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Repair and Evolution of *nef* In Vivo Modulates Simian Immunodeficiency Virus Virulence

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Experimental evidence from the simian immunodeficiency virus (SIV) model of AIDS has shown that the *nef* gene is critical in the pathogenesis of AIDS. Consequently, *nef* is of considerable interest in both antiviral drug and vaccine development. Preliminary findings in two rhesus macaques indicated that a deletion of only 12 bp found in the overlapping *nef*/3′ long terminal repeat (LTR) region (9501 to 9512) of the SIVmacC8 molecular clone was associated with reduced virus isolation frequency. We show that this deletion can be repaired in vivo by a sequence duplication event and that sequence evolution continues until the predicted amino acid sequence of the repair is virtually indistinguishable from that of the virulent wild type. These changes occurred concomitantly with reversion to virulence, evidenced by a high virus isolation frequency and load, decline in anti-p27 antibody, substantial reduction in the CD4/CD8 ratio, and development of opportunistic infections associated with AIDS. These findings clearly illustrate the capacity for repair of small attenuating deletions in primate lentiviruses and also strongly suggest that the region from 9501 to 9512 in the SIV *nef*/3′ LTR region is of biological relevance. In addition, the ability of attenuated virus to revert to virulence raises fundamental questions regarding the nature of superinfection immunity.

Similarities between simian and human immunodeficiency viruses (SIV and HIV) in genome sequence and structure, gene regulation, biology, and disease induction make SIV infection of macaques a valid model of human HIV infection (reviewed in reference 51). The *nef* gene of primate lentiviruses, which is absent from all other animal lentiviruses, is situated at the 3′ end of the genome and overlaps the U3 region of the 3′ long terminal repeat (3′ LTR). *nef* is expressed early in the virus replication cycle (29) and encodes a myristylated, predominantly membrane-associated protein, the function of which remains controversial (10). Early studies suggested that *nef* acts as a negative regulatory factor of virus replication and transcription (1, 7, 41), but these findings were questioned (20, 30), and later studies demonstrated a positive effect of *nef* on the rate of viral replication (13, 39, 54). Nef may also downregulate interleukin-2 induction (36) and inhibit the induction of the transcription factors NF-κB and AP-1 (4) and was reported to possess both GTP-binding and GTPase activities (18), though these findings were subsequently refuted (25, 38, 40). Additionally, sequence comparisons have revealed limited homology with motifs seen in a range of diverse proteins (reviewed in references 21 and 49), leading to suggestions of other potential biological activities. Less controversially, results from studies in cell lines (5, 17, 18) and more recently with *nef* transgenic mice (37, 50) have established that *nef* downregulates CD4 expression, although the role of this in vivo remains unclear.

The *nef* gene products of both HIV and SIV are dispensable for replication in vitro (27, 35, 52). However, elegant experiments with the SIV model have shown that a functional *nef* gene is critical in vivo for full pathogenic potential. Rapid reversion of a premature stop codon in SIV *nef* occurs in vivo, resulting in a high virus load and rapid disease progression (28). In contrast, an SIV *nef* deletion mutant replicates only poorly in vivo and does not induce disease (28). Analysis of HIV *nef* variants obtained directly from clinical tissue also shows strong pressure for retention of an open *nef* gene (6, 12, 22), suggesting that HIV *nef* plays a similarly crucial role in vivo. The apparent vital role of *nef* in pathogenesis has made it an attractive target for attenuation in the design of model live attenuated vaccines. Recent studies have demonstrated that an attenuated SIV strain with a 182-bp deletion in *nef* can protect against subsequent challenge with virulent SIV (11).

Preliminary studies in our laboratory with two molecularly cloned viruses derived from SIVmac32H (an isolate derived by in vivo passage of SIVmac251 [9]) demonstrated that one, SIVmacC8, shows an attenuated phenotype when inoculated into two Chinese rhesus macaques relative to the other, SIVmacJ5, in terms of virus isolation frequency and absence of sequence variation in *nef* 10 months after infection (44). In contrast to SIVmacC8, SIVmacJ5 is known to be pathogenic, resulting in a higher-grade infection and AIDS-like pathology (19, 44). These two clones are isogenic except for a deletion and six nucleotide substitutions in SIVmacC8 relative to SIVmacJ5, all occurring in the 3′ LTR region. In the *nef*/3′ LTR overlapping region, SIVmacC8 has a 12-bp in-frame deletion (nucleotides 9501 to 9512) as well as three nucleotide substitutions, two of which are nonsynonymous changes (nucleotides 9646 and 9819, G to A and A to G, respectively), resulting in R to K and T to A changes at amino acids 191 and 249, respectively. A synonymous nucleotide substitution (G to A) is seen at nucleotide 9815. In the noncoding region, single base substitutions occur at positions 9931, 10061, and 10122 (A to G, A to G, and A to C, respectively). The positions of these changes relative to known regulatory elements are summarized in Fig. 1.

Seven rhesus macaques were each infected with $10^{4.7}$ 50% tissue culture infective doses of SIVmacC8 by intravenous in-

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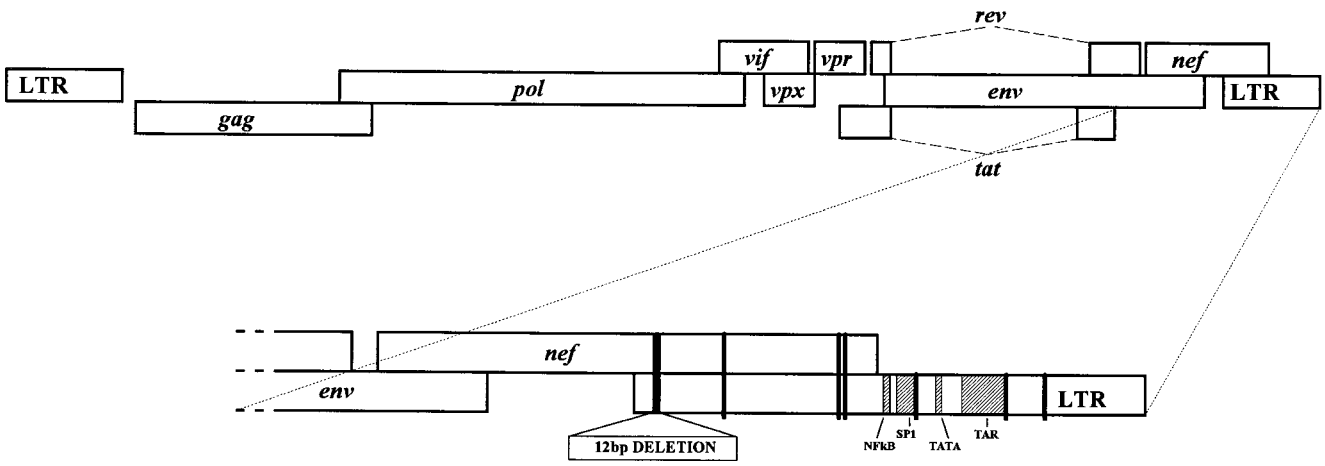


FIG. 1. Genetic structure of SIV. The expanded region shows differences seen in SIVmacC8 relative to SIVmacJ5. Sequence variation between these two molecular clones is confined to the extreme 3′ end of the genome. The location of the 12-bp deletion in SIVmacC8 is shown, and the six further nucleotide substitutions are shown as thick vertical lines. Of the three substitutions in the overlapping *nef*/3′ LTR, two encode conservative amino acid substitutions in *nef* and one is a silent change. Locations of major regulatory elements are indicated (NFκB, NFκB binding site; SP1, SP-1 binding sites; TATA, TATA box; TAR, *trans* activation response element). Details of the origins and construction of these clones have been published elsewhere (44).

oculation. All macaques were colony bred within the United Kingdom from animals of Indian origin and housed according to the Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989). Virus isolation was performed at intervals following infection. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by centrifugation on Ficoll Paque (Pharmacia, Milton Keynes, United Kingdom) at 1,500 × *g* for 25 min following 50% dilution in RPMI 1640. The interfacial band was removed, and the PBMC were washed in RPMI 1640 containing 10% fetal calf serum, counted, and cocultured with the human T-cell line C8166 in 25-cm^2 flasks. Medium and C8166 cells were replenished every 3 to 4 days, and the total culture volume was maintained at approximately 15 ml. Up to and including week 21, all cultures contained at least 2×10^6 PBMC, with the exception of animal 29R at week 10, when a low cell yield meant that only 1.2×10^6 cells were available. Subsequently, all cultures contained 10^6 PBMC. Cultures were maintained for at least 28 days or until cytopathic effect was seen. Culture results were confirmed by detection of SIV antigens by indirect immunofluorescence staining with simian anti-SIV serum. Within 17 weeks of infection with SIVmacC8, six of seven macaques became either persistently negative (11R and 19R) or intermittently positive (28R, 29R, 55R, and 57R) for virus isolation (Fig. 2A). In contrast, one animal (45R) remained persistently virus isolation positive.

At week 33, the proportion of virus-infected PBMC was determined by serial dilution. Separated cells were diluted from 1×10^6 to 4×10^5 and subsequently in fivefold steps to 130 cells per culture, and each culture was duplicated. The 50% endpoints were calculated by using the Kärber formula, and the results are expressed as the number of infected cells per 10^6 PBMC. These results confirmed that the number of virus-infected PBMC was markedly higher in animal 45R (at least 583-fold higher) than in other animals of the group (Fig. 3). Thus, in terms of virus recovery, the input attenuated SIVmacC8 in animal 45R showed a phenotype characteristic of the more virulent SIVmacJ5 virus (19, 45).

The clinical progression of AIDS has been associated with a decline in the anti-core antibody titer (16, 34). Antibody titers to the SIV p27 core antigen were determined in all animals by enzyme-linked immunosorbent assay (ELISA) with recombi-

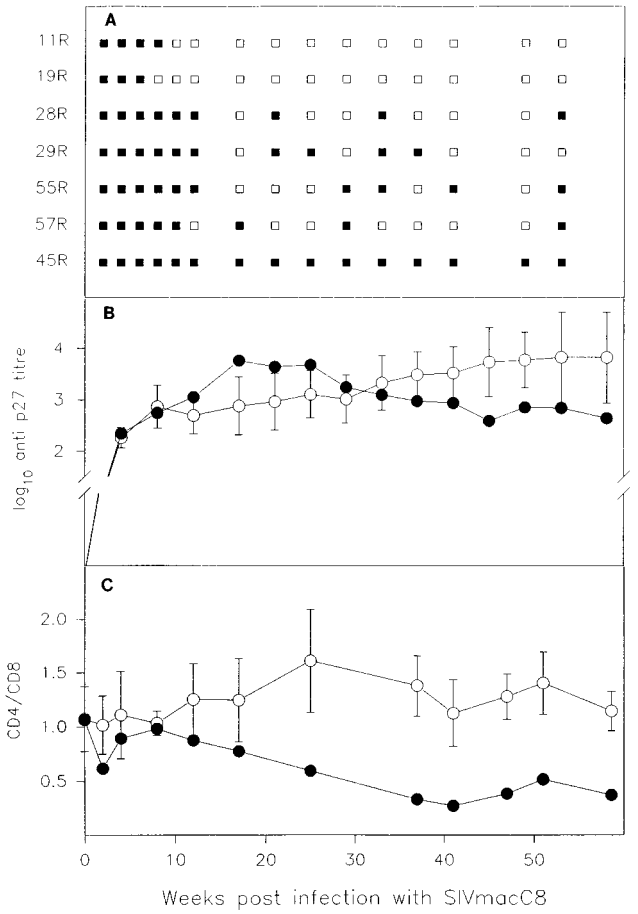


FIG. 2. Characterization of SIVmacC8 behavior in rhesus macaques. (A) Virus isolation profile following infection: ■, isolation positive; □, isolation negative. (B) Anti-SIV p27 titers in rhesus 45R (●) compared with those for the rest of the group (group mean ± 1 standard deviation [SD]) (○). (C) CD4/CD8 ratio in rhesus 45R (●) compared with that in the rest of the group (group mean ± 1 SD) (○).

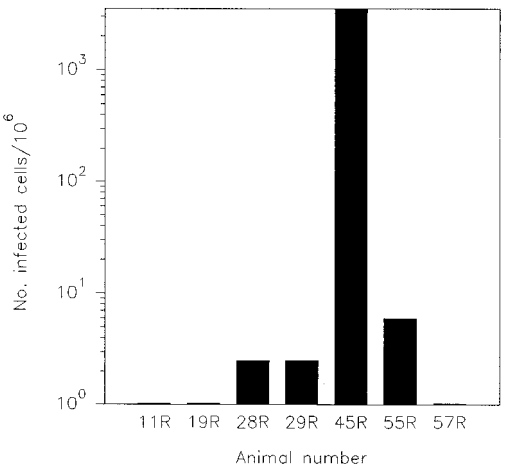


FIG. 3. Determination of virus-infected PBMC load at 33 weeks after infection with SIVmacC8. PBMC were diluted serially and cocultivated with C8166 cells as for standard virus isolation. Duplicate samples were analyzed for cell concentration, and the 50% endpoint was determined. Results are expressed as the number of virus-infected cells per 10^6 PBMC.

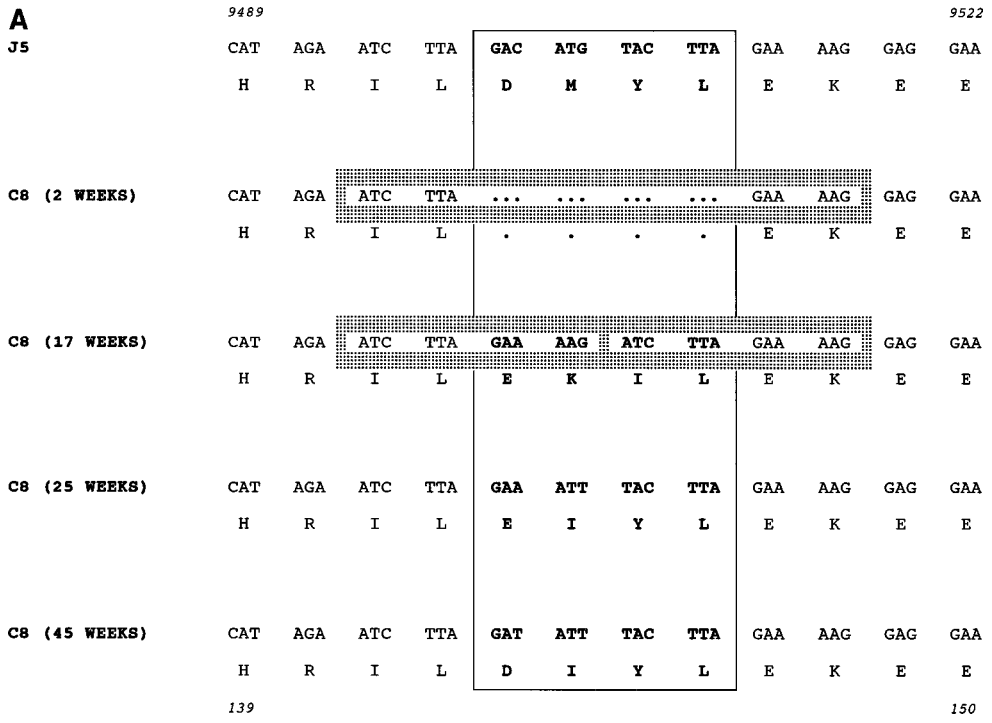
nant LacZ-p27 fusion protein essentially as described by Almond et al. (2) except that bound immunoglobulin G (IgG) was detected by using horseradish peroxidase-conjugated rabbit anti-monkey IgG (Sigma). The anti-SIV p27 antibody titer in animal 45R initially rose above the mean titer of the rest of the group. This higher response is more characteristic of infection with nonattenuated virus and is consistent with high virus load. Subsequently, after 17 weeks of infection, titers fell steadily, eventually declining to levels considerably below those of the rest of the group (Fig. 2B).

PBMC phenotyping was performed at intervals following infection. A whole-blood method was used to label PBMC with fluorescein-conjugated murine monoclonal antibodies specific for CD4 (OKT4; Ortho Diagnostic Systems Ltd., High Wycombe, United Kingdom) and CD8 (Dk25; Dako, High Wycombe, United Kingdom). Volumes (50 μl) of heparinized (5 to 10 IU/ml) whole blood were mixed with 15 μl of labeled monoclonal antibody or an isotype-matched control and incubated at room temperature for 1 h. After washing in 3 ml of phosphate-buffered saline (PBS), cells were suspended in 1 ml of Immunolyse solution (Coulter, Luton, United Kingdom) and mixed vigorously for 30 s. Subsequently, 3 ml of PBS containing 1% formaldehyde was added, and the cells were centrifuged for 8 min at 400 × *g* before resuspension in 1 ml of this solution. After standing overnight at room temperature, cells were analyzed with an EPICS Profile 1 flow cytometer (Coulter). The lymphocyte population was delineated by means of a forward scatter versus 90° light scatter histogram. A bitmap placed around this population was then used to gate the log fluorescence signals for analysis on single-parameter histograms, and 15,000 events were accrued. Total leukocyte counts and blood smear differential counts were determined manually. A sharp decline in the CD4/CD8 ratio was seen in animal 45R (Fig. 2C) beginning 12 weeks after SIVmacC8 infection, providing a further indication of disease progression. By 25 weeks postinfection, CD4 cell numbers had fallen to 55% of the preinfection level, and by 58 weeks, shortly before autopsy, the CD4 count was 0.5×10^6 cells per cm^3, which represents 28% of the preinfection value.

Consistent with the development of AIDS, the clinical condition of animal 45R declined rapidly, and necropsy and histopathology, 63 weeks after infection, revealed lymphocyte depletion from lymph nodes and spleen, colitis, small intestinal cryptosporidiosis, and *Pneumocystis carinii* pneumonia. Infection with *Cryptosporidium* sp. was detected by examination of sections stained by hematoxylin and eosin. Immunostaining with anti-*P. carinii* monoclonal antibody (M77S; Dako) in conjunction with streptavidin-biotin-peroxidase and diaminobenzidine was used to identify *P. carinii* in lung sections. All other animals in the group remained healthy.

In order to investigate sequence changes which might explain the reversion to virulence of SIVmacC8 in animal 45R, we determined almost the complete nucleotide sequence of the *nef*/3′ LTR region of PBMC-associated provirus at intervals following infection. DNA extractions from isolated PBMC were performed essentially as described previously (33), and a nested PCR procedure used to amplify proviral sequences. PCR amplification of the *nef*/3′ LTR region was performed by standard procedures (24) with 2×10^4 to 1×10^5 cell equivalents of DNA. The primer sequences used are shown with the position in the SIVmacJ5 clone sequence in parentheses. The outer primer pair were 5′-GGGATAATAGGACTATAAT-3′ (7479 to 7497) and 5′-GGGTCGACTGCTAGGGATTTTCC TGC-3′ (10260 to 10277). Second-round PCRs were performed by transferring 5 μl of the first-round PCR product to a fresh PCR with the primers 5′-CGGGATCCCTGACCTAC CTACAATATG-3′ (9059 to 9077) and 5′-GGGTCGACAG CAGAAAGGGTCCTAAC-3′ (10229 to 10246). Some of these primers included restriction enzyme sites (underlined) to facilitate potential cloning of PCR products. PCR products were sequenced directly following purification by agarose gel electrophoresis and recovery on DEAE filters as described previously (15). The inner PCR primers described above and a series of internal primers corresponding to the SIVmacJ5 sequence (44) were used as sequencing primers. Commercial sequencing kits (modified T7 polymerase, Sequenase Version 2.0 kit; United States Biochemicals) were used according to the manufacturer's instructions with the modification that PCR products were denatured in the presence of the appropriate sequencing primer by heating at 100°C for 8 min, followed by annealing at room temperature for 0.5 min, prior to initiating the extension reaction. Electrophoresis and autoradiography of sequencing gels were performed by standard procedures (46).

No changes from the input virus were detected in the sequence obtained from provirus recovered at 2 and 8 weeks postinfection. However, a direct duplication of the 12 bp flanking the SIVmacC8 deletion had occurred by 17 weeks (Fig. 4A). This event restored the size of this region to that seen in the more virulent SIVmacJ5 virus and effectively "repaired" the 4-amino-acid deletion with the predicted sequence EKIL. Analysis at later time points revealed that the consensus sequence of the repair continued to evolve rapidly towards the SIVmacJ5-like sequence. By 25 weeks postinfection, the predicted amino acid sequence of the repair (EIYL) showed only two conservative amino acid changes relative to the virulent SIVmacJ5 sequence (DMYL). Evolution continued over the following 20 weeks, with the gradual conversion of E143 to D143 (Fig. 4B), and, by week 45, brought the consensus predicted amino acid sequence (DIYL) even closer to that of SIVmacJ5. Interestingly, the evolved sequence in the SIVmacC8 cognate provirus used a different aspartic acid codon (GAT) than that used in SIVmacJ5 (GAC) (Fig. 4A), suggesting that evolution in this region is driven by pressure to restore Nef function rather than any potential function of this region as part of the 3′ LTR. This is consistent with recent reports that upstream U3 sequences are deleted in the absence of a functional, intact *nef*


A
9489
9522
J5
CAT AGA ATC TTA GAC ATG TAC TTA GAA AAG GAG GAA
H R I L D M Y L E K E E
C8 (2 WEEKS)
CAT AGA ATC TTA ... ... GAA AAG GAG GAA
H R I L . . . . E K E E
C8 (17 WEEKS)
CAT AGA ATC TTA GAA AAG ATC TTA GAA AAG GAG GAA
H R I L E K I L E K E E
C8 (25 WEEKS)
CAT AGA ATC TTA GAA ATT TAC TTA GAA AAG GAG GAA
H R I L E I Y L E K E E
C8 (45 WEEKS)
CAT AGA ATC TTA GAT ATT TAC TTA GAA AAG GAG GAA
H R I L D I Y L E K E E
139
150

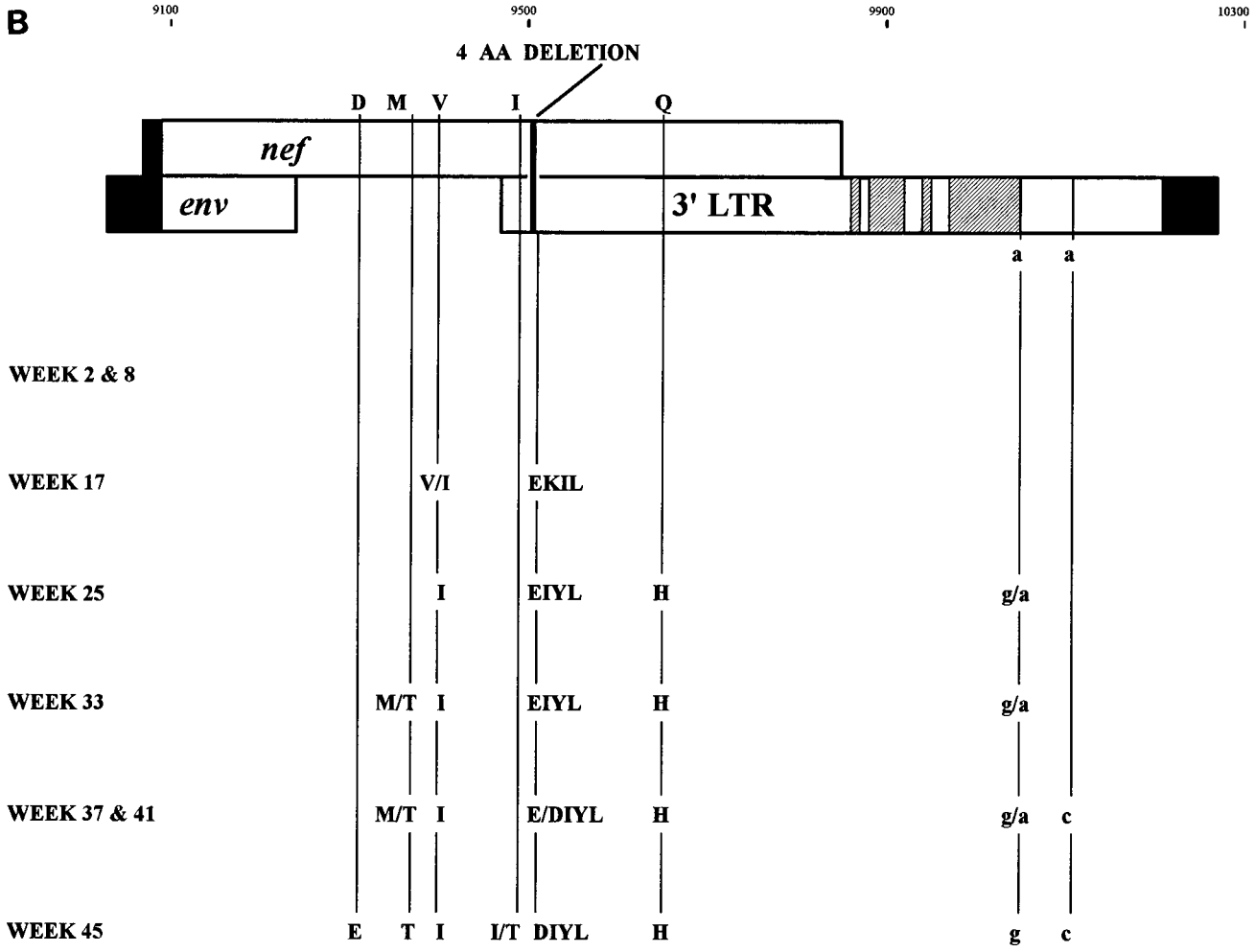

B
9100
9500
9900
10300
4 AA DELETION
D M V I Q
nef
env
3' LTR
a a
WEEK 2 & 8
WEEK 17 V/I EKIL
WEEK 25 I EIYL H g/a
WEEK 33 M/T I EIYL H g/a
WEEK 37 & 41 M/T I E/DIYL H g/a c
WEEK 45 E T I I/T DIYL H g c

FIG. 4. (A) Repair of the SIVmacC8 deletion in rhesus 45R by duplication of flanking sequence and subsequent evolution within the repair to restore SIVmacJ5-like sequence. Sequence corresponding to the 12-bp deletion in SIVmacC8 relative to SIVmacJ5 is shown boxed and highlighted. Duplicated sequence is shown blocked. The italicized numbering above the figure represents the nucleotide number in the complete SIVmacJ5 sequence (44), while that below the figure represents the predicted amino acid number within the *nef* open reading frame. (B) Schematic representation of other sequence changes accumulated over the time course of infection in rhesus 45R. Amino acid (AA) changes in Nef are shown in capital letters. No synonymous nucleotide substitutions were seen in this consensus sequence. Nucleotide substitutions within the noncoding 3′ LTR are shown in lowercase letters. When a mixed sequence population was apparent at a particular time point this is indicated, e.g., M/T. Regions of the genome shown in black were not sequenced in this study.

gene (31) and that extensive mutagenesis of upstream U3 does not significantly alter replicative capacity or virulence in vivo (23), suggesting that the primary function of the overlapping *nef*/3′ LTR region is as a *nef* coding sequence. Sequencing of the SIVmacC8 deletion region from all other animals (except 11R, which was PCR negative by this time) at 33 weeks (29R) or 37 weeks (19R, 28R, 55R, and 57R) postinfection confirmed that the original deletion associated with the parent SIVmacC8 virus had been maintained.

Figure 4B summarizes changes occurring elsewhere in the *nef*/3′ LTR region of provirus isolated from animal 45R over the 45-week time course. Only limited sequence evolution was apparent, with a further five changes in predicted amino acid sequence accumulating over time. Interestingly, two of these (M-T103 and Q-H196) correspond to changes previously noted in vivo following infection with virulent SIVmacJ5 but not SIVmacC8 virus (44). No synonymous nucleotide changes were seen. Importantly, the three substitutions in SIVmacC8 *nef* relative to SIVmacJ5 (Fig. 1) remained stable, confirming that the 12-bp deletion represents the attenuating change. Thus, although the possibility of changes elsewhere in the SIV genome cannot be formally discounted, it appears highly probable that repair of the 12-bp deletion is directly responsible for the change in phenotype of the SIVmacC8 in 45R. Little sequence variation was seen in the 3′ LTR region downstream of the *nef* open reading frame, and no changes were seen in regulatory elements. The two nucleotide substitutions seen in this region (Fig. 4B) corresponded to the two 3′-most changes seen in the SIVmacC8 molecular clone relative to SIVmacJ5 (Fig. 1). Nucleotide sequencing of the provirus from the cells transfected with the SIVmacC8 molecular clone revealed that the SIVmacC8 input virus had already reverted to sequence characteristic of SIVmacJ5 at these points. Unexpectedly, in the course of infection of rhesus 45R, these bases returned to the nucleotides characteristic of the SIVmacC8 molecular clone.

Reversion of attenuating single-nucleotide changes in SIV and HIV has been reported previously (28, 42). The results reported here demonstrate the capacity for repair of small attenuating SIV deletions in vivo and concomitant reversion to virulence and emphasize the requirement for the use of multiple, well-characterized deletions in putative live attenuated vaccines (14). A recent report of the in vitro reversion of an HIV-1 integration-defective U5 deletion mutant to partial function by extension of the original deletion (53) further highlights the capacity of these viruses for repair. Previous studies have shown that small sequence duplication events, such as that seen in the provirus isolated from rhesus 45R, are not uncommon in *nef* isolated from HIV-1 not subjected to in vitro passage and may result from reverse transcriptase strand switching (3, 6, 49). Indeed, a similar duplication event appears to have occurred in another animal of this cohort (29R) much later in infection. At 49 weeks postinfection, the *nef* sequence showed an almost perfect duplication (11 of 12 bp) of the four codons immediately upstream of the SIVmacC8 deletion in this animal (Fig. 5). Analysis of plasmid clones generated from 29R SIV DNA at an intermediate time point (week 41) indicated a provirus population distribution of 65% C8 parent virus to 35% "repaired" virus. Prior to this event, virus isolation was intermittent but became invariably positive by 61 weeks after infection. This animal is now being monitored for signs of disease progression and further sequence evolution.

It has been reported that additional *nef* deletions arise in macaques infected with a SIVmac molecular clone containing a 182-bp out-of-frame deletion in *nef* (SIVmac239Δ*nef*) constructed so as not to interfere with the overlapping envelope gene and 3′ LTR (31). Similarly, a defective *nef* gene isolated from an HIV-1-infected long-term survivor accumulated further substantial *nef* deletions over time (32). We have reported previously that SIVmacC8 may also give rise to an additional *nef* deletion in vivo, resulting in a frameshift and premature termination of Nef (44). However, this was found only in a single clone 1 month after infection and was not detected at 10 months postinfection. The apparent low frequency of addi-

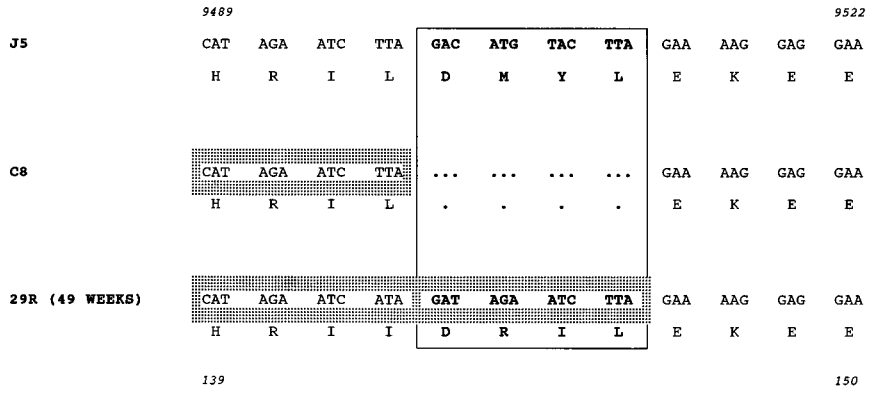


FIG. 5. Repair of SIVmacC8 deletion in rhesus 29R at 49 weeks after C8 infection. Sequence corresponding to the 12-bp deletion in SIVmacC8 relative to SIVmacJ5 is shown boxed and highlighted. Probable duplicated sequence is shown blocked. The italicized numbering above the figure represents the nucleotide number in the complete SIVmacJ5 sequence (44), while that below the figure represents the predicted amino acid number within the *nef* open reading frame.

tional *nef* deletion events suggests that the minimally deleted SIVmacC8 *nef* may have some residual function in vivo. SIVmacC8 Nef can be detected in infected cells by immunofluorescent staining with monoclonal antibodies (26), and macaques infected with SIVmacC8 generate *nef*-specific cytotoxic T lymphocyte responses (48). Although it is possible that the duplication per se in 45R may have partially restored *nef* function reduced or lost as a result of the 12-bp deletion in SIVmacC8, there is clearly a strong selective pressure imposed on the repaired sequence for evolution towards SIVmacJ5-like sequence. This observation indicates that the region from 9501 to 9512 (amino acids 143 to 146) or closely associated regions are crucial for Nef function in vivo. In this respect, it is notable that the regions immediately N-terminal to the SIVmacC8 deletion, especially ILD (141 to 143) part of one of four "*nef*-defining regions" in this polymorphic gene, and a glycine-rich motif (amino acids 127 to 131) are very highly conserved in both HIV and SIV isolates (22, 48). Despite this, no definite function has yet been ascribed to this region, though it has been identified as having homology to nucleotide-binding domains (18, 47, 49).

The emergence of virus following *nef* sequence repair poses interesting questions regarding protective immune responses. Daniel et al. (11) reported that animals infected with SIVmac239Δ*nef* were protected from subsequent intravenous challenge with virulent virus. In this study, animals 45R, 55R and 57R were challenged intravenously with 100 50% monkey infectious doses of SIVmacJ5 at 41 weeks after infection with SIVmacC8. Although virus was isolated intermittently after challenge from animals 55R and 57R and from 45R on every occasion tested, reflecting the prechallenge virus isolation status, in no case could challenge virus proviral DNA be detected in PBMC. It was possible to distinguish SIVmacJ5 from SIVmacC8 and C8 cognate virus by the presence of an *Rsa*I restriction endonuclease site in SIVmacJ5 *nef*, at the point where the 12-bp deletion occurs in SIVmacC8 (43). Repair of the SIVmacC8 sequence did not restore this restriction site. Furthermore, there was no evidence of an anamnestic antibody response following challenge. In contrast, challenge virus was readily detected in four naive macaques challenged at the same time. Virus was isolated from two of these animals as early as 1 week after challenge and from all four animals at 2 weeks postchallenge and at two weekly intervals thereafter. The virus challenge results confirm and extend the observations of Daniel et al. (11) and show that macaques previously infected with attenuated virus resist superinfection with a high dose of challenge virus. The mechanism of superinfection immunity is unknown. Neutralizing antibody against SIVmacJ5 was detected in all SIVmacC8-infected animals in serum taken on the day of challenge (8), and antibodies against all major viral structural components were detectable by Western immunoblotting. The status of cellular immune responses is under investigation.

Taken together, the results presented here show that virulent virus can evolve in vivo even in the presence of an immune response that is apparently able to elicit protection from challenge. It is possible that the evolved virus population arises through immune escape from a response driven specifically from infection with the attenuated virus. Alternatively, the dynamics of infection may determine outcome, so that the first infecting virus population modulates the viability of incoming virus, perhaps at the level of a critical target cell population. In conclusion, these results define a small region of the SIV *nef* gene, the maintenance of which is important for the persistence of high virus loads and expression of full pathogenic potential. The modulation of SIV virulence by repair of the *nef* lesion and subsequent selection of sequence variants highlight the remarkable potential of a retrovirus population for rapid genetic adaptation in vivo.

Nucleotide sequence accession numbers. The sequences described in this report have been deposited in GenBank under accession numbers X86724 to X86732.

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