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Human Immunodeficiency Virus Type 1 Envelope Gene Structure and Diversity In Vivo and after Cocultivation In Vitro†

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Nested-primer polymerase chain reaction (PCR) has been applied to the molecular cloning of 4.6-kb half-genome fragments of human immunodeficiency virus type 1 (HIV-1) taken directly from the peripheral blood mononuclear cells (PBMC) of an individual with neurological symptoms of HIV-1 infection. In a similar manner, gp120-coding portions of the envelope gene were cloned after PBMC from the same blood sample were cocultivated with uninfected PBMC for 28 days. The complete 1.6-kb nucleotide sequence of the gp120 gene was determined from each of 35 clones examined. Two of 13 (15%) PBMC-derived gp120 genes and 3 of 22 (14%) coculture-derived gp120 genes were defective as a result of frameshifts and an in-frame stop codon(s). Mean diversity between individual gp120-coding sequences in PBMC was fivefold greater (3.24%) than after coculture (0.65%). A predominant sequence or "strain" was found after coculture that was distinct from the diverse viral genotypes detected in vivo and therefore was selectively amplified during in vitro propagation. Multiple distinct third variable (V3) regions encoding the principal neutralizing domain of the envelope protein were detected in PBMC-derived genes, suggesting the presence of immunologic diversity of HIV *env* genes in vivo not reflected in the cocultured virus sample. The large size of the HIV fragments generated in this study will permit analysis of the diversity of immunologic reactivity, gene function, and pathogenicity of HIV genomes present within infected individuals, including the functional significance of the loss of diversity that occurs upon coculture.

Human immunodeficiency virus type 1 (HIV-1), a member of the lentivirus subfamily of retroviruses, has largely been studied as virus isolates, that is, after replication in cell culture. HIV-1 isolates from a given individual appear to change over the course of the disease, as measured in functional assays of replication kinetics and syncytium formation. During the asymptomatic phase of infection, virus isolates generally display slow-low replication and syncytium-inducing characteristics; this is followed by rapid-high characteristics during symptomatic phases of infection (reviewed in reference 13).

The appearance of variants with rapid-high growth characteristics has precedent in the feline leukemia virus-FAIDS variant of feline leukemia virus (38, 52, 53). The late-appearing feline leukemia virus-FAIDS variants have been shown to be more pathogenic than the earlier-appearing virus and to be replication defective, leading to the suggestion that cultivation of virus prior to analysis leads to the selective loss of pathogenic variants because of defectiveness, acute cytopathicity, or inability to grow in the particular tissue culture system employed (46). Alternatively, in vitro propagation may increase the representation of viruses of high replicative capacity that are released from the suppressive pressures imposed by the immune system (68). Analysis of simian immunodeficiency virus (SIV) provided examples of tissue culture adaptation leading to selection of variant viruses with truncated open reading frames encoding the *env* proteins (3, 20, 28) and selection for extension of the *env* and *nef* open reading frames in vivo (20, 27). Thus, tissue culture adaptation can partially obscure the true coding potential of these genetically complex retroviruses. Similarly, analysis of HIV-1 has revealed the possibility that *vpr* and perhaps other genes are inactivated when virus is adapted to culture (6). Last, primary HIV-1 isolates and laboratory strains differ in their ability to be neutralized by soluble CD4 (9). These considerations led to and continue to fuel uncertainty about the relationship of cultured virus to that found in vivo and about how these differences might affect our understanding of the pathogenic and immunologic diversity of HIV (9, 20, 46).

The differences between HIV genomes present in vivo and after culture adaptation are difficult to address since viral DNA is generally in too low amount in most tissues to be analyzed by conventional molecular cloning techniques based on bacteriophage lambda and plasmid cloning technol-

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ogy (18, 32, 58) and because of the instability of these sequences upon propagation in *Escherichia coli*-based host-vector systems (10, 26). The extreme sensitivity of polymerase chain reaction (PCR) can be enhanced to the point of reliable single-molecule sensitivity by using nested-primer PCR (16, 39, 61). In the latter procedure, amplified products are subjected to a second series of amplification cycles with primers which anneal within the previously amplified product. PCR and nested PCR analyses coupled with DNA sequence analyses have revealed the presence of diverse viral quasispecies in vivo, differences in the quantitative representation of HIV sequences found in vivo, and, after coculture, a loss of diversity in the *tat* gene upon propagation, a variable frequency of defective genomes, differential representation of *env* third variable (V3) sequences in different tissues, and independent fluctuation of different regions of the viral genome (11, 17, 35, 75).

The functional significance of these differences is more difficult to assess since they have to date been evaluated outside of the context of the complete interactive unit within the genome (e.g., the entire gene or regulatory protein-coding sequence together with its responsive element) within which alterations in one gene or region of a gene could be compensated for by changes elsewhere, resulting in new or preserved functions. It is known, for example, that recognition of some epitopes can be affected by protein sequence alterations at linearly distant sites within a protein or within associated proteins (41, 48), including alteration of the recognition of the principal neutralization domain of the HIV-1 envelope protein by changes elsewhere (41). These considerations led us to develop a means of PCR amplifying the entire HIV genome in as few segments as possible so that changes in one region could be evaluated within the context of as much of the genome as was technically possible to obtain. We have succeeded in amplifying 5- to 6-kb half-genome fragments of SIV after brief virus amplification in culture, which could then be reassembled into infectious, pathogenic virus (10). In this report, we extend this technology to the amplification and cloning of half-genome fragments of HIV-1 directly from the peripheral blood mononuclear cells (PBMC) of an infected individual and show that single molecules of HIV of this size may be detected through the use of nested PCR and serial dilution of template DNA.

We also describe analysis of the DNA sequence encoding the exterior envelope glycoprotein (gp120) as it is present in PBMC and after 4 weeks of coculture of an aliquot of the same PBMC with PBMC from an uninfected donor. Choice of the gp120 gene arises from the knowledge that changes within it are responsible for major differences in host range, cytopathicity, susceptibility to serum neutralization, and cytotoxic T-cell (CTL) responses (5, 64, 74, 76). The gp120 gene is also the most variable of the HIV-1 genes, and the degree of sequence variation in distinct virus isolates is growing at a rate of up to about 1% per year (2, 63). Understanding variation of this protein is therefore thought to be crucial for the understanding of disease progression and for the design of multivalent vaccines against HIV infection.

MATERIALS AND METHODS

Patient description. The PBMC sample under study was taken in April 1989 from a 27-year-old male Caucasian homosexual (referred to as patient MA) infected with HIV-1 and living in Massachusetts. Patient MA has been infected with HIV-1 since late 1984 or early 1985, and he reports having a single sexual partner since early 1984 with infrequent sexual contacts since 1985. Two reports on the initial clinical course of this patient have been published (patient 2 in reference 23 and patient 1 in reference 22). The clinical manifestations of his HIV infection have been predominantly limited to neurological problems; during his primary syndrome, he developed aseptic meningitis which was self-limited. He has had persistent, mild neuropsychiatric problems and developed a transient myelopathy-neuropathy syndrome in mid-1987. He was hospitalized in September 1988 for an investigation of headaches for which no specific etiology was found and which resolved spontaneously. Intermittent visual dimming due to a mild optic neuropathy occurred in 1989. The patient had not received antiviral therapy before the time the PBMC sample was taken. His CD4 lymphocyte counts fluctuated between 346 and 893/mm^3 between 1985 and 1989.

Virus culture and viral DNA isolation. Whole-blood samples were obtained in heparinized tubes, and PBMC were separated by Ficoll-Hypaque density centrifugation. DNA was isolated directly from patient MA's PBMC and from a 4-week coculture. PBMC (10^7) were cocultivated with 5×10^6 PBMC from an HIV-1-seronegative donor in RPMI 1640 medium containing 2 μg of Polybrene (Sigma) per ml and 10% interleukin-2 (Electronucleonics), supplemented with penicillin (250 U/ml), streptomycin (250 μg/ml), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (10 mM), L-glutamine (2 mM), and 20% heat-inactivated fetal calf serum (GIBCO). PBMC from the seronegative donor had been previously harvested by Ficoll-Hypaque centrifugation and stimulated for 3 to 4 days with 10% interleukin-2 and 10 μg of phytohemagglutinin-P (Difco) per ml in RPMI 1640 medium supplemented as above.

Cultures were maintained with a twice weekly addition of fresh medium and a weekly addition of 5×10^6 fresh prestimulated PBMC. Cultures were tested twice weekly for extracellular HIV-1 p24 antigen by enzyme-linked immunosorbent assay (Dupont, NEN Research Products, Boston, Mass.) and were considered positive if two consecutive determinations had optical densities more than twice the mean of the negative control wells, and with the second positive value greater than the first (62). High-molecular-weight DNA was purified by a standard proteinase K digestion-phenol extraction technique and an automated nucleic acid extractor (Applied Biosystems Inc.).

PCR primers and conditions. Oligonucleotide primer sequences were chosen from regions in the central part of the HIV-1 genome and from the 3′ long terminal repeat downstream of the end of the (HIV-1) transcriptional unit in U5. Each primer was evaluated for its potential for mispriming with itself, with other primers, and within the targeted amplified region using the program PRIMER (12a). The efficacy of amplification for each primer pair was measured by using a dilution series of linearized HIV-1 clone HXB2-pSP62 DNA (10^7 to 10^{-1} molecules) in 1 μg of uninfected human placenta carrier DNA. The following four primers displayed high sensitivity and specificity and were utilized for amplification and cloning of HIV-1 sequences in PBMC DNA. First round: KK-pol-1b, 5′-GAGTCTAGATGGAAA GGTGAAGGGGCAGTAGTA (corresponding to positions 4955 to 4978 of the HXB2 genome); and Td-4N, 5′-GGTCT GAGGGATCTCTAGTTACCAGAGTC (positions 9689 to 9661). Second round: KK-pol-3d, 5′-TAGGCGGCCGCACA GANGGCAGGTGATGATTGTGT (positions 5047 to 5070); and KK-R-4b, 5′-CAGGCGGCCGCGGCAAGCTTTATTG AGGCTTAAG (positions 9623 to 9601). *Not*I restriction

enzyme sites (underlined above) were incorporated near the 5′ ends of the second-round primers to facilitate subsequent cloning. The product of the second round of amplification extends from the 3′ end of the *pol/vif* gene through to the U5 region in the 3′ long terminal repeat.

The same first-round primers were used to amplify viral sequences from cultured PBMC. Second-round primers were: JM-11a, 5′-CTCGAGCTCCTGAAGACAGTCAGAC TCATCAAG (positions 5999 to 6028); and JM-12, 5′-CTC CTAAGAACCCAAGGAACAGAGCTCC (positions 7796 to 7768). The *Sac*I restriction enzyme sites (underlined) are found at this position in several HIV-1 isolates and were incorporated to facilitate subsequent cloning. The 1.8-kb product of the second round of amplification extends from 197 bases 5′ to the start of the gp120 gene and ends 6 bases into the gp41-coding sequence.

PCR mixtures consisted of 100 mM Tris-Cl (pH 8.3), 50 mM KCl, 0.01% gelatin, 0.2 mM each of the four deoxynucleoside triphosphates, 2.2 mM $MgCl_2$ (round 1) or 1.8 mM $MgCl_2$ (round 2), 10 pmol of each primer, a total of 1 μg of DNA (round 1; pHXB2 plasmid dilutions each contained 1 μg of human placental DNA), and 2 U of ampliTaq polymerase (Perkin-Elmer Cetus) in a volume of 100 μl. Amplification of sequences derived from culture-adapted virus differed in the second round with respect to $MgCl_2$ (2.0 mM) and primer concentration (20 pmol each). Following round 1 amplification, 2.5 μl was removed and used as target DNA for amplification in round 2 in a final volume of 100 μl. Both rounds of amplification were conducted for 35 cycles in a thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of three steps: denaturation (94°C, 45 s), primer annealing (55°C, 2 min), and extension (72°C, 4 min), with a final extension at 72°C for 10 min. Southern blot hybridization of amplified products was performed using as a hybridization probe a nick-translated fragment corresponding to positions 5079 to 9598 of the HXB2 genome.

Molecular cloning, transfection, and plasmid DNA purification. PCRs were precipitated following the addition of 50 μl of 7.5 M ammonium acetate and 150 μl of isopropanol, centrifuged, washed twice with 70% ethanol, and dried. DNA pellets were redissolved in TLE (10 mM Tris [pH 8.3], 0.1 mM EDTA) and digested with either *Not*I or *Sac*I. These reaction mixtures were separated on a 1% preparative agarose gel, and a band of the appropriate size was excised, extracted, and purified with Gene-Clean (Bio 101).

Purified fragments were ligated (56) to the plasmid vector pSP73NS (modified from pSP73 [Promega Biotechnology] by insertion of *Not*I and *Sfi*I sites in the polylinker), which was predigested with *Not*I or *Sac*I and treated with calf intestinal alkaline phosphatase (New England BioLabs). Following ligation, the reaction mixture was precipitated with 1 volume of isopropanol in a final concentration of 2.5 M ammonium acetate, spun, washed with 70% ethanol, resuspended in 5 μl of H_2O, and introduced into JM109 cells by electroporation (56). Cells were incubated in SOC medium (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) for 1 h at 37°C, plated on LB-carbenicillin agar plates, and left overnight at 30°C. Colonies were picked and grown in 10-ml cultures containing LB-ampicillin overnight at 30°C. Clones with inserts of roughly appropriate size, as judged by restriction enzyme digestion and gel electrophoresis, were propagated a second time in larger batches and used for DNA sequencing.

DNA sequencing and analysis. Plasmids were sequenced by using doubled-stranded DNA templates with Sequenase-2 kits (United States Biochemicals) with ^{35}S-dATP (Dupont, NEN Research Products) and minor modifications of the procedures recommended by the manufacturer. DNA samples were denatured in 0.4 N NaOH–0.4 mM EDTA for 5 min, primers (0.7 to 1 pmol) were added, and the mixture was neutralized with 0.64 M sodium acetate (pH 5.3). The mixture was precipitated with 5 volumes of 95% ethanol, centrifuged, washed with 70% ethanol, and dried. Pellets were resuspended in 8 μl of double-distilled H_2O, and subsequent reactions were performed according to the supplier's recommendations. Sequences were read manually from X-ray film exposures of 6% polyacrylamide-urea wedge sequencing gels.

Basic sequence manipulations were performed on a SUN 4/260 minicomputer using the IG-suite programs (Intelligenetics). Sequences were aligned by using the IG program GENALIGN and then refined by using the Multiple Alignment Sequence Editor (12). Alignments were analyzed by the program DOTS (59a), which performs and prints out aligned sequence sets, divergence analysis, tallied mutational changes, and synonymous/nonsynonymous changes.

Nucleotide sequence accession numbers. DNA sequences have been deposited in GenBank under the accession numbers M79342 to M79354 and in the Human Retrovirus and AIDS Sequence Data Base (40).

RESULTS

PCR amplification and cloning of large DNA fragments from rare proviral targets. We first addressed the possibility that PCR amplification of half-viral-genome-sized fragments (~5 kb; [10]) could be used to provide sufficient material for cloning HIV present at very low levels in target DNA, such as in the PBMC of persons infected with HIV-1. Recent studies suggest that HIV-1 proviruses are present in as few as 1 in 10^5 to 10^6 PBMC in vivo and that the majority of infected cells contain a single provirus (21, 61). To establish the sensitivity of the technique, we used a modified PCR procedure utilizing nested PCR primers, with amplification performed in two steps (7, 39, 45). Following the first 35-cycle PCR amplification step, a small aliquot (2.5 μl was used here) was removed and used as target DNA for a second round of amplification along with primers from a region of the HIV genome within those used for the first-round reaction (nested primers). This procedure restores preferential amplification of viral DNA fragments compared with nonspecific sequences amplified from cellular DNA, a problem of most significance with long products and at very low target HIV DNA levels in the first round of amplification.

To successfully clone fragments using single-round PCR to amplify 5-kb fragments under the conditions we employed, approximately 10^5 copies of the target molecule would be required per μg of DNA, or about 1 copy per cell equivalent of DNA (Fig. 1). This level of sensitivity is insufficient to allow direct recovery from most biopsy materials from HIV-1-infected individuals. Single-round PCR was also previously found to be inadequate for consistent isolation of 1-kb fragments from SIV-infected, asymptomatic macaques (20). However, when a second round of PCR amplification was performed with nested primers, sufficient amounts of DNA could be amplified from as little as one molecule of viral DNA in a background of 1 μg of cellular DNA, or one viral copy in about 1.5×10^5 or more cells (Fig. 1). However, most of the oligonucleotide primers we exam-

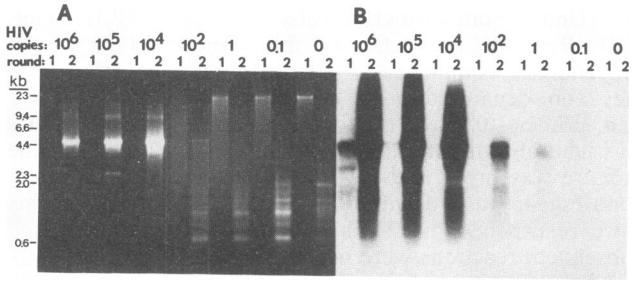

FIG. 1. PCR amplification of the 3′ half of the HIV-1 genome from sequential dilutions of pHXB2 plasmid DNA in uninfected cell DNA. (A) Ethidium bromide-stained gel; (B) Southern blot analysis of the same gel with an HIV-1-specific hybridization probe. Lanes 1 and 2 represent first- and second-round PCR, respectively.

ined failed to amplify HIV sequences at this level of sensitivity under the conditions we described (data not shown).

To determine the applicability of the procedure for use with patient PBMC, we next attempted direct amplification of the 3′ half of the HIV-1 genome from DNA isolated from PBMC of an HIV-1-infected but currently asymptomatic individual. Interestingly, the expected 4.6-kb HIV-1 DNA fragment was observed in most but not all second-round PCR products, and the quantity of specific product generated varied in duplicate reactions (Fig. 2). In contrast, consistent results were obtained with duplicate second-round reactions from a given first round (data not shown). These results suggest that some stochastic event, likely taking place during the early cycles of the first-round reaction, is responsible for determining the major products produced in a given reaction. The smaller products could be derived from mispriming or from amplification of preexisting deleted viral genotypes, and we found evidence of both mechanisms in this study (data not shown). By either mechanism, the shorter products would be expected to be selectively amplified and thereby to decrease the yield of full-length products.

Yield-optimized PCR conditions were used to amplify and clone 4.6-kb products from high-molecular-weight DNA extracted from PBMC. Twenty stable clones with inserts in the range of 4.6 kb were obtained, and of these, 13 had the potential to encode full-length gp120 (12 are shown in Fig. 3), whereas the 7 additional clones contained large deletions. Cloning efficiency of these fragments was extraordinarily low given that a total of about 1,800 insert-bearing clones

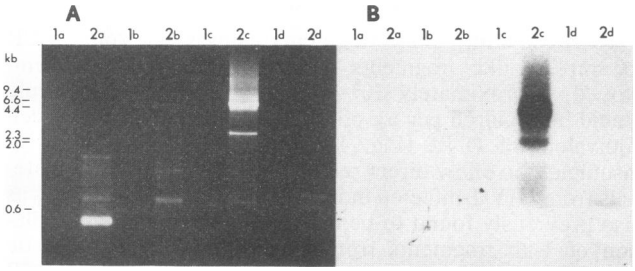

FIG. 2. PCR amplification of the 3′ half of the HIV-1 genome from DNA isolated from PBMC from patient MA. (A) Ethidium bromide-stained gel; (B) Southern blot analysis of the same gel with an HIV-1-specific hybridization probe. Lanes 1 and 2 represent first- and second-round PCR, respectively. a through d correspond to duplicate reactions done simultaneously.

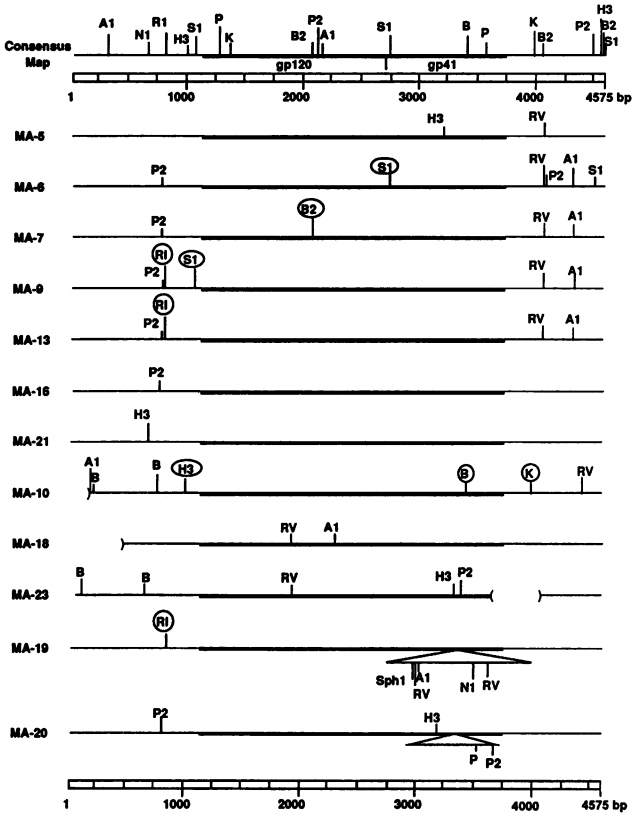

FIG. 3. Restriction site maps of 12 PBMC-derived 3′-half-genome clones of HIV-1 from patient MA. Sites found in greater than 50% of the clones examined are indicated in the consensus map at the top of the figure. Only divergent sites are indicated in the maps of individual clones; circled sites are missing from the clone indicated. Restriction sites indicated correspond to *Acc*I (A1), *Bam*HI (B), *Bgl*II (B2), *Eco*RI (R1), *Eco*RV (RV), *Hin*dIII (H3), *Nco*I (N1), *Pst*I (P), *Pvu*II (P2), and *Sac*I (S1). None of the clones were cut by *Bgl*I, *Cla*I, *Sal*I, or *Sph*I. Regions bounded by parentheses correspond to the approximate location of deletions. Thick lines correspond to the gp120- and gp41-coding portions of the envelope gene. Broad triangles below the maps of MA19 and -20 correspond to the position of insertion of nonviral sequences. Sequence analysis identified the insertion in clone MA19 as the bacterial insertion element-like sequence IS*10*-left flanking transposon Tn*10* (19) and that in MA20 as insertion element IS*1* (38a, 43).

were examined in plasmid minipreparations of DNA. The sequences responsible for the instability were not mapped; however, two clones with approximately correctly sized inserts were found to contain bacterial insertion elements integrated near the 3′ end of the gp41-coding sequence of *env* (Fig. 3). Similarly low cloning efficiency occurred when we attempted cloning 4.6-kb viral DNA from PBMC cocultivated with interleukin-2-stimulated, uninfected donor PBMC for 1 month. However, when we used second-round primers to produce only 1.8-kb fragments encompassing just the gp120 gene, cloning efficiency was high and each of 24 clones examined was stable. Two contained evident deletions, but none contained evident bacterial insertion elements.

Restriction site analysis, defectives, and hypermutation. Restriction site analysis of 12 approximately full-length clones with 16 restriction endonucleases revealed that each clone was distinct (Fig. 3). Furthermore, the complete nucleotide sequence of the 1.6-kb gp120 region of all 35

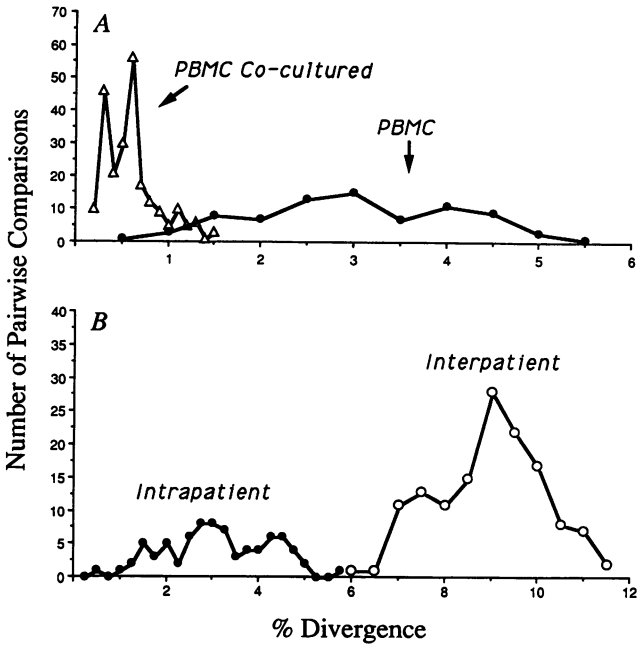

FIG. 4. Pairwise comparison of divergence between individual gp120 sequences obtained from patient MA (intrapatient) and from 16 reported non-African HIV-1 viruses (interpatient). Data correspond to those reported in Table 1. (A) Patient MA. Cocultured sequence data plotted in 0.1% intervals; PBMC data plotted in 0.5% intervals. (B) PBMC from patient MA plotted in 0.25% intervals; interpatient data plotted in 0.5% intervals.

clones containing potentially complete gp120 genes was determined, and no two identical sequences were found. Two of 13 (15%) PBMC clones were defective as a result of three in-frame stop codons (MA10) and a frameshift (MA23), whereas 3 of 22 (14%) coculture-derived clones were defective because of frameshifts (MA211, MA203, and MA220); clone MA220 also contained an in-frame stop codon. Thus, circulating blood cells in this patient appeared to contain no higher percentage of defective gp120 genes than viruses replicating in 28-day coculture.

G→A hypermutation occurred only over short regions of the genes (data not shown). For MA10, a 190-nucleotide stretch was found where 12 G→A mutations occurred in 15 GpG dinucleotides, resulting in TAG termination codons at all three TGG (Trp) codons found in the uninterrupted sequences.

Short-term coculture reduces gp120 gene diversity. The nucleotide sequence diversity was determined between and within patient MA strains and other groups of HIV-1 gp120 genes. A fivefold-higher level of diversity was observed in patient MA PBMC gp120 genes than after 4 weeks of coculture (Fig. 4A and Table 1). The average diversity among PBMC-derived sequences was 3.24% (6.50% at the amino acid level), versus 0.65% (1.25% at the amino acid level) after coculture. Thus, as previously shown for *tat* gene sequences (35), cocultivated viruses do not quantitatively represent HIV-1 populations in vivo.

HIV-1-MA can be included as a member of the non-African subgroup of HIV-1, since the average degree of divergence is 10% versus 17% when compared with African isolates (Table 1). MA is nonetheless one of the most divergent non-African HIV-1 viruses described to date: clone MA10 differs from CDC42 by 12.17%, the highest degree of diversity observed within the non-African HIV-1 subgroup, and the average difference between MA and other non-African isolates is greater than the previous intraisolate average of 8.94%.

The range of intrapatient variation (1.5 to 5.8%) did not quite overlap the range of interpatient variation (>6.4%; Table 1 and Fig. 4B). This analysis suggests that most or all HIV-1-MA sequences are derived from one strain; that is, the patient was infected by a single independent strain or that at least only one strain was predominant in the blood at the time of sampling. It is also possible that newly infecting strains could have blended in through recombination with preexisting viruses (65).

Increased diversity within variable regions. As examined along the length of the gp120 gene, variation between PBMC-derived clones and, to a lesser extent, the coculture-derived clones is highest within what has been recognized as variable regions of gp120 between isolates of HIV (37, 65). Thus, the viral quasispecies within an individual is qualitatively similar to that found between individuals. All deletions and duplications and six of the eight inactivating mutations detected were in variable regions. The disparity between virus found in PBMC and after coculture is also most evident through analysis of variable regions (Fig. 5 to 7). For example, 16 of 22 cocultured sequences are identical at the amino acid level in the V1-V2 region; however, there are no two identical sequences among the 13 PBMC sequences (Fig. 5). Only two coculture sequences are identical to PBMC clone MA14 in this region. Cocultured sequences are most variable in the V4-V5 region (Fig. 6). Sequences containing an insertion of an asparagine codon at position in 433 were present in all PBMC-derived clones examined (5 of 22) but uncommon following cocultivation. Similarly, only two cocultured sequences, MA218 and MA204, were identical to PBMC-derived sequences (MA7 and MA14, respectively) over this region.

TABLE 1. Sequence diversity between HIV-1 gp120 genes

Source (*n*)	Avg and range of diversity[a] (%)			
	Coculture	PBMC	Non-African	African
Coculture (22)	0.65 (0.25–1.57)			
PBMC (13)	2.63 (1.06–4.86)	3.24 (0.50–5.79)		
Non-African (17)[b]	10.30 (8.46–11.98)	10.06 (5.91–12.17)	8.94 (6.46–11.4)	
African (6)[b]	17.15 (15.64–19.77)	17.09 (14.54–19.75)	16.75 (14.16–20.5)	14.66 (7.73–20.6)

[a] Diversity is reported as a percentage of mismatched bases and was calculated by pairwise calculation of all matched and mismatched nucleotides. Insertions and deletions were not counted.

[b] Sequences were obtained from the Human Retroviruses and AIDS data base (40) and corresponded to the following isolates: non-African, HXB2 (LAI), BAL1, MN, ALA1, JRCSF, BRVA, SC, JH32, CDC42, OYI, SF2, HAN, NY5, JFL, ADA, WMJ12, RF; African, ELI, Z2, NDK, JY1, MAL, Z321.

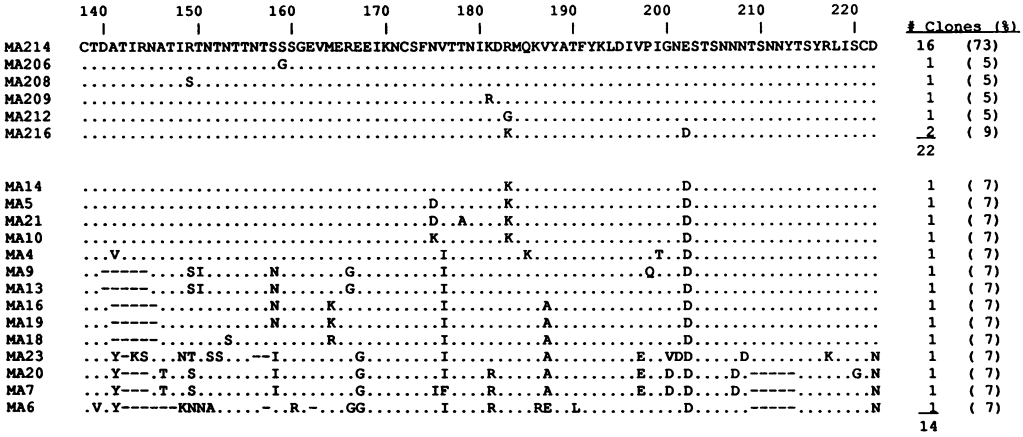

FIG. 5. Deduced gp120 sequences in the first and second variable regions.

No variation was found relative to the amino acid residues identified in HIV isolate HXB2c2 as important for CD4 binding (44, 69). These include residues 282 [256], 283 [257], 392 [368], 394 [370], 408 [384], 457* [427], 460* [430], 477* [447], 487* [457], 509 [477], and 514 to 516 [482 to 484] of the full MA sequence alignment (sequences in brackets correspond to positions in the HXB2c2 gp120 protein according to Olshevsky et al. [44]; residues marked with an asterisk are double underlined in Fig. 6). Furthermore, none of the amino acid changes observed in an overlapping region shown to be critical for CD4 binding (underlined in Fig. 6) have been found to affect CD4 association (8, 30). However, only the Lys→Arg change found in the MA set at position 451 of defective clone MA211 had been evaluated previously (8) and in the context of the HIV-1-LAI (71) envelope gene.

Variable region three (V3) corresponds to the location of the principal neutralization domain in HIV-1 gp120 (51), and changes in this region can also change the cellular tropism of the virus (24, 42, 59) as well as murine CTL specificity (67). No PBMC-derived clone analyzed (note that a larger data set was examined over this region: 23 coculture- and 20 PBMC-derived clones) encodes a V3 peptide identical to the coculture consensus sequence, and only coculture-derived clone 218 encodes a peptide identical to the prevalent sequence found in PBMC (Fig. 7).

There are a total of 26 to 30 glycosylation sites in the MA clones, 30 in the coculture consensus sequence. Only 11 of 30 sites (37%) are conserved in all PBMC-derived clones, and of these, only 4 are conserved in all non-African isolates. By comparison, HXB2 has 24 sites, all of which are glycosylated (31).

DISCUSSION

One motivation to undertake this study was to evaluate the extent of genetic variation in the HIV-1 surface protein gene present in the peripheral blood of an infected individual and after what is a routine PBMC coculture procedure normally used to obtain and evaluate HIV strains (15). The patient under study has reported to have never received

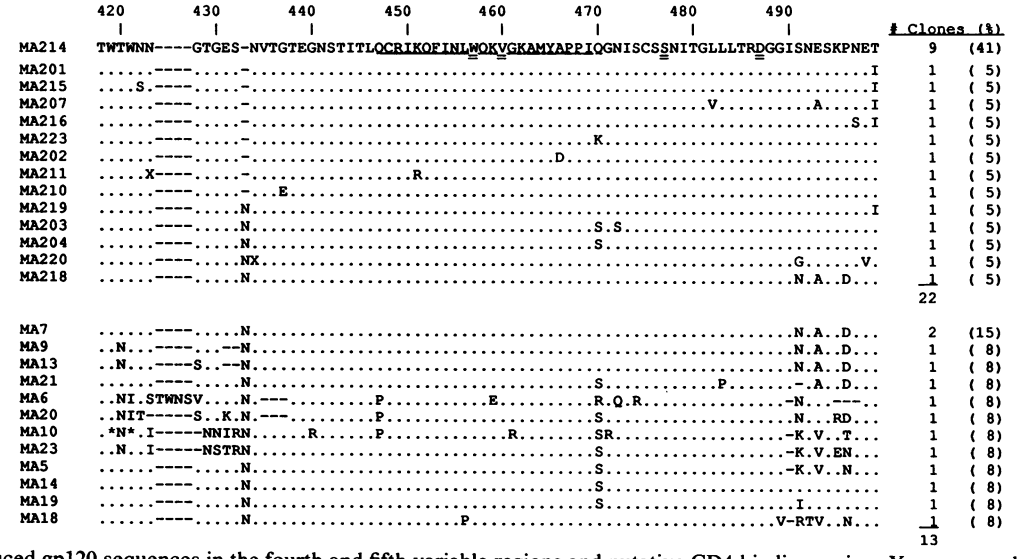

FIG. 6. Deduced gp120 sequences in the fourth and fifth variable regions and putative CD4-binding region. X corresponds to a frameshift in MA211. Asterisks (*) correspond to stop codons in MA10. Underlined sequences are described in the text.

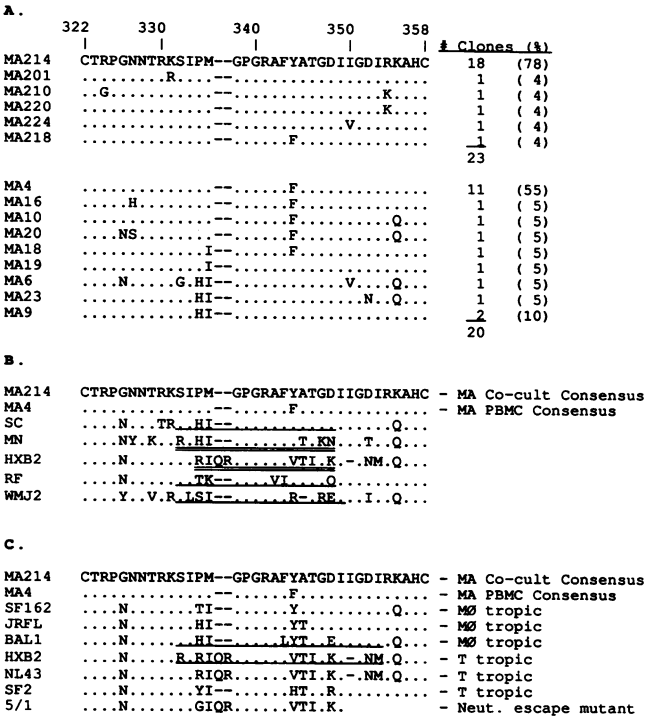

FIG. 7. Deduced gp120 sequences in the third variable region. (A) MA clones. (B, C) MA consensus sequences compared with HIV data base sequences. Underlined sequences are described in the text. mø, macrophage.

anti-HIV medication; thus, no selection due to drugs, e.g., zidovudine, is likely to have occurred. The selection observed is therefore due to the infecting inoculum and intrapatient pressures, and hence the spectrum of variation provides information about the natural development of HIV infection. It is likely that this individual was infected in late 1984 or early 1985, since he developed a primary HIV syndrome in March 1985, 6 weeks after his latest sexual contact (23). This means that the virus population had more than 4 years to evolve in the patient before sampling. By the time of sampling, the patient had experienced several neurological syndromes likely related to HIV infection, but he has not otherwise experienced a significant deterioration in his medical condition.

Our findings confirm and extend the results of others that indicate that culturing virus alters our view of the viral genomic variants present within a quasispecies (35). The cocultured virus isolate under study was clearly from the same individual as the PBMC-derived virus since the average degree of divergence between the two sets was 2.63% (range, 1.06 to 4.86%), whereas the next closest non-African relative was 8.46% divergent compared with the cocultured virus and 5.91% divergent compared with the PBMC set (Table 1). We found that gp120 genes of viruses present after 4 weeks of coculture had a fivefold-lower level of genetic diversity and that the predominant virus found in vitro was not detected in vivo. The greatest outlyer (most divergent) within the coculture set (MA218, average diversity of 1.32%; range, 1.19 to 1.57%), was the closest link to the PBMC-derived sequences (average diversity of 2.45%; range, 1.06 to 4.66). Conversely, the closest link to the cocultured viruses (and MA218) among the PBMC-derived set was MA16 (average diversity of 1.40%; range, 1.06 to 1.65%). In contrast to being an outlyer, MA16 was the closest to the consensus among the PBMC-derived set (average diversity of 2.44%; range, 1.19 to 4.53%). Only two other PBMC-derived sequences had mismatches of less than the 1.57% maximal diversity observed between the coculture-derived sequences (MA14, average diversity of 1.54%; range, 1.32 to 1.80%; MA19, average diversity of 1.51%; range, 1.40 to 1.78%). Thus, viruses most representative of the integrated forms of HIV-1 DNA in PBMC are poorly represented after 28 days of coculture. Several explanations are possible to account for the origin of the viruses predominating in culture. (i) They may have faster replicating properties but were differentially suppressed in vivo by the immune system, analogous to a proposed origin of the rapidly replicating viruses present at late-stage disease (68); (ii) the culture system may have imposed selective pressures that resulted in the preferential amplification of certain viral genotypes, as demonstrated for SIV (3, 20, 28); (iii) they could have been derived from the few cells in the PBMC that may have been actively replicating virus at the time of sampling; (iv) they could be enriched in replication-competent viruses, whereas the proviruses present in PBMC may correspond to remnants of earlier abortive or noncytopathic infections (either of the latter two hypotheses would suggest that the PBMC sequences could represent an archeological record of prior infection of the cell population); (v) they could have originated from free virus present in the serum, potentially produced by cells other than the PBMC, and transiently attached to PBMC membranes and thus carried over into coculture. We are unable to distinguish among the possibilities mentioned above with the current information available; however, the clones of half-genome-sized fragments obtained in this study will permit experimental testing of some of these hypotheses upon reconstruction into full-length provirus.

We found that circulating blood cells in this patient appeared to contain no higher percentage of obviously defective gp120 genes (those with frameshifts and/or stop codons) than viruses replicating in coculture. A previous study of HIV-1 *tat* genes examined after amplification of PBMC and cocultured virus also failed to reveal a discrepancy between the frequency of obvious defectives in the two data sets (35). We and others have found that inactivating mutations cluster in regions of hypermutation (e.g., MA10). If we consider only the frequency of obviously defective genes, then we would predict that the proviruses under study would have approximately one inactivating mutation per genome (i.e., at least 50% of the virus population would be defective). This would be an underestimate, however, since some defectives would be expected to be found as a result of incompatible amino acid substitutions.

Previous studies which revealed G→A hypermutation found a twofold-higher or greater overall percentage of defectives (17, 35, 70), whereas studies in which hypermutation was not detected found inactivating mutations at a rate twofold or more lower than we observed (2, 60). We and the others cited above have relied on PCR for viral DNA amplification and therefore have excluded from analysis viral genomes which have deletions or significant mismatches in regions encompassing primer-annealing sites. However, defective viral genomes of internally deleted and full-length structure were implicated in feline and murine AIDS models (1, 4, 46, 47), leading to the concern that defectives may play an important role in the pathogenesis of AIDS (46, 73). A recent study of HIV-1 unintegrated circular

viral DNA isolated directly from the brain of an infected individual by lambda cloning revealed that only 4 of 10 clones harbored complete proviruses, only one of which formed transmissible virus upon transfection (32). Furthermore, cells infected with HIV in the peripheral blood retain their $CD4^+$ phenotype, in contrast to cells infected in vitro (50, 57). Thus, even if a defective or latent provirus is found within a cell, its potential role in the pathogenesis of AIDS should not be dismissed since these cells can presumably be infected with replication-competent virus and undergo recombination to generate new intragenic viral assortants (65). In summary, the potential role of defective genomes in the pathogenesis of AIDS remains to be elucidated.

A second motivation for this study was to provide materials with which to evaluate antigenically and pathogenically distinct viruses expected to be found to exist simultaneously in the host, as has been found in animal models for AIDS (14, 46). Our finding that viral sequences in coculture are relatively homogeneous is consistent with previous studies which have indicated that virus strains isolated (cultured) from individuals can be relatively pure antigenically, including their neutralization characteristics (25, 49, 55). However, within the PBMC viruses, the greater variation observed was clustered into the previously described variable regions (65).

To date, the most extensively analyzed portion of the envelope gp120 gene is the third variable region, or V3 (29, 40). V3 encodes the principal type-specific neutralization epitope of the virus (51, 55), and changes in regions which include V3 can also determine CTL specificity (66, 67) and cellular tropism (24, 42, 59). No amino acids found in the MA set were unique in the V3 sequence data base described to date (29, 40); however, some were rare. For example, the Pro-Met (PM) sequence at positions 334 to 335 (Fig. 7A) is found infrequently (9 and 7%, respectively), and the combination had not been observed previously. The Phe (F) at position 344, which is characteristic of PBMC virus, is quite rare in the data base, having been found at a frequency of 2%. The latter amino acid could also be quite critical to immune recognition since Berzofsky and colleagues (67) have shown that conversion between Tyr and Val yields reciprocal murine CTL specificities in the context of 15-residue peptides from the MN and IIIB (HXB2) isolates of HIV-1 (double underlined in Fig. 7B). Furthermore, they found that CTL recognizing the MN peptide also recognized the corresponding SC peptide (underlined in Fig. 7B) (67), which is identical to that found in three PBMC-derived clones from patient MA. In contrast, the MN sera did not recognize the RF or WMJ-2 peptides and the IIIB-specific CTL recognized only the IIIB sequence (underlined in Fig. 7B) (67).

V3 encodes the type-specific principal neutralizing domain of the virus, and divergence within this region has been shown to result in escape from neutralization (33, 34). However, changes within the envelope but outside the V3 region also affect neutralization, including recognition of the V3 domain (34, 54). V3 peptides and sera raised against them can provide type-specific blocking activity of virus infection and fusion, allowing distinction between the MN, RF, and HXB2 sequences (25). Sequences within as well as surrounding the conserved central GPG affect neutralization sensitivity, including single amino acid substitutions such as an Arg-to-Gly change at position 334 in mutant 5/1 (34) (Fig. 7C). Last, V3 can also encode determinants for host range. For example, substitution of a central portion of V3 between the Bal and HXB2 viruses will result in a reversal of macrophage versus T-cell tropism (24) (underlined in Fig. 7C).

In summary, intrapatient variation in gp120 was qualitatively similar to the interpatient variation that has been observed to date. This, together with the findings of others which demonstrate significant biologic diversity with subtle mutational changes in the envelope gene, suggests the potential for significant intrapatient functional diversity which can be best explored through the use of reconstructed viruses which permit examination of *env* function within the context of the entire protein in which these changes have occurred naturally.

The desire to evaluate the functional consequences of viral genetic variation in the context of the genome in which these sequences reside resulted in our efforts to develop methods of efficient large-fragment PCR. However, the efficiency we obtained for cloning these fragments was extremely low, and some clones we did obtain acquired deletions upon regrowth in *E. coli*. We had no success cloning stable 4.6-kb fragments from cocultivated virus, but stable plasmids were formed at high frequency when just the gp120-coding sequence was amplified and cloned from cocultivated virus. At least 2 of 12 ~4.6-kb PBMC-derived clones we studied contained bacterial insertion elements in the HIV transmembrane (TM)-coding region, suggesting that this very low frequency insertion event (it had otherwise never been observed in more than 6 years of use of this host and vector in our laboratory) increased the stability of the plasmid and thereby resulted in a selective advantage, perhaps through interruption of the HIV open reading frame which produced a toxic product in the bacterial host. Support for such a mechanism is provided by the finding that peptides derived from the C-terminal portion of lentiviruses, including HIV-1, are toxic for bacterial as well as mammalian cells (36). SIV (unpublished data) and equine infectious anemia virus (55a) are also stably propagated with very low efficiency in bacteria; thus, the possibility that cloned lentivirus sequences undergo further selection in procaryotic hosts must also now be considered.

Selection in bacteria would be harder to detect by using standard bacteriophage lambda cloning technology since these sequences are not analyzed until after a few rounds of propagation and screening and there is selective pressure to maintain only those clones which retain inserts of a minimum size. The use of low-copy-number plasmid cloning vectors (55a, 72) and a spontaneous derivative of the JM109 cells used in this study (19a) has been found to greatly increase the cloning efficiency of certain SIV genomes that had previously been intractable to molecular cloning (14a, 19b) and may increase the efficiency of cloning large fragments of HIV-1 genomes in the future.

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