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Persistence of Simian Immunodeficiency Virus Mne Variants upon Transmission

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In macaques infected with a clone of simian immunodeficiency virus (SIV) Mne, viral variants consistently evolve multiple new potential glycosylation sites in the first variable region (V1) prior to the development of AIDS. In the present study, we asked whether viruses with these glycosylation sites persist when they are transmitted to a naive macaque. Variants that evolved after transmission to a recipient macaque were compared with virus that evolved in the donor, which had been infected by cloned SIV Mne. Upon transmission, the specific serine/threonine-rich motifs potentially encoding novel O-linked glycosylation site(s) in V1 were conserved in virus isolated from lymph node, spleen, and liver tissue from the recipient. There was some accumulation of changes in V3 of envelope in virus from the recipient, whereas changes in this region were not observed in virus from the donor macaque. Some variants detected in the tissue of the recipient at necropsy were most closely related to viruses present in the donor inoculum even though these particular variants were not detected early after infection in the recipient's peripheral blood mononuclear cells. Overall, virus with the predominant V1 sequences associated with progression to disease are transmitted to and persist in the recipient animal.

Infection of macaques with simian immunodeficiency virus (SIV) provides a model system in which to study viral variation throughout the course of infection and disease in individuals infected with a virus with a known sequence. Several groups have examined temporal genetic variation of the SIV envelope (*env*) gene in macaques infected with pathogenic clones of SIV (2, 6, 12). These studies demonstrated that variation within *env* occurs primarily in discrete variable regions, several of which are analogous to the hypervariable regions of the human immunodeficiency virus type 1 (HIV-1) *env* gene. One principal difference between SIV and HIV-1, however, is that the third variable region (V3), a region coding for the principal neutralizing epitope of the HIV-1 envelope (4, 9, 13, 16), is relatively conserved in SIV (2, 6, 12). In studies which correlated SIV Mne *env* variation with disease progression, it was shown that variation is largely confined to V1 prior to the development of AIDS and to V1 and V4 after prolonged disease (11, 12). These studies have demonstrated a consistent pattern of *env* changes during infection with a clone of SIV Mne. The most extensive divergence of late variants of SIV Mne from the inoculum within *env* was observed in V1 and was concentrated in two hypervariable regions. Within these hypervariable regions, amino acid changes to serine and threonine residues predominate. These changes lead to sites which may code for potential O-linked and N-linked glycosylation modifications. Similar sequence motifs in V1 evolved in four SIV Mne-infected macaques that developed simian AIDS (11).

We were interested in determining what happens when an animal is infected with virus representing late variants of SIV Mne. Of particular interest is whether the common changes seen in the SIV Mne *env* gene as macaques progressed to disease, particularly the threonine/serine-rich sequences in V1, persist in virus transmitted to another macaque. In addition, we wanted to determine if the nature of the infecting virus governed the overall pattern of variation in *env*. To address these questions, we characterized *env* sequences in peripheral blood mononuclear cells (PBMCs) and tissues from a macaque (the recipient macaque) infected with a pool of variant SIV genomes passaged directly from a macaque (the donor macaque) in the early stages of simian AIDS. In addition, we characterized *env* sequences from tissue at necropsy from the donor macaque to compare continued *env* variation in a single animal with variation after in vivo passage.

In vivo passage of virus. To address these questions, samples were available from an earlier study performed by Hu et al. (5) that generally fit our experimental criteria. A donor macaque (*Macaca nemestrina* M89152) was inoculated intravenously with approximately five macaque infectious doses of SIV Mne E11S, a biological clone of SIV Mne (1). SIV Mne CL8 was molecularly cloned from a library of genomic DNA from the E11S cell line. The *env* sequence of SIV Mne E11S has been determined and is essentially identical to that of SIV Mne CL8 (14). Macaque M89152 seroconverted at 4 weeks postinoculation (p.i.), displayed transient fever and lymphadenopathy beginning at 2 weeks p.i. and anemia at 63 weeks p.i., and died at 106 weeks p.i., with wasting and oral shigella. At 43 weeks after initial virus challenge, during the period when the animal's $CD4^+$ lymphocytes were in rapid decline, an uncloned mixture of PBMCs (2×10^7 cells) and lymph node cells (2×10^7 cells) was used to intravenously inoculate an SIV-negative recipient animal (*M. nemestrina* M90076). SIV-specific antibody was detected in this animal by enzyme-linked immunosorbent assay as early as 2 weeks p.i. The recipient macaque displayed lymphadenopathy, fever, and anemia beginning 2 weeks p.i. and died at 38 weeks p.i. with B-cell lymphoma. Typically, animals infected with the biological clone E11S develop AIDS 1 to 3 years p.i. (8, 11).

Cloning of SIV *env* genes. Genomic DNA was purified from cells of the donor macaque (M89152) that were used as the inoculum (PBMCs and lymph node tissue from 43 weeks p.i.) and from this macaque's spleen and liver tissue at necropsy.

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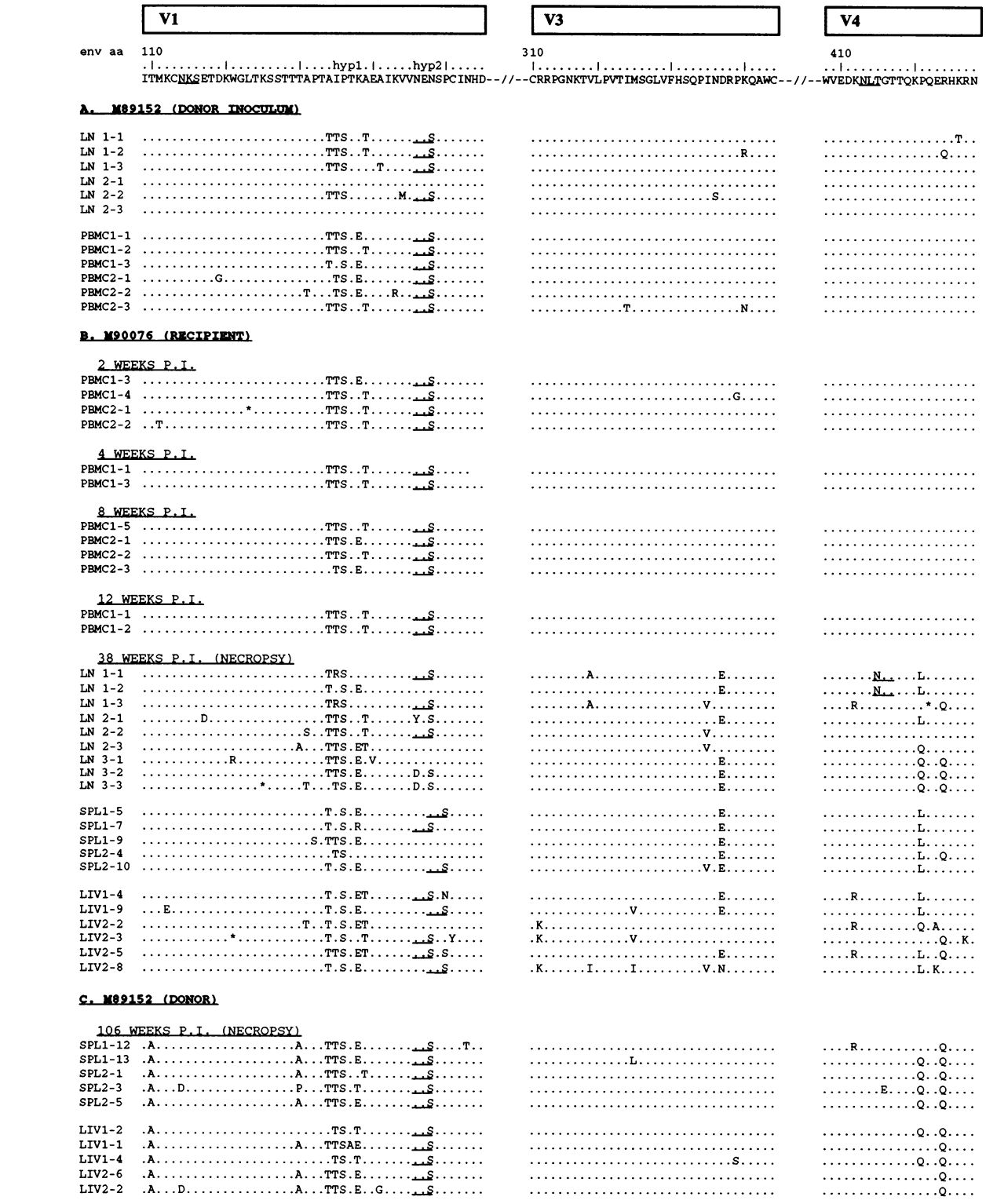

FIG. 1. Comparison of the predicted amino acid (aa) sequence of SIV Mne CL8 with those of SIV variants present in the tissues of the donor (M89152) and recipient (M90076) macaques. The amino acid sequences of previously defined V1, V3, and V4 of SIV Mne CL8 *env* (12) are shown in single-letter code, with double slashes to indicate where the sequence is discontinuous; the envelope amino acid number for SIV Mne CL8 (GenBank number M32741) is shown. The two previously defined hypervariable regions in V1 (V1-hyp1 [amino acids 134 to 139] and V1-hyp2 [amino acids 146 to 150]) are indicated above the amino acid sequence. Predicted amino acid differences in the clones from macaques M89152 and M90076 are shown below. Dots, no change in amino acid; underlined amino acids, a consensus sequence for N-linked glycosylation (NXT/S); asterisk, stop codon. Clones were isolated from PBMCs and from lymph node (LN), spleen (SPL), and liver (LIV) tissues. Clones are clustered by independent PCRs (1-, 2-, or 3-). (A) Clones derived from the donor macaque (M89152) PBMC and lymph node DNA at 43 weeks p.i.; (B) clones derived from the recipient macaque (M90076) PBMC DNA at 2, 4, 8, and 12 weeks p.i. and lymph node, spleen, and liver DNAs at 38 weeks p.i.; (C) clones derived from the donor macaque (M89152) spleen and liver DNAs at 106 weeks p.i.

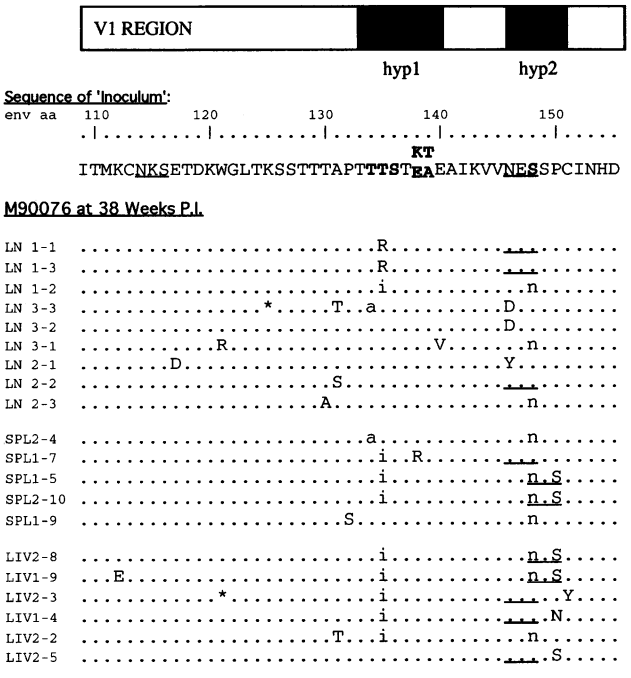

FIG. 2. Comparison of the predicted V1 amino acid (aa) sequences of the predominant SIV variants isolated early after infection of the recipient macaque (M90076) with clones isolated at necropsy. The two most common genotypes of variants isolated early after inoculation of macaque M90076 are shown on the top line. Amino acid differences from SIV Mne CL8 are represented by boldface type. Two predominant V1-hyp1 genotypes are found at 2 weeks p.i. in the recipient macaque (M90076), and both motifs are represented in the sequence of the inoculum shown on the top line; thus, the two inoculum sequences include two amino acids at positions 138 and 139. Variation of clones from the recipient animal (M90076) at necropsy (38 weeks p.i.) from either of the inoculum sequences is shown below. Amino acids found in SIV Mne CL8 but not in the inoculum sequence are in lowercase letters. Abbreviations are as described in the legend to Fig. 1.

Genomic DNA was purified from recipient (M90076) PBMCs at 2, 4, 8, and 12 weeks p.i. and from lymph node, spleen, and liver tissue at necropsy. SIV *env* sequences, spanning the majority of gp120, were amplified from genomic DNA and cloned into a M13 phage vector using primers and methods described previously (11). Multiple gp120 sequences from each sample were typically obtained from two independent PCR amplifications.

Sequence diversity in the donor inoculum. We analyzed V1, V3, and V4 of *env* from multiple clones derived from the donor inoculum (Fig. 1). Changes in V1 are concentrated in two previously defined hypervariable regions (V1-hyp1 and V1-hyp2) (11). Within V1-hyp1, there are extensive changes toward threonine and serine residues that may be targets for potential O-linked glycosylation (11). Within V1-hyp2, an Asn→Ser change codes for a new potential N-linked glycosylation site in four of six lymph node- and six of six PBMC-derived clones. There are few changes from the parental SIV Mne clone outside the hypervariable regions of V1 or in V3 or V4. Thus, the pattern of *env* variation at this time point is similar to what is seen in other SIV Mne-infected animals at or prior to the onset of immunodeficiency (12).

Envelope variation in PBMC-derived clones and lymph node-derived clones is generally similar, indicating that both cell types harbor a similar proviral population. Clones from both cell types possess the same predominant V1-hyp1 motif, TTSTKT. Overall, there are five unique V1-hyp1 sequences among the PBMC-derived clones and three unique V1-hyp1 sequences from lymph node tissue. However, two of six clones derived from lymph node tissue are identical to SIV Mne CL8 in V1, V3, and V4. Envelope sequences corresponding to wild-type SIV Mne have been isolated from the lymph node tissue of another macaque infected with SIV Mne E11S (11).

Sequence diversity in the recipient macaque. Clones were derived from macaque M90076 PBMCs at 2, 4, 8, and 12 weeks p.i. (Fig. 1B). We chose to isolate two to four clones from several early time points rather than multiple clones from a single PCR at one time point because previous experiments suggested that cloning from multiple PCRs gives the best representative of proviral diversity (15). Each of the 12 clones are similar to the predominant *env* genotypes detected in the donor inoculum. The similarity of these clones to the donor inoculum suggests that the proviral population in the donor is characteristic of the virus that is being transmitted to the recipient animal during infection with cell-associated virus. The diversity of clones from these time points is limited. There is one predominant SIV variant containing a TTSTKT motif in V1-hyp1 that is seen in 9 of 12 clones. This V1-hyp1 motif was the most common genotype seen in the donor inoculum and was detected in both PBMCs and lymph node tissue. A second V1-hyp1 motif, TTSTEA, is seen in 2 of 12 clones and was detected at 2 weeks p.i. In V1-hyp2, all 12 clones possess the Asn→Ser change, which leads to a potential N-linked glycosylation site. There is only one amino acid change in V3 and none in V4.

Clones were derived from lymph node, spleen, and liver tissue from the recipient animal at necropsy (Fig. 1B). The changes to serine and threonine residues that evolved in V1-hyp1 in the donor animal are conserved, despite considerable variation elsewhere in V1 as well as in V3 and V4. Figure 2 illustrates variation in V1 of *env* if we consider the virus with the two predominant V1-hyp1 motifs seen at 2 weeks p.i. as the parental sequence. At necropsy, changes in V1 are more scattered than what was observed at earlier time points. The threonine and serine residues in V1-hyp1 are generally conserved; the only exceptions are at amino acids 134 and 135. However, the predominant differences at these positions are the same as amino acids found in the wild-type SIV Mne clone, and both the alanine at position 134 and the isoleucine at position 135 are found in variant clones from the donor inoculum (Fig. 1A [clones PBMC2-1 and PBMC1-3]), suggesting that the viruses with these amino acids in the donor macaque (M89152) may have been transmitted. The threonine and serine at positions 134 and 136 are conserved in 90 and 100% of the clones, respectively. In contrast, only about half of the clones from lymph node, spleen, and liver tissue have the previously ubiquitous potential N-linked glycosylation site in V1-hyp2. Thus, while there is continued variation in V1-hyp2 and elsewhere, the serine/threonine-rich V1-hyp1 is relatively conserved.

The other regions that we analyzed display numerous amino acid differences compared with earlier time points (Fig. 1B). The changes observed in the recipient macaque (M90076) in V4 include the shift of an N-linked glycosylation site (in two of nine clones from lymph node tissue) that was also observed in one animal infected with SIV Mne CL8 (12). Other predominant changes in V4 were to amino acids frequently found in other SIV isolates at conserved positions (11). While it is difficult to draw conclusions from a single animal, variation in

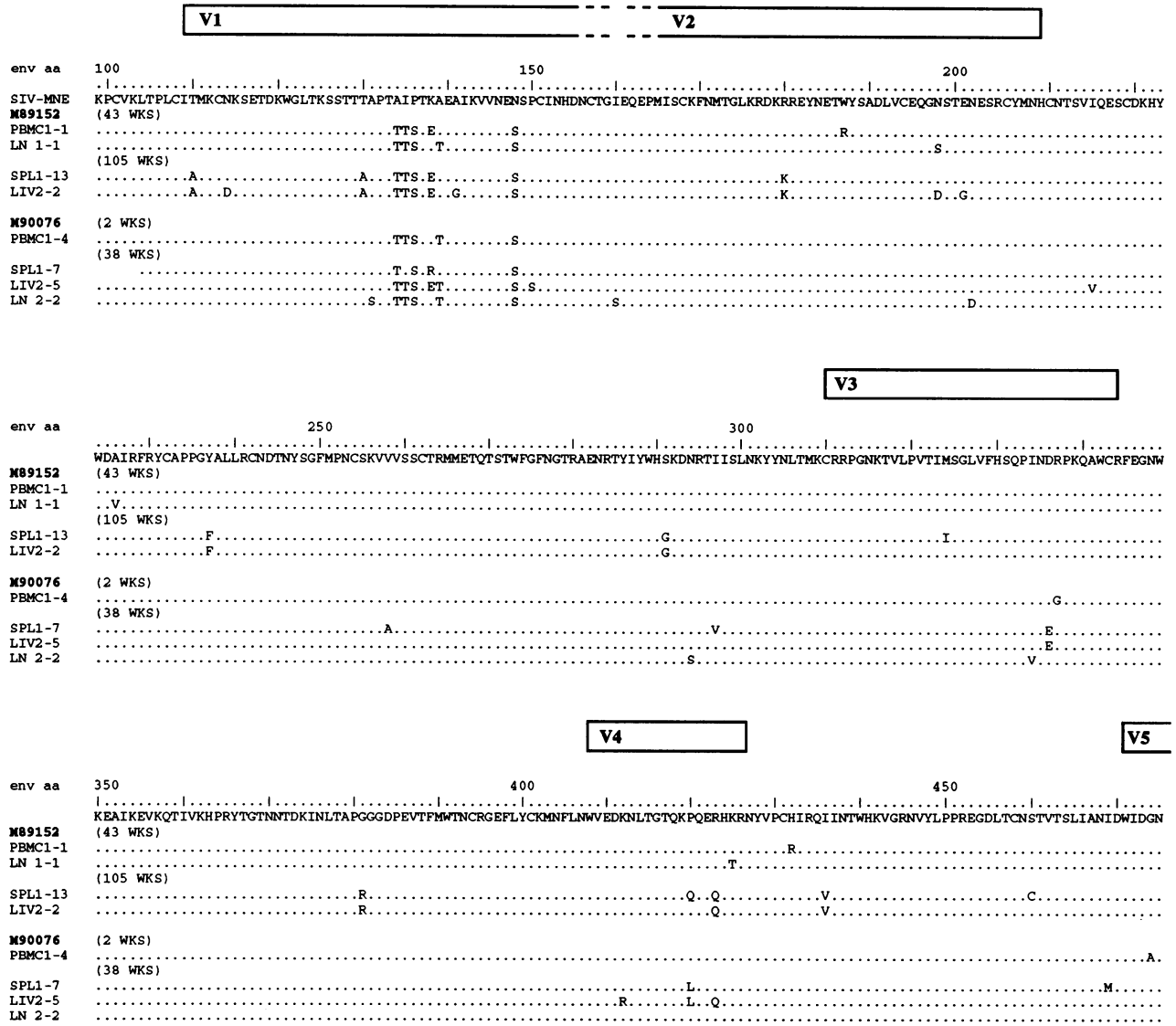

FIG. 3. The complete predicted amino acid (aa) sequence of the amplified region of *env* from randomly selected SIV variants compared with the sequence of SIV Mne CL8. Clones were chosen from all cell types from both animals. The arrangement of sequences and other abbreviations are similar to those described in the legend to Fig. 1.

V3 was more extensive than has been observed in previous studies of SIV Mne-infected macaques. Many of the common amino acid changes observed in V3 were not seen in the SIV Mne variants from any of the four animals analyzed previously or the donor animal in this study. However, the most common change, Asp-337→Glu, (seen in six of nine lymph node clones, five of five spleen clones, and three of six liver clones) was detected as a minor population in PBMC-derived clones from very late time points in one SIV Mne-infected individual which survived more than 2 years with AIDS (11, 15). A glutamic acid at this position is found in other SIV isolates, including SIVsmH-4 and SIVsmmPBj. Unlike in our previous study (11), we observed no changes adjacent to one of the conserved cysteines just outside the V3 loop (data not shown).

Envelope variation in the donor macaque at necropsy. To compare *env* variation of virus as it continued to replicate in the donor macaque with variation of virus in the recipient macaque, clones were derived from the donor macaque's spleen and liver tissue at necropsy, 106 weeks p.i. (Fig. 1C). In general, this animal harbors a virus population that is similar to what is seen late in infection in other animals infected with cloned SIV Mne. That is, variation in V1 is concentrated, but not limited to, the hypervariable regions. All 10 clones have the potential N-linked glycosylation site in V1-hyp2. Variation in V4 is limited, by and large, to two sites in which variation has been observed previously (11, 12) and is similar to changes seen in the recipient macaque tissue at necropsy. V3 is very conserved. The lack of variation in V3 and conservation of the N-linked glycosylation site in V1-hyp2 are striking differences between variation in the donor and the recipient. There is no further evolution in these regions in virus from the donor macaque, despite the fact that the donor macaque was infected for 25 weeks longer (from the time of transmission) than the recipient macaque.

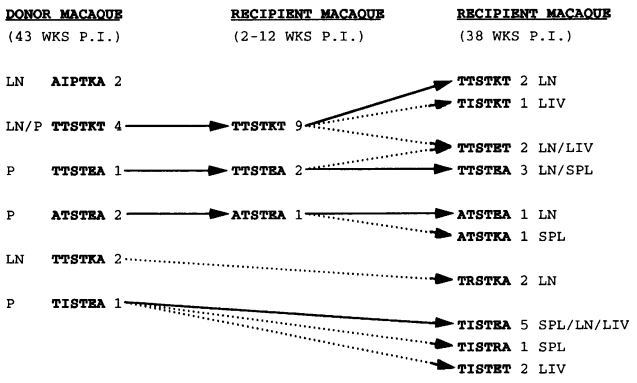

FIG. 4. Comparison of V1-hyp1 amino acid sequences between donor (M89152) and recipient (M90076) macaques. The three columns present V1-hyp1 sequences (*env* amino acids 134 to 139) of clones amplified from PBMC (P) and lymph node (LN) DNAs from the donor macaque inoculum (at 43 weeks p.i.), from PBMC DNA from 2 to 12 weeks p.i. from the recipient macaque, and from PBMC, lymph node, spleen (SPL), and liver (LIV) DNAs from the recipient macaque at necropsy (38 weeks p.i.), respectively. Immediately following each V1-hyp1 amino acid sequence is the number of clones which contain this specific genotype. Solid lines between columns indicate the probable parental sequence, on the basis of identity of the V1-hyp1 sequence. Some clones from the recipient macaque at necropsy are not identical to a clone from either the donor inoculum or the recipient at 2 to 12 weeks p.i. The most likely parental sequence(s) is identified by a dotted line; these clones differ from each other by a single amino acid in V1-hyp1.

In a study of *env* variation in SIVmac239-infected rhesus macaques, Kodama et al. reported little variability in V1 and high variability in the region between V1 and V2 and from V3 through C3 in brain, gut, lung, and lymph cells (7). In that study, there was little V1 variation in tissue; taken with the results presented here, we hypothesize that this may reflect the fact that V1 of SIVmac239 *env* already has the threonine/serine-rich sequence that evolves during SIV Mne CL8 or E11S infection (11). It may be that once an appropriate V1 sequence evolves, then variation in V3 (and other regions) is allowed. Although it is possible that different cells or tissues harbor different viral variants, our data suggest that the viral variants that are observed in tissue are similar to those found in PBMCs. Variants found in tissue were similar to variants found in PBMCs at late stages of infection in each of four other macaques that were infected with cloned SIV Mne (11). The similarity between tissue- and PBMC-derived variants was also observed in the donor macaque PBMCs and lymph node tissue at 43 weeks p.i.

Variation within gp120. V1 and V4 of SIV Mne *env* were defined as the most variable regions on the basis of analysis of PBMC-derived variants from macaques infected with cloned virus (12). To determine if there were new variable regions in tissue-derived variants or in variants after transmission, we determined the complete nucleotide sequence of the amplified region of *env* from one clone from each animal and each tissue type. The amino acid sequence of this region was compared with the sequence of SIV Mne CL8 (Fig. 3). In all four cell types that were examined, the most dramatic variation in gp120 relative to the parental SIV Mne clone that infected the donor macaque is concentrated in the V1 variable domain of *env*. Approximately 55% (48 of 86) of the observed amino acid changes map to V1, 26% (22 of 86) map to the regions corresponding to V2, V3, V4, and V5, and 19% (16 of 86) map to the remainder of *env*. About 97% of the 333 nucleotide changes found in V1 and V4 from all of the clones encoded an amino acid change, indicating strong selection for changes in these regions, consistent with earlier results (2, 12). Analysis of changes at the nucleotide level from all cell types (data not shown) confirms the high rate of G→A and A→G transitions that have been reported previously (2, 3, 6, 12).

Some donor variants not detected in PBMCs early after transmission are detected in tissue at necropsy. One predominant *env* genotype and two minor species were detected in PBMCs early after the infection of the recipient macaque despite the fact that the donor inoculum contained a diverse population of virus. This suggests that there may be some form of selection limiting the diversity of virus found in PBMCs after inoculation. Characterization of HIV-1 *env* genotypes from primary infection has shown that the initial virus population is homogenous within an individual (10, 17–19). These reports suggested that this selection is due to selected transmission of particular genotypes. Our data suggest two possibilities. The first is that only a subset of the viral population is transmitted and that the diversity of sequences seen at necropsy is due to the convergent evolution of this subset to viral genotypes similar to those seen in the donor. Alternatively, multiple genotypes are transmitted, but only a subset of the population is expressed early in infection. In this model, the diverse pool of virus persists and is expressed at later time points. This latter theory would account for the similarity of many of the V1-hyp1 genotypes seen at the death of the recipient animal with clones from the donor inoculum. Figure 4 traces the evolution of the V1-hyp1 sequences found in the recipient macaque at necropsy. Half of these V1-hyp1 sequences are related more closely to sequences found in the donor inoculum than sequences found in PBMCs early after inoculation of the recipient macaque. For example, virus with the V1-hyp1 sequences TISTEA, TISTRA, and TISTET isolated from three tissues from the recipient macaque at necropsy are most similar to virus from the donor inoculum with the V1-hyp1 sequence TISTEA. The fact that these viral genotypes, originally found in the donor's PBMCs, are detected in the liver, lymph node, and spleen tissue of the recipient suggests that posttransmission analysis of PBMC-derived virus may not reflect the diversity of the viral population in other tissues early in infection.

Conclusions. On the basis of envelope sequence, the predominant SIV variants associated with progression to disease persist when they are transmitted to an uninfected animal. Notably, changes in V1 of envelope in late variant viruses seen after SIV Mne CL8 or E11S infection, which may provide site(s) for O-linked glycosylation, were maintained upon passage in a single macaque. In the single macaque analyzed here, after transmission there was more variation in regions outside V1, especially V3, than we have seen in other macaques infected with cloned SIV Mne. The examination of viral genomes in the recipient macaque suggests that the variants found in PBMCs early may not represent the diversity of the transmitted virus population. Additional SIV transmission studies that include analysis of viral genomes present early in tissues are necessary to fully address this issue. Such studies may be particularly relevant, because the complexity of transmitted viruses may influence strategies for effective vaccines.

Nucleotide sequence accession numbers. The nucleotide sequences of subgenomic portions of each of the unique SIV *env* genes presented in Fig. 1 and 3 have been submitted to GenBank (accession numbers U06277 to U06422).

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