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

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Monoclonal antibody SF8/5E11, 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 mangabey monkeys (SIVsm) (12, 33), and mandrills (SIVmnd) (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) SF8/5E11 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 SF8/5E11, 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 [^{35}S]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 [^{35}S]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

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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% suspension of K-PAS. The samples were incubated for 18 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 SF8/5E11 for 1 h at 37°C and inoculating the mixture onto 5×10^5 CEMx174 cells or HUT-78 cells in 1 ml of RPMI 1640 medium containing 10% fetal bovine serum, 1% heat-inactivated MAb SF8/5E11, 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 2.5×10^4 CEMx174 cells with 10^4 SIVmac-infected CEMx174 cells in the presence or absence of 10 μl of MAb SF8/5E11 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 clone has been subcloned into two separate DNA segments by using a unique, centrally located SphI site (24). This generated two pBS (Stratagene) 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 C_{18} 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) (41). PCR amplifications were performed with an automated DNA Thermal Cycler (Perkin-Elmer Cetus) for 20 cycles (each consisting of a 1-min 94°C denaturation, a 2-min 55°C annealing, and a 5-min 72°C extension step), followed by a single 10-min extension at 72°C. Then 1 μM each primer, 2.5 U of Taq polymerase, 1.5 mM $MgCl_2$, and 200 μM each deoxynucleoside triphosphate were used in the PCR reaction mixture.

Site-specific mutagenesis by overlap extension (SOE) requires introduction of nucleotide changes within oligonucleotide PCR primers and a secondary PCR amplification to incorporate changes into an internal site within the amplified DNA fragment (21, 22). Two mutants with defined substitutions within the envelope TMP of SIVmac239 were generated by using pairs of complementary mutagenic oligonucleotide primers representing nucleotides 8491 to 8517 (38). Primer 5'-CCA AAG TGG AAC AAT GA̲T̲ ACT TGG CAA-3' and its complementary oligonucleotide were used to generate mutant p239SpE3'tm-1, and primer 5'-CCA G̲A̲T̲ TGG AAC AAT GA̲T̲ ACT TGG CAA-3' and its complement generated mutant p239SpE3'tm-2. Underlined letters identify nucleotide changes that were introduced. The outer set of oligonucleotide primers consisted of 5'-AGTGCA

GAGGTGGCAGAACT-3' (8050 to 8069) and 5'-TGTCCCT CACAAGAGAGTGAGCTCAAGCCC-3' (9220 to 9249). Two segments (8050 to 8517 and 8491 to 9249) were generated in separate reactions, each containing one set of outer and inner mutagenic primers and 0.1 μg of plasmid p239SpE3'. For overlap extension by PCR, amplified products from the first round of PCR which shared overlapping sequences at the ends to be joined were purified from an agarose gel and mixed in a subsequent PCR reaction containing additional outer primer pairs. The resulting PCR-generated recombinant products were purified from an agarose gel and restricted with ClaI and BglII, and the fragments were inserted into the ClaI-BglII site of p239SpE3'. Since the recombinant plasmid (p239ΔBglII) lost the 2.8-kb BglII fragment containing the 3' long terminal repeat region through the cloning step, the fragment was reinserted to generate p239SpE3'tm-1 and p239SpE3'tm-2. The nucleotide sequence surrounding the site of SOE in the mutant clones was confirmed by primer-directed dideoxy-chain termination sequencing (42).

DNA transfection. An SIVmac239 left-half clone (p239SpSp5') was ligated with parental and mutant right-half clones after digestion with SphI 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 6544 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 9279 to 9298), while the second round was performed with 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 data base under accession number M33262.

RESULTS

Strict strain specificity of MAb SF8/5E11. Previously it was demonstrated that MAb SF8/5E11 reacted with the TMP of
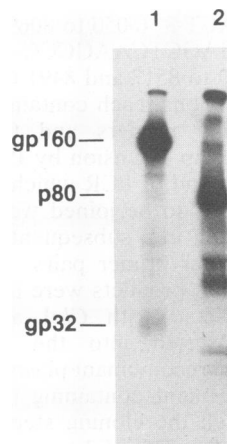
FIG. 1. Immunoprecipitation of SIVmac251 unglycosylated en-
velope precursor with MAb SF8/5E11. CEMx174 cells infected with
cloned SIVmac251 were treated with no inhibitor (lane 1) or 3 µg of
tunicamycin per ml (lane 2) and labeled for 5 h with [³⁵S]methionine.
Radiolabeled protein in the cell lysates was immunoprecipitated
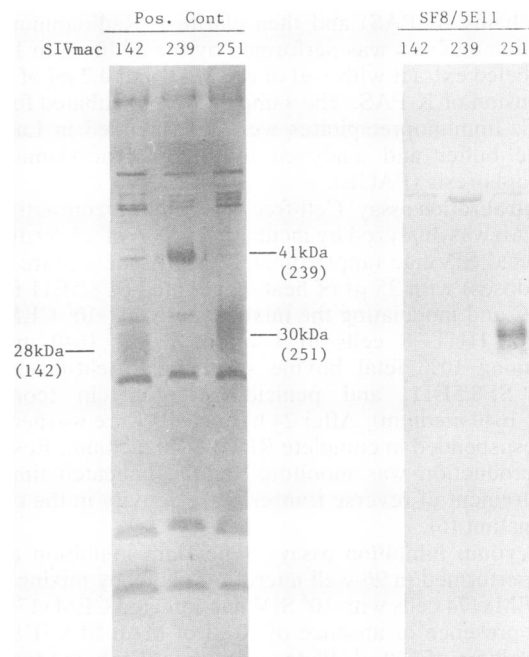with MAb SF8/5E11 and analyzed by SDS-PAGE.

FIG. 2. Strain-specific reactivity of MAb SF8/5E11 to cloned
SIVmac251 TMP by immunoblotting. The SIVmac142, SIVmac239,
and SIVmac251 cloned viruses were concentrated from the culture
supernatants and analyzed for immunoblot reactivity, using SIV-
mac-positive rhesus monkey sera (Pos. Cont) and MAb SF8/5E11.
The size of each TMP is indicated.

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 dolichol phosphate-linked
oligosaccharide precursor (10), the MAb precipitated a poly-
peptide 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 SIVmac
(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-posi-
tive cell line CEMx174 (Fig. 2) (27). The MAb reacted
strongly with the 30-kDa TMP of cloned SIVmac251. How-
ever, 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 immunofluores-
cence 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 neu-
tralize cloned SIVmac239 virus. The MAb was also exam-
ined 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 SIV-
mac251-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 neutraliz-
ing 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 previ-
ously 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
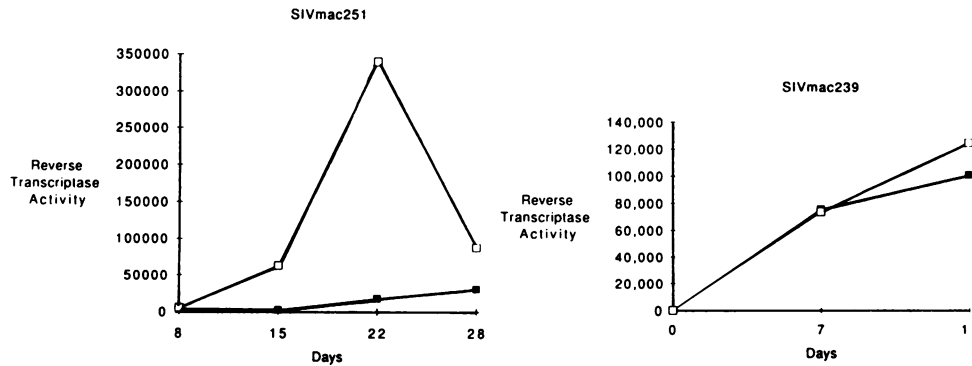on this region. To verify that the strain specificity of the

FIG. 3. Cell-free virus neutralizing activity of MAb SF8/5E11. Approximately 200 tissue culture infectious doses of cloned SIVmac251 and SIVmac239 were incubated with (■) or without (□) MAb SF8/5E11 for 1 h at 37°C and inoculated onto cultured cells as described in Materials and Methods. Resultant SIV production was monitored by assay of reverse transcriptase activity in the culture supernatant.

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 8×10^4 phages onto Escherichia coli Y1090 cells. After plaques developed, nitrocellulose filters impregnated with isopropyl-β-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 SIVmac 239tm-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.

TABLE 1. Syncytium inhibition by SF8/5E11

Cloned virus	Syncytium formation[a]	
	+SF8/5E11	−SF8/5E11
SIVmac251	−	+++
SIVmac239	++	++
SIVmac142	+++	+++

[a] 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.

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                          10            20            30            40
SIVmac251 G V F V L G F L G F L A T A G S A M G A A S L T L T A Q S R T L L A G I V Q Q Q
SIVmac142                                                 V
SIVmac239

                          50            60            70            80
251 Q Q L L D V V K R Q Q E L L R L T V W G T K N L Q T R V T A I E K Y L K D Q A Q
142                                                   S
239

                          90           100           110          120
251 L N A W G C A F R Q V C H T T V P W P N A S L T P D W N N[D]T W Q E W E R K V D
142                                                        [E]
239                                                   K    [E]

                         130           140           150          160
251 F L E E N I T A L L E E A Q I Q Q E K N M Y E L Q K L N S W D V F G N W F D L A
142       A                                                                     T
239

              ┌───────── Putative anchor domain ─────────────┐
                         170           180           190          200
251 S W I K Y I Q Y G I Y[V]V V G V I L L R I V I Y I V Q M L A K L R Q G Y R P V F
142                       [I]I                                  R
239                       [I]  V

251 S S P P S Y F *
142             Q
239             Q
```
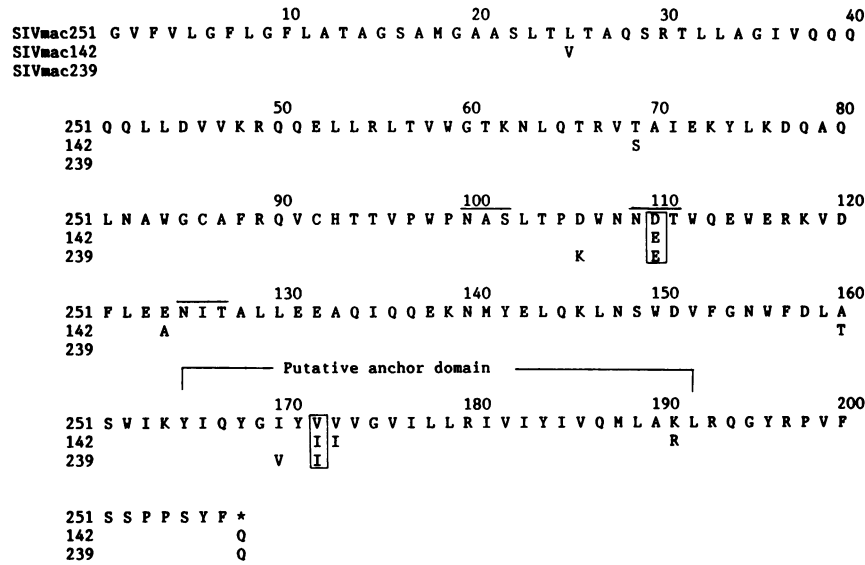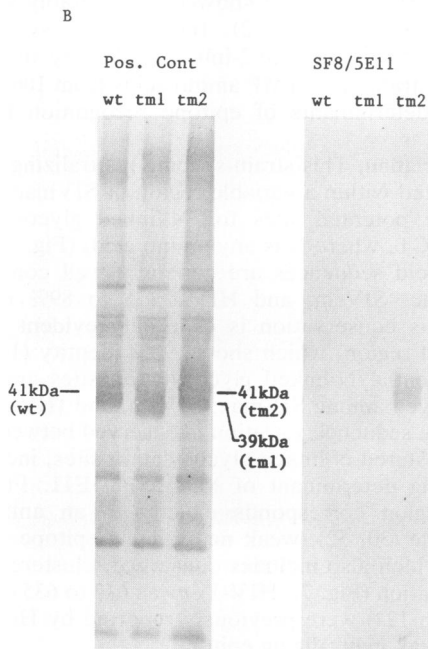
FIG. 4. Sequence comparison of TMP regions of three SIVmac molecular clones. The predicted amino acid sequences that differ from SIVmac251 are shown. Overlines indicate potential sites for N-linked glycosylation. Translational in-frame stop codon in SIVmac251 is indicated by an asterisk. Boxes indicate 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

A	105	106	107	108	109	110	111
SIVmac251	Pro	Asp	Trp	Asn	Asn	Asp	Thr
SIVmac142	Pro	Asp	Trp	Asn	Asn	Glu	Thr
SIVmac239wt	Pro	Lys	Trp	Asn	Asn	Glu	Thr
SIVmac239tm1	Pro	Lys	Trp	Asn	Asn	[Asp]	Thr
SIVmac239tm2	Pro	[Asp]	Trp	Asn	Asn	[Asp]	Thr

B

Pos. Cont   SF8/5E11

wt tm1 tm2   wt   tm1 tm2

41kDa—   —41kDa
(wt)   ⎱(tm2)
                          ⎰39kDa
                          (tm1)

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 p239SpSp5' after digestion with *Sph*I 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 TMP is indicated. Pos. Cont, SIVmac-positive rhesus monkey serum; wt, parental, wild-type SIVmac239.
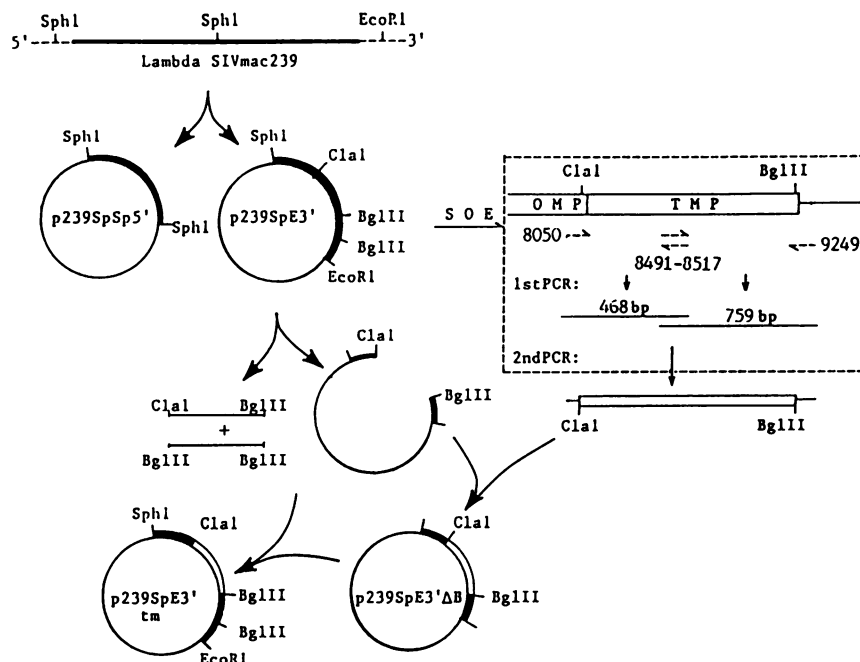
FIG. 6. Construction of mutants by using SOE. Plasmid p239SpE3′ was used as template DNA for SOE. Defined nucleotide changes were introduced in complementary inner oligonucleotide primers (8491 to 8517), and the mutations were incorporated into an internal site of the amplified DNA fragment by secondary PCR amplification as outlined in Materials and Methods. OMP, Outer membrane protein.

in the envelope have properties of predicted high antigenicity (30, 45), pressures from the host immune response to infection may select for variant virus strains. In fact, the RP135 epitope in hypervariable region V3 of gp120 has been identified as a major, strain-specific neutralization epitope of HIV-1 (14, 37, 40). Alignment of the predicted envelope protein sequences of SIVmac, SIVsm, and HIV-2 also shows alignment of conserved and variable domains and apparent conservation of overall structure (3, 15, 18, 54). The strain-specific neutralizing determinant described in this report is located within a variable region of the TMP that includes conserved sites for N-linked glycosylation and is flanked by conserved domains. This region exactly corresponds to the antigenic, slightly variable (30, 52), weak neutralizing epitope (20) in the TMP of HIV-1 which also has conserved N-linked glycosylation sites.

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

TABLE 2. Syncytium inhibition of mutant SIVmac239tm-2 by SF8/5E11

Virus	Syncytium formation[a]	
	+SF8/5E11	−SF8/5E11
SIVmac239	++	++
SIVmac239tm-1	+	+
SIVmac239tm-2	−	++

[a] CEMx174 cells were cocultivated with CEMx174 cells infected with parental cloned SIVmac239 and with mutants SIVmac239tm-1 and SIVmac239tm-2 in the presence or absence of MAb SF8/5E11. The extent of syncytium formation was evaluated at 24 h.

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
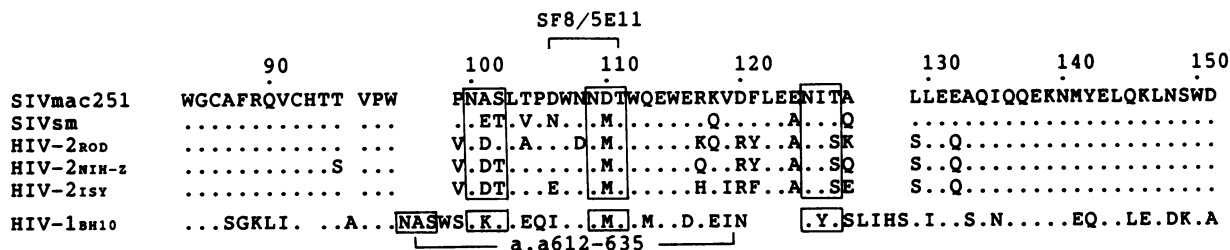
FIG. 7. Amino acid sequence variation around potential N-linked glycosylation sites of the TMP of SIVmac, SIVsm, and HIV-2. The predicted amino acid sequences of the TMP region of SIVsm (18), HIV-2$_{ROD}$ (15), HIV-2$_{NIH-Z}$ (54), HIV-2$_{ISY}$ (11), and HIV-1$_{BH10}$ (50) are aligned with those of SIVmac251. The predicted amino acid sequences that differ from SIVmac251 are shown. Boxes indicate potential sites for N-linked glycosylation. The determinant of recognition by MAb SF8/5E11 is indicated. Envelope protein aa 612 to 635 in HIV-1 correspond to TMP aa 101 to 124 of HIV-1.

N-linked carbohydrates in this region may also play a role in early stages of virus entry, possibly in recognition of a 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 reproduced 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 tat 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-
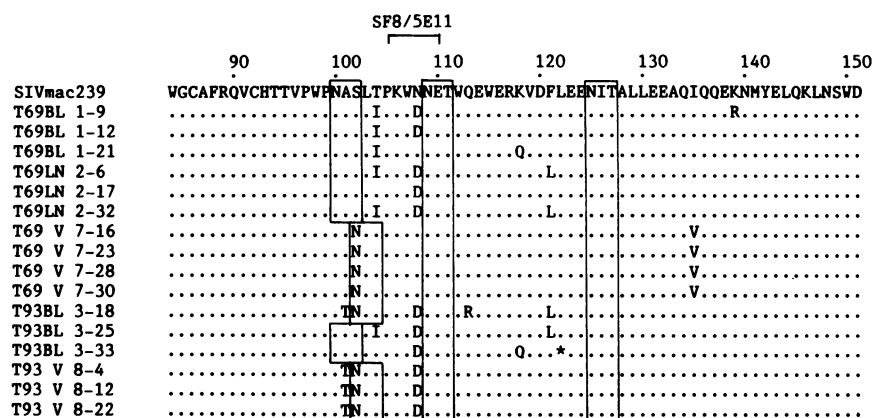

FIG. 8. In vivo genetic drift of the SIVmac TMP variable region. T69 and T93 refer to the number of weeks postinfection of cloned SIVmac239. Sources of DNA for PCR amplification were blood (BL), lymph node (LN), and recovered virus (V). The predicted amino acid sequences that differ from SIVmac239 are shown. Potential sites for N-linked glycosylation are indicated by boxes. The asterisk indicates a translational in-frame stop codon.

ment and will hopefully shed light on fundamental principles of virus entry, replication, and pathogenesis.

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