig. 3A). The reaction products from amplification with primers PS A to PS D were subjected to amplification with nested primer sets PS B-SI 1-SI 2, PS C-SI 3-SI 4 and PS B-PS C. Primer set PS B-PS C supports amplification of V3 from both SI and NSI isolates and was thus used as a positive control. For practical reasons, two combinations of two SI-specific oligonucleotides were used rather than four reaction mixtures with one SI-specific oligonucleotide.

To optimize PCR annealing temperatures for SI-specific primer sets, we used a set of four virus isolates which had V3 sequences that perfectly matched any one of the four SI-specific oligonucleotides (Fig. 3B, upper panel). V3 sequences from isolates ACH-320.2a.5 and ACH-479.7 are recognized by PS B in combination with SI 1 and SI 2, whereas the V3 sequences from ACH-168.7 and HIV-IIIb are recognized by PS C in combination with SI 3 and SI 4. In the lower panel of Fig. 3B, results of PCR amplification with the three primer sets are shown. Primer set PS B-SI 1-SI 2 amplified V3 from ACH-320.2a.5 (Fig. 3B, lane 13) and ACH-479.7 (lane 12) but not the other two isolates (lanes 10 and 11) at 48°C. Primer set PS C-SI 3-SI 4 supported amplification of V3 from ACH-168.7 (Fig. 3B, lane 7) and HIV-IIIb (lane 6) but not the other two isolates (lanes 8 and 9) at 54°C. With DNA isolated from PBMC infected with three NSI HIV-1 isolates, V3 could be amplified with primer set PS B-PS C but not primer sets PS

B-SI 1-SI 2 and PS C-SI 3-SI 4 (data not shown). Lower annealing temperatures resulted in amplification of V3 sequences with mismatches at the 3' ends of the primers (data not shown). Primer set PS B-PS C supported amplification of V3 from all four isolates (Fig. 3B, lanes 1 to 4) within the range of annealing temperatures tested (37 to 58°C). For further experiments, the annealing temperature for primer set PS B-SI 1-SI 2 was 48°C and that for primer sets PS B-PS C and PS-C-SI 3-SI 4 was 54°C.

**Limited effect of genotype diversity on selective PCR amplification of SI V3 sequences.** Since in naturally occurring HIV-1 isolates V3 DNA sequences may be highly heterogeneous, we next determined whether nucleotide differences at positions flanking the 3' ends in the 20-mer oligonucleotide annealing sites resulted in failure to be amplified by PCR. In our set of HIV-1 isolates, a maximum of two nucleotide differences was observed in the primer annealing site for SI isolates with basic residues at position 320. In SI isolates with basic residues at position 306, three or fewer nucleotide differences were observed, with the exception of isolates from one individual (Ams-164) that had up to seven substitutions. The results of PCR amplification of V3 from these SI isolates are shown in Fig. 3C, together with the DNA sequences of the primer recognition sites. One or two nucleotide substitutions downstream of codon 320 in the primer site for primers SI 1 and SI 2 did not affect PCR amplification (Fig. 3C; lane 8, Ams-32; lane 7, Ams-55). For PCR amplification with primers SI 3 and SI 4, three nucleotide substitutions were tolerated (Fig. 3C; lane 11, ACH-373.38.5; lane 10, Ams-42), whereas six nucleotide substitutions resulted in a loss of PCR amplification (lane 9, Ams-164.18). Nucleotide substitutions in the primer recognition sites did result in a significant reduction in band intensity (e.g., in Fig. 3C, lanes 10 and 11), probably as a result of less efficient annealing. Nevertheless, band intensities were still sufficient for visualization on agarose gels stained with ethidium bromide. PCR analysis with primer set PS B-PS C resulted in efficient amplification for all virus isolates (Fig. 3C, lanes 2 to 6), confirming the presence of proviral DNA in the samples. Thus, since sequence variability in the primer annealing sites of our HIV-1 isolates was limited, a diagnostic assay based on specific amplification of SI viruses by PCR seems to be feasible.

## DISCUSSION

Monitoring of the SI capacities of virus isolates is now routinely performed for large cohorts of seropositive individuals to provide markers for disease progression and for evaluation of the efficacy of antiviral therapy by using MT2 cells or phytohemagglutinin-stimulated PBMC as indicator cells (12, 17). The disadvantages of these laborious assays are the requirements for highly specialized laboratory facilities and personnel to work with high titers of HIV-1 and the need for viably frozen purified lymphocytes. The HIV-1 V3 genotype may be used as an alternative marker for the SI phenotype. The PCR-based assay with discriminative oligonucleotides described here is simple and fast, allowing analysis of multiple samples at the same time, and it can be made applicable for analysis of viral

TABLE 1. Comparison of nucleotide usage at the last position in codon 306 and the first position in codon 320 in V3 from 249 virus isolates

Isolate phenotype	No. of isolates with indicated nucleotide usage	
	A or G in codon 306 and/or A in codon 320	C or T in codon 306 and C, G, or T in codon 320
NSI	9 <sup>a</sup>	182
SI	55	3

<sup>a</sup> All nine NSI HIV-1 isolates had A as the first nucleotide in codon 320, resulting in positively charged amino acid residues in five of nine isolates and neutral amino acid residues in four of nine isolates.

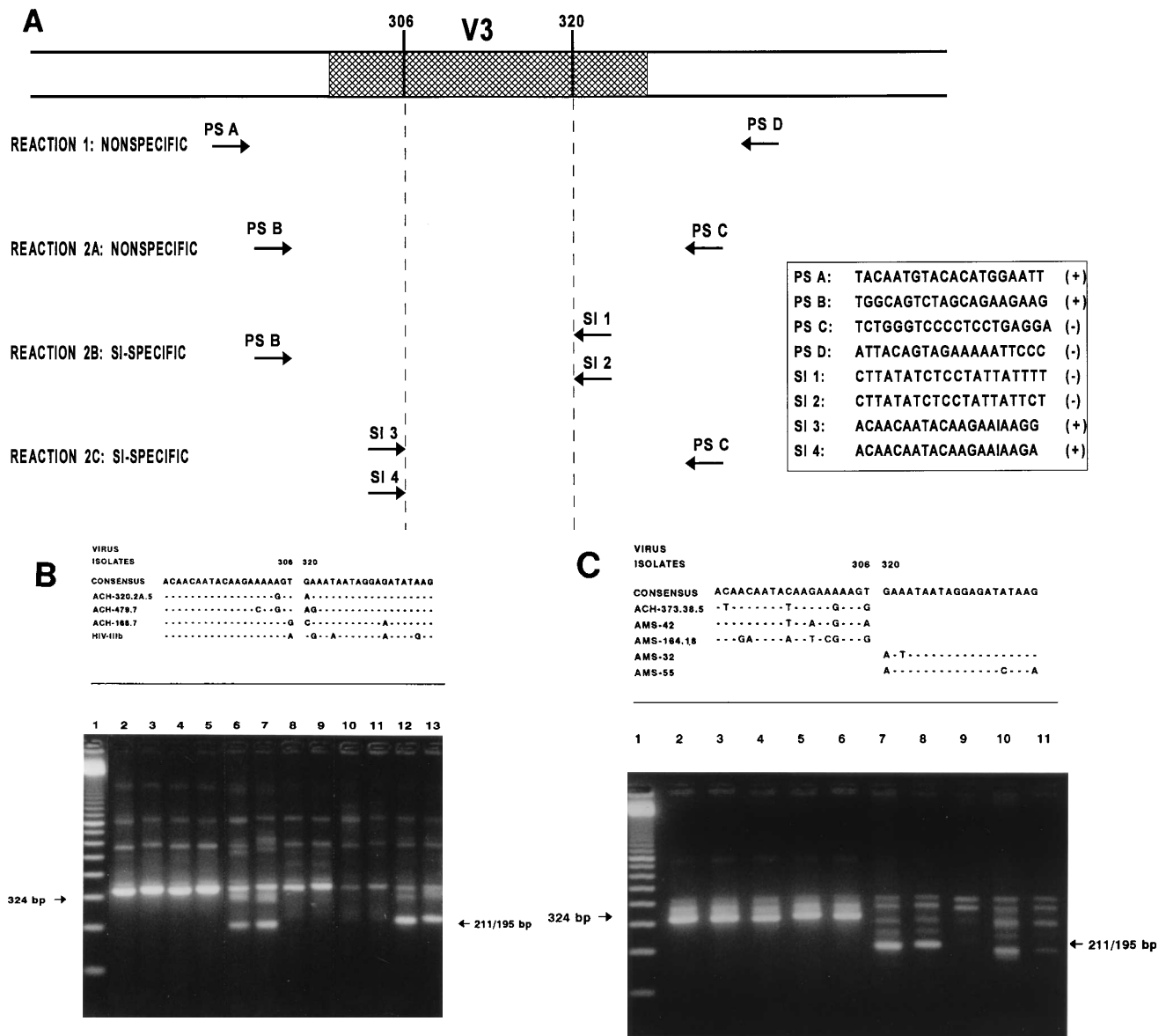


FIG. 3. (A) Schematic representation of primers used for discriminative PCR analysis. Primers PS A to PS D recognize relatively conserved sequences outside the V3 loop, whereas SI 1 to SI 4 recognize SI-specific DNA sequence variation at positions 306 and 320 in V3. PS A and PS D were used for initial PCR, and nested PCR analysis was performed with either the nonspecific primers PS B-PS C or the SI-specific primers SI 1-SI 2 or SI 3-SI 4 in combination with PS B or PS C, respectively. Primer sequences are shown with their orientations (+, sense; -, antisense). (B) Upper panel, primer recognition sequences of V3 from four virus isolates used for evaluation of PCR analysis with SI-specific primers. Sequences are aligned with the type B consensus sequence. Lower panel, analysis of PCR products (amplified DNA) on a 3% NuSieve agarose gel stained with ethidium bromide. Lane 1, 100-bp DNA molecular size marker (GIBCO/BRL); lanes 2 to 5, 6 to 9, and 10 to 13, products from PCR with primers PS B-PS C, PS C-SI 3-SI 4, and PS B-SI 1-SI 2, respectively; lanes 2, 6, and 10, HIV-IIIb; lanes 3, 7, and 11, ACH-168.7; lanes 4, 8, and 12, ACH-479.7; lanes 5, 9, and 13, ACH-320.2a.5. (C) Upper panel, primer recognition sequences of five virus isolates used for testing the effect of mismatches in primer sites for PCR analysis with SI-specific primers. Sequences are aligned with the type B consensus sequence. Lower panel, analysis of PCR products (amplified DNA) on a 3% NuSieve agarose gel stained with ethidium bromide. Lane 1, 100-bp DNA molecular size marker (GIBCO/BRL); lanes 2 to 6, 7 and 8, and 9 to 11, products from PCR with primers PS B-PS C, PS B-SI 1-SI 2, and PS C-SI 3-SI 4, respectively; lanes 2 and 7, Ams-55; lanes 3 and 8, Ams-32; lanes 4 and 9, Ams-164.18; lanes 5 and 10, Ams-42; lanes 6 and 11, ACH-373.38.5.

phenotype in either serum (RNA) or cell (DNA or RNA) samples from seropositive individuals with high sensitivity. With the SI-specific primer sets, V3 sequences were amplified from eight of nine SI and none of three NSI HIV-1 isolates tested. On the basis of the DNA sequence analysis presented in Table 1 and the fact that two mismatches downstream of codon 320 and three mismatches upstream of codon 306 still allow PCR amplification with the SI-specific primers, V3 genotype analysis by PCR would result in proper prediction of viral

phenotype for 51 of 58 SI HIV-1 isolates obtained from 23 of 26 individuals and 182 of 191 NSI HIV-1 isolates obtained from 58 of 63 individuals. Clade B-classed HIV-1 isolates from geographical regions other than The Netherlands have V3 sequences similar to those in the present study and demonstrate a similar correlation with SI capacity (3, 15, 16), indicating that the PCR with sequence-specific primers may be widely applicable. Moreover, De Wolf et al. recently reported that the correlation of SI phenotype with positively charged amino acid

residues in V3 was also observed for HIV-1 isolates classed in clades A and C to F (6). This suggests that sequence comparison of large numbers of HIV-1 variants classed in clades other than B may enable the design of SI-specific oligonucleotides for PCR-based diagnostics of the HIV-1 phenotype of non-type B viruses, confirming the study presented here.

PCR-based diagnostic assays that discriminate single-nucleotide substitutions in certain target genes have already been designed for other applications (24). Such assays require extreme optimization and proper controls, since minor fluctuations in annealing temperature or concentration of ingredients may result in false-positive or false-negative results.

The PCR-based assay as presented in Fig. 3 may require certain modifications for most efficient routine usage. Ideally, PCR with SI-specific primers should be made applicable to uncultured, uncloned patient material, which may contain mixtures of SI and NSI HIV-1 strains. Direct analysis of SI V3 genotypes in patient PBMC by PCR therefore requires a thorough comparison with culture-based assays with regard to sensitivity and selectivity. For routine use, it would be beneficial if the number of PCR tests per sample could be reduced by combining the SI-specific reactions in one reaction. Moreover, primers may be designed in the reverse orientation if required to improve sensitivity and selectivity. Third, inosine residues and relatively stable mismatched T·G base pairs may be incorporated in the primers to ensure amplification of heterogeneous V3 sequences. Such modifications may lead to a PCR-based assay for HIV-1 phenotype determination directly from uncultured blood or plasma samples from HIV-1-infected individuals.

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