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Toshiaki Kodama

Dawn P. Wooley
Wright State University - Main Campus, dawn.wooley@wright.edu

Daniel P. Silva

Fulvia DiMarzo Veronese

Ronald C. Desrosiers

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Strain-Specific Neutralizing Determinant in the Transmembrane Protein of Simian Immunodeficiency Virus

TOSHIKII KODAMA,1 DAWN P. W. BURNS,1 DANIEL P. SILVA,1 FULVIA DI MARZO VERONESE,2 AND RONALD C. DESROSIIERS1*

New England Regional Primate Research Center, Harvard Medical School, One Pine Hill Drive, Southborough, Massachusetts 01772,1 and Advanced Bioscience Laboratories, Inc., Kensington, Maryland 208952

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Monoclonal antibody SF8/SE11, which recognizes the transmembrane protein (TMP) of simian immunodeficiency virus of macaque monkeys (SIVmac), displayed strict strain specificity. It reacted with cloned and uncloned SIVmac251 but not with cloned SIVmac142 and SIVmac239 on immunoblots. This monoclonal antibody neutralized infection by cloned, cell-free SIVmac251 and inhibited formation of syncytia by cloned SIVmac251-infected cells; these activities were specific to cloned SIVmac251 and did not occur with the other viruses. Site-specific mutagenesis was used to show that TMP amino acids 106 to 110 (Asp-Trp-Asn-Asn-Asp) determined the strain specificity of the monoclonal antibody. This strain-specific neutralizing determinant is located within a variable region of SIVmac and human immunodeficiency virus type 2 (HIV-2) which includes conserved, clustered sites for N-linked glycosylation. The determinant corresponds exactly to a variable, weak neutralizing epitope in HIV-1 TMP which also includes conserved, clustered sites for N-linked glycosylation. Thus, the location of at least one neutralizing epitope appears to be common to both SIVmac and HIV-1. Our results suggest a role for this determinant in the viral entry process. Genetic variation was observed in this neutralizing determinant following infection of a rhesus monkey with molecularly cloned SIVmac239; variant forms of the strain-specific, neutralizing determinant accumulated during persistent infection in vivo. Selective pressure from the host immune response in vivo may result in sequence variation in this neutralizing determinant.

The simian immunodeficiency viruses (SIVs) are nonhuman primate lentiviruses related to the human immunodeficiency viruses (HIV-1 and HIV-2), the causative agents of AIDS in humans. SIV has been isolated from several species of Old World primates, including rhesus monkeys (SIVmac) (6), African green monkeys (SIVagm) (35), sooty mangabeys (SIVsm) (12, 33), and mandrills (SIVmdm) (47). The extensive similarity of SIV to HIV in genetic organization and biological properties suggests that SIV systems are ideally suited for study of AIDS pathogenesis and vaccine strategies. Analysis of nucleotide sequence homology and antigenic cross-reactivity has demonstrated that SIVmac and SIVsm are closely related to HIV-2 (3, 9, 15, 18). The three viruses SIVmac, SIVsm, and HIV-2 likely comprise a discrete subgroup of primate lentiviruses. Thus, SIV genetic relationships also provide clues to the evolution, origin, and possible interspecies transmission of lentiviruses in primates.

One of the striking features of HIV-1 is the extent of genetic variation among virus isolates (8, 16, 53). Variation is largely concentrated in the outer membrane protein (gp120) of the envelope (8, 16, 45), which is a major target of host immune responses. The transmembrane protein (TMP) of HIV-1 is relatively conserved in its sequence (16, 45). A major neutralizing epitope in HIV-1 has been localized to one of the variable regions of gp120, the V3 cysteine loop (14, 37, 40). In addition, neutralizing epitopes have been identified in other regions of the gp120 molecule (19, 20) and in the TMP (4, 5, 20). Neutralizing epitopes in SIV have not yet been identified. The extent to which neutralizing epitopes in SIV correspond to those in HIV-1 is not known.

In this report, we identify an SIVmac neutralizing determinant defined by monoclonal antibody (MAb) SF8/SE11 and show that it corresponds to a neutralizing epitope in HIV-1 TMP. The determinant is located in a variable region of the TMP which includes conserved, clustered sites for N-linked glycosylation. Furthermore, genetic drift in this neutralizing determinant was observed in a persistently infected rhesus monkey that was previously inoculated with molecularly cloned SIVmac239 and had a strong antibody response to the infecting virus.

MATERIALS AND METHODS

MAb. MAb SF8/SE11, which recognizes the TMP of SIVmac, was previously isolated by using detergent-disrupted uncloned SIVmac251 as an immunogen (49). The immunoglobulin subclass of the MAb was determined to be G1, and it was used as mouse ascitic fluid.

Radiolabeling of cells and immunoprecipitation assay. CEMx174 cells infected with cloned SIVmac251 were labeled with [35S]methionine, and immunoprecipitations were performed as previously described (48). For tunicamycin treatment, cells were suspended for 1 h in methionine-free medium containing 3 μg of tunicamycin per ml and subsequently labeled for 5 h at 37°C with [35S]methionine (200 μCi/ml). The same concentration of tunicamycin was maintained during the labeling period. Labeled cells were washed and disrupted in phosphate-buffered saline containing 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 0.5% sodium deoxycholate. The lysates were preabsorbed with protein A-Sepharose bound to rabbit antiserum to mouse κ
light chains (K-PAS) and then clarified. Radioimmunoprecipitation analysis was performed by the addition to 1 ml of the labeled extract with 1 μl of the MAb and 0.2 ml of a 10% sucrose solution in K-PAS. The samples were then incubated for 2 h at 4°C. Immunoprecipitates were resuspended in Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Neutralization assay. Cell-free virus neutralizing activity of the MAb was analyzed by incubating 100 μl of a 1:50 dilution of cloned SIVmac (approximately 200 tissue culture infectious doses) with 25 μl of heat-inactivated SF8/SE11 for 1 h at 37°C and inoculating the mixture onto 5 × 10⁵ CEMx174 cells or HUT-78 cells in 1 ml of RPMI 1640 medium containing 10% fetal bovine serum, 1% heat-inactivated MAb SF8/SE11, and penicillin-streptomycin (complete RPMI 1640 medium). After 24 h, the cells were washed once and resuspended in complete RPMI 1640 medium. Resultant SIV production was monitored at the indicated times by measurement of reverse transcriptase activity in the culture supernatant (6).

Syncytium inhibition assay. Syncytium inhibition assays were performed in 96-well microtiter plates by mixing 2.5 × 10⁴ CEMx174 cells with 10⁵ SIVmac-infected CEMx174 cells in the presence or absence of 10 μl of MAb SF8/SE11 in a total volume of 100 μl. Plates were then incubated for 24 to 48 h at 37°C, and the extent of syncytium formation in each well was evaluated by microscopic examination.

SIVmac molecular clones. The infectious lambda clones designated SIVmac142, SIVmac251, and SIVmac239 have been described previously (3, 25, 34). The SIVmac239 lambda clones have been subcloned in two sets of plasmid subclones containing the left half (p239SpSp5') and the right half (p239SpE3') of the viral genome.

Oligonucleotide synthesis. Oligodeoxyribonucleotides were synthesized by the phosphoramidite method on a Cyclone DNA synthesizer (Biosearch), using reaction columns containing derivatized CPG support (Milligen) and purified with C₁₈ Sep-Pak cartridges (Millipore).

Construction of mutants by PCR. Mutants in the viral envelope gene were derived from plasmid p239SpE3' by site-specific mutagenesis using polymerase chain reaction (PCR) amplification. The samples were incubated for 18 h by a unique, centrally located Spht site (24). This generated two pBS (Stratagene) plasmid subclones containing the left half (p239SpSp5') and the right half (p239SpE3') of the viral genome.

DNA transfection. An SIVmac239 left-half clone (p239SpSp5') was ligated with parental and mutant right-half clones after digestion with Spht and transfected into CEMx174 cells with DEAE-dextran (34). Virus replication was monitored by measurement of reverse transcriptase activity in the culture supernatant. Virus particles concentrated from the culture supernatant were used for immunoblot analysis (27).

Analysis of envelope variability in vivo. Total cell DNA was prepared from peripheral blood mononuclear cells at 69 and 93 weeks postinfection and from a lymph node biopsy taken at 69 weeks from a rhesus monkey persistently infected with molecularly cloned SIVmac239. A fraction of the lymph node taken at 69 weeks and a fraction of the peripheral blood mononuclear cells taken at 93 weeks were each cocultivated with CEMx174 cells for a total of 51 and 41 days, respectively, and the recovered virus was used to prepare Hirt supernatant DNA. The total cell and Hirt supernatant DNAs were used as templates in PCR to specifically amplify full-length env sequences between nucleotides 5644 and 9249 (38). For the total cell DNAs, two rounds of 30 PCR cycles were performed. The first round of PCR was performed with an outer set of oligonucleotide primers (6474 to 6493) and 9249 to 9220, followed by an inner set of primers (6544 to 6573 and 9220 to 9249) complementary to the amplified product of the first round. For the Hirt supernatant DNAs, only the inner set of primers was used in one round of PCR. The inner primers contained SstI sites for cloning. The PCR reaction conditions were in accordance with those of the manufacturer (Perkin-Elmer Cetus), and the following cycle profile was used: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. An autoextension of 10 s was added to each PCR cycle, and a 10-min final extension at 72°C was added to the last cycle. The 2.7-kb amplified env fragments were restricted with SstI and cloned into PBS' (Stratagene) for subsequent double-stranded DNA sequence analysis.

Nucleotide sequence accession number. The SIVmac239 sequence reported here has been filed with the GenBank database under accession number M33262.

RESULTS

Strict strain specificity of MAb SF8/SE11. Previously it was demonstrated that MAb SF8/SE11 reacted with the TMP of
uncloned SIVmac251 but did not cross-react with SIVsm or SIVmne (49). Therefore, the MAb seemed to recognize a specific epitope in SIVmac TMP. The MAb predominantly precipitated envelope precursor (gp160) from metabolically labeled CEMx174 cells infected with cloned SIVmac251 (Fig. 1, lane 1). After tunicamycin treatment of the same cells to block the synthesis of the dolichyl phosphate-linked oligosaccharide precursor (10), the MAb precipitated a polypeptide of 80 kDa, which is approximately the predicted size of the unglycosylated form of the envelope precursor of SIVmac251 (Fig. 1, lane 2). Therefore, SF8/5E11 recognized an epitope located in the protein backbone of SIVmac TMP, and carbohydrates were apparently not involved in the MAb recognition.

Reactivity with three molecular clones of SIVmac was analyzed by immunoblotting to examine whether the epitope recognized by the MAb was commonly present in the human CD4-positive cell line CEMx174 (Fig. 2). The SIVmac142 and SIVmac251 cloned viruses have truncated forms of TMP (28 to 30 kDa; Fig. 2), reflecting the presence of in-frame stop codons in their TMP coding regions (3, 27, 34); the SIVmac239 infectious clone does not have a premature in-frame stop codon in its TMP coding region, and it produces virus with full-length (41-kDa) TMP following short-term culture in the human CD4-positive cell line CEMx174 (Fig. 2) (27). The MAb reacted strongly with the 30-kDa TMP of cloned SIVmac251. However, the MAb reacted only very weakly with TMP of cloned SIVmac142 (the reactivity cannot be seen in Fig. 2), and it did not react detectably with cloned SIVmac239.

To determine whether the epitope recognized by the MAb in the TMP was expressed on the surface of cells infected with SIVmac251, we performed a live-cell immunofluorescence assay. CEMx174 cells infected with SIVmac251 were incubated with a 1:100 dilution of SF8/5E11, washed, and then incubated with fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G. Cells were further washed and examined under a fluorescence microscope. Expression of the epitope recognized by the MAb was clearly detected on the surface of the infected cells (data not shown).

To examine potential biological activity of the MAb, two assays were performed. In a cell-free virus neutralization assay (Fig. 3), cloned SIVmac251 and cloned SIVmac239 were used to infect HUT-78 cells and CEMx174 cells, respectively. MAb SF8/5E11 exhibited neutralizing activity with cloned SIVmac251 cell-free virus, but it did not neutralize cloned SIVmac239 virus. The MAb was also examined for its ability to inhibit syncytium formation between uninfected and infected CEMx174 cells (Table 1). Inhibition of syncytium formation by SF8/5E11 was also strain specific. The MAb inhibited syncytium formation with cloned SIVmac251-infected cells, but it did not prevent syncytium formation with cloned SIVmac142- and cloned SIVmac239-infected cells. Thus, the strict strain specificity of the MAb with respect to reactivity on immunoblots paralleled results with the biological assays.

**Identification of determinants of strain specificity.** These data indicated that the MAb recognized a specific neutralizing determinant within a variable region of the SIVmac TMP. We compared the TMP sequences of the three SIV mac infectious clones to obtain insight into the epitope recognized by MAb SF8/5E11. The sequences of SIVmac142 TMP (3) and SIVmac239 TMP (27) have been previously published. We sequenced DNA in the TMP region of the SIVmac251 infectious clone and compared it with the previously published sequences (Fig. 4). Sequence differences at only two sites could possibly explain the specificity of the MAb. Valine at TMP amino acid (aa) 172 was unique to the SIVmac251 infectious clone. However, this amino acid is located in the putative membrane-spanning domain and would not be a likely candidate for recognition by the MAb. Aspartate at TMP aa 110 was also unique to the SIVmac251 infectious clone, so we focused our additional experiments on this region. To verify that the strain specificity of the
MAB was due to the amino acid differences in this region. SOE was used to change individual TMP amino acids in SIVmac239 at positions 110 and 106. In SIVmac239tm-1, the Glu at TMP aa 110 in SIVmac239 was changed to Asp, i.e., the amino acid that is present at this location in SIVmac251 (Fig. 5A). This was accomplished by changing a G to T at bp 8508 in the SIVmac239 sequence. Since SIVmac239 also differs from SIVmac251 at TMP aa 106, a second mutant of SIVmac239, called SIVmac239tm-2, was constructed in which both Glu-110 → Asp and Lys-106 → Asp changes were introduced (Fig. 5A). The strategy for site-specific mutagenesis is outlined in Fig. 6 and in Materials and Methods. The mutant forms were confirmed by DNA sequencing. Plasmid DNAs with the described mutations were transfected into CEMx174 cells, and virus production was monitored by assay of reverse transcriptase activity. The mutant viruses were fully replication competent. Virus particles were concentrated from the culture supernatant 2 weeks after transfection and analyzed by immunoblotting. Although the predicted molecular weights of the TMP polypeptide backbones of SIVmac239, SIVmac239tm-1, and SIVmac239tm-2 are essentially the same, SIVmac239tm-1 TMP was approximately 2 kDa smaller than wild type and SIVmac239tm-2 by SDS-PAGE (Fig. 5B). SIVmac239tm-1 showed only weak reactivity to MAB SF8/5E11. However, SIVmac239tm-2 with changes at both positions 106 and 110 reacted strongly with the MAB, as strongly as SIVmac251 (Fig. 5B). Thus, the TMP amino acid sequence Asp-Trp-Asn-Asn-Asp at position 106 to 110 determines the strain-specific reactivity and neutralization by SF8/5E11.

Samples (1 μg) of overlapping peptides 25 amino acids in length spanning this region (SIVmac251 TMP aa 86 to 110 and 103 to 127) were spotted onto nitrocellulose and tested for reactivity to MAB SF8/5E11. SIVmac-positive rhesus monkey sera reacted with both peptides. However, specific reactivity of the MAB to these peptides containing the 106 to 110 region of SIVmac251 could not be demonstrated (data not shown). A lambda gt11 expression library containing 100-bp average inserts from the full-length genome was screened by plating approximately $8 \times 10^4$ phages onto Escherichia coli Y1090 cells. After plaques developed, nitrocellulose filters impregnated with isopropyl-b-D-thiogalactopyranoside (IPTG) were overlayed and incubated overnight. The filters were blocked and reacted with SF8/5E11 for 1-h. SF8/5E11 failed to react positively with any of the E. coli recombinant proteins (data not shown).

CEMx174 cells were infected with parental cloned SIVmac239 and with mutants SIVmac239tm-1 and SIVmac239tm-2, and by 4 weeks more than 80% of the cells expressed viral antigens. The virus-infected cells were cocultivated with uninfected CEMx174 cells in the presence or absence of MAB SF8/5E11. Similar extents of syncytium formation were observed in cultures infected with parental SIVmac239 and mutants SIVmac239tm-2. However, cells infected with SIVmac239tm-1 showed considerably weaker syncytium formation (Table 2). Inhibition of syncytium formation with SIVmac239tm-2-infected cells by the MAB further demonstrated that TMP amino acids from 106 to 110 were critical determinants of epitope recognition by the MAB.

**Genomic variation.** This strain-specific neutralizing determinant is located within a variable region of SIVmac which includes three potential sites for N-linked glycosylation (N-X-S or N-X-T, where X is any amino acid) (Fig. 7). The TMP amino acid sequences are generally well conserved among SIVmac, SIVsm, and HIV-2 (75 to 89% overall homology); this conservation is especially evident in the amino-terminal region, which shows 90% identity (18, 54). The three potential N-linked glycosylation sites are completely conserved among SIVmac, SIVsm, and HIV-2 (Fig. 7), but extreme sequence variation is observed between and around the clustered N-linked glycosylation sites, including the neutralizing determinant of MAB SF8/5E11. Furthermore, this region corresponds exactly to an antigenic, slightly variable (30, 52), weak neutralizing epitope (20) in HIV-1 TMP which also includes conserved, clustered sites for N glycosylation (Fig. 7). HIV-1 env aa 612 to 635 (HIV-1 TMP aa 101 to 124) were previously reported by Ho et al. (20) to be a weak neutralizing epitope.

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**TABLE 1. Syncytium inhibition by SF8/5E11**

<table>
<thead>
<tr>
<th>Cloned virus</th>
<th>Syncytium formation&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>+SF8/5E11</td>
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<tr>
<td>SIVmac251</td>
<td></td>
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<tr>
<td>SIVmac239</td>
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<td>SIVmac142</td>
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<sup>a</sup> CEMx174 cells were mixed with cloned SIVmac-infected CEMx174 cells in the presence or absence of MAB SF8/5E11. The extent of syncytium formation was evaluated at 48 h.
FIG. 4. Sequence comparison of TMP regions of three SIVmac molecular clones. The predicted amino acid sequences that differ from SIVmac251 are shown. Overlines indicate potential sites for N-linked glycosylation. Translational in-frame stop codon in SIVmac251 is indicated by an asterisk. Boxes indicate the positions of amino acids that are unique to SIVmac251.

We next investigated whether genetic changes accumulated in this region following persistent infection of a rhesus monkey. Rhesus monkey 243-86 was infected with molecularly cloned SIVmac239, developed a strong, stable antibody response, and remained persistently infected. We analyzed 16 clones isolated 69 and 93 weeks postinfection directly from blood, directly from a lymph node biopsy, and from recovered virus (Fig. 8). All 16 clones displayed amino acid substitutions arising from individual point mutations within and around this neutralizing determinant. In this region, 69% (43 of 62) of the nucleotide substitutions were first- and second-base changes which changed the amino acid. Eleven of sixteen clones exhibited an Asn-to-Asp conversion at aa 108 within the determinant defined by SF8/5E11. Interestingly, all seven clones from recovered virus and one clone from peripheral blood mononuclear cells had Asn at aa 102 instead of the parental Ser; this amino acid substitution resulted in displacement of one of the potential N-linked glycosylation sites two codons downstream.

DISCUSSION

The env genes of HIV-1 exhibit significant heterogeneity at both the nucleotide and deduced amino acid levels. Amino acid variations appear as clustered mutations interspersed with polypeptide segments that are highly conserved (45, 52). The conserved regions likely contribute to the stability of a constant structure and function. Since variable regions

FIG. 5. Identification of the neutralizing determinants recognized by MAb SF8/5E11. (A) Site-specific mutagenesis of SIVmac239. The sequences of oligonucleotide primers and strategy for SOE are outlined in Materials and Methods. In SIVmac239tm-1, the Glu at aa 110 was changed to Asp by changing a G-to-T at bp 8764 in the SIVmac239 sequence. In addition to this mutation, the Lys at aa 106 was changed to Asp in SIVmac239tm-2. (B) Reactivity of mutant SIVmac239 with MAb. Mutants p239SpE3'tm-1 and -tm-2 were ligated with plasmid p239SpSs5' after digestion with SpeI and transfected separately into CEMx174 cells (See Fig. 6). The virus was concentrated from the culture supernatants and analyzed for immunoblot reactivity with MAb SF8/5E11. The size of each TMA is indicated. Pos. Cont, SIVmac-positive rhesus monkey serum; wt, parental, wild-type SIVmac239.
The SIVmac239 molecular clone causes fatal immunodeficiency in approximately 50% of rhesus monkeys within 1 year of infection (24). The strength of the antibody response in infected monkeys can be predictive of the outcome of infection; monkeys with weak antibody responses die within 6 months with AIDS (7, 24). However, longer-term survivors with strong antibody responses remain persistently infected (7, 24). Recent results suggest that genetic variants in env are selected during persistent infection of rhesus monkeys with molecularly cloned SIVmac239 (2). All 16 clones that were analyzed in the current study exhibited extensive sequence changes within and around the strain-specific neutralizing determinant defined by MAb SF8/5E11 (Fig. 8). Thus, this variable, strain-specific, neutralizing determinant is likely to be a critical target or to interact with a critical target of the host immune response; flexibility of sequence in this region may facilitate continued survival and persistence of the virus in the presence of an active immune response. For two other lentiviruses, equine infectious anemia virus and visna virus, progressive changes in the envelope gene have been suggested to be directly responsible for the periodic or chronic course of the disease (32, 36, 43).

The env gene products of HIV and SIV are heavily glycosylated proteins, and potential sites of N-linked glycosylation are well conserved (45, 52). The importance of N-linked glycosylation for early stages of HIV-1 entry has been described previously (17, 31, 51). Sequence analysis predicts three potential sites for N-linked glycosylation in the TMP of SIVmac. These three sites, which are well conserved in SIVmac, SIVsm, and HIV-2, all cluster around the neutralizing determinant defined by SF8/5E11. Although considerable variation in the SIV TMP neutralizing determinant was observed in vivo in our current study, all 16 clones preserved sites for N-linked glycosylation, including the 8 clones in which one glycosylation site was displaced (Fig. 8). Tunicamycin treatment demonstrated that carbohydrate moieties are not involved in recognition by the MAb. Since the MAb neutralized viral infectivity and inhibited syncytium formation, the aa 106 to 110 region of the TMP is likely to be important for the viral entry process.

Whether
N-linked carbohydrates in this region may also play a role in cellular receptor or in fusion with host cell membranes, is currently under investigation.

At present, it is not obvious why the size of the TMP and the ability of virus to form syncytia were altered by the mutation at TMP codon 110 in SIVmac239tm-1. The single amino acid substitution at TMP position 110 could possibly affect the extent or nature of the glycosylation and thus alter its mobility through SDS-polyacrylamide gels. It is tempting to speculate whether the reduced capacity for syncytium formation by SIVmac239tm-1 may be related to altered glycosylation in the aa 106 to 110 region of the TMP.

Our results indicate that SIVmac TMP aa 106 to 110 are essential for formation of the epitope recognized by MAb SF8/5E11. However, we were not able to demonstrate specific reactivity of MAb SF8/5E11 to 25-amino-acid-long peptides spanning the region of interest nor to E. coli recombinant libraries making viral fusion proteins. It is still possible that the MAb directly recognizes sequences around TMP positions 106 to 110, but this recognition may be highly dependent on secondary structure that is not present in synthetic peptides or E. coli recombinant protein. Alternatively, it is possible that MAb SF8/5E11 recognizes a complex conformational epitope or a distal epitope whose secondary structure is influenced by the 106 to 110 region. Recently, a murine MAb to HIV-1 gp41 was suggested to require a contiguous, conformational epitope for recognition (23).

Sequence variation in the 106 to 110 region of SIV TMP was much more limited in the seven clones from recovered virus than in the clones directly obtained from peripheral blood or lymph node (Fig. 8). Therefore, there may be cell culture selection for particular variants in this region during the virus recovery and expansion process. Meyerhans et al. have presented evidence for cell culture selection for individual rat gene variants (29), while Simmonds et al. observed no cell culture selection for individual gag variants (44).

Several functional epitopes in the TMP of HIV-1 have been identified previously, including a fusion domain (13, 28), neutralization epitope (4, 5, 20), immunosuppressive domain (26, 39), and cytotoxic T-lymphocyte epitope (46). The fusion domain of SIVmac has been localized to the amino terminus of the TMP (1), just as in HIV-1. However, other functional domains of SIV TMP have not been identified. The strain-specific neutralizing determinant in the variable region of SIVmac TMP demonstrated in this report corresponds in location to a variable, neutralizing epitope previously identified in HIV-1. Thus, the location of at least one neutralizing epitope appears to be common to both SIVmac and HIV-1. Continued studies along these lines will be important for validating SIV models for vaccine develop-
ment and will hopefully shed light on fundamental principles of virus entry, replication, and pathogenesis.

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