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Simian Immunodeficiency Virus in Which *nef* and U3 Sequences Do Not Overlap Replicates Efficiently In Vitro and In Vivo in Rhesus Macaques

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The *nef* genes of human immunodeficiency virus and simian immunodeficiency virus (SIV) overlap about 80% of the U3 region of the 3′ long terminal repeat (LTR) and contain several essential *cis*-acting elements (here referred to as the TPI region): a T-rich region, the polypurine tract, and attachment (*att*) sequences required for integration. We inactivated the TPI region in the *nef* reading frame of the pathogenic SIVmac239 clone (239wt) by 13 silent point mutations. To restore viral infectivity, intact *cis*-regulatory elements were inserted just downstream of the mutated *nef* gene. The resulting SIV genome contains U3 regions that are 384 bp shorter than the 517-bp 239wt U3 region. Overall, elimination of the duplicated Nef coding sequences truncates the proviral genome by 350 bp. Nonetheless, it contains all known coding sequences and *cis*-acting elements. The TPI mutant virus expressed functional Nef and replicated like 239wt in all cell culture assays and in vivo in rhesus macaques. Notably, these SIVmac constructs allow us to study Nef function in the context of replication-competent viruses without the restrictions of overlapping LTR sequences and important *cis*-acting elements. The genomes of all known primate lentiviruses contain a large overlap between *nef* and the U3 region. We demonstrate that this conserved genomic organization is not obligatory for efficient viral replication and pathogenicity.

The *nef* gene is characteristic of primate lentiviruses and important for the full pathogenic potential of both human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) (11, 24, 27). A number of Nef functions that might increase virulence and allow the maintenance of high virus burdens have been described (reviewed in references 14 and 32). Nef downmodulates class I MHC and CD4 cell surface expression (3, 9, 15, 30, 35). Furthermore, Nef increases the infectivity of viral particles and stimulates viral replication in primary peripheral blood mononuclear cells, macrophages, and tonsillar histocultures (1, 8, 12, 16, 28, 31, 36). Recent studies suggest that most or all of these Nef activities are functionally independent and contribute to efficient viral replication and persistence in vivo (4, 6, 19, 20, 29, 37).

The use of replication-competent primate lentiviruses is obligatory to assess the effect of specific alterations in Nef on viral replication and pathogenicity. Also, the effects of Nef on cell surface expression of class I MHC and CD4 molecules and on signal transduction pathways are ideally investigated in infected cells. However, a detailed structure-function analysis is complicated because the *nef* open reading frame (ORF) overlaps several *cis*-acting elements and the U3 region in the viral long terminal repeat (LTR). A T-rich sequence (22), the polypurine tract (PPT), and the viral attachment (*att*) site at the 5′ end of the U3 region are essential for lentiviral replication (reviewed in reference 39). Mutations in the corresponding Nef region cannot be made without changing these critical *cis*-acting elements. Furthermore, the entire 3′ half of the *nef* gene overlaps the U3 region of the LTR. Therefore, after reverse transcription (RT), changes in the C-terminal half of Nef might have unexpected effects on transcriptional 5′ LTR activity, which could affect the results of replication and infectivity assays.

Previous findings suggest that 332 to 407 bp of U3 sequences upstream of the major core enhancer and promoter elements (US sequences) serve mainly as a *nef* coding sequence (21, 26, 27, 33). After infection of rhesus macaques with an SIVmac239 variant containing deletions in the *nef* unique region, additional deletions accumulate over time in the US region (26). Similar U3 deletions were selected in a long-term survivor of HIV-1 infection in whom only *nef*-deleted proviral sequences could be detected (27). Furthermore, an SIV variant containing a large number of nucleotide changes in the US region which did not affect the predicted *nef* coding sequence showed normal pathogenic potential in infected rhesus macaques (21). These SIVmac and HIV-1 variants did not contain most of the wild-type upstream U3 sequences. However, they were either attenuated, because of a deleted *nef* ORF (26, 27), or they maintained the *nef*-LTR overlap and the wild-type length of the U3 region as well as the essential *cis*-regulatory elements in the *nef* gene (21).

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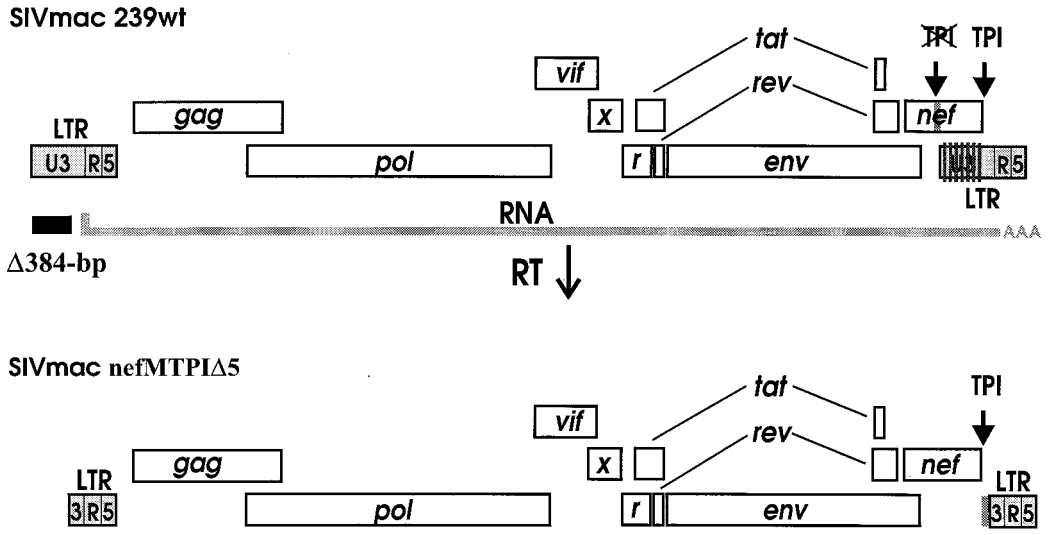

FIG. 1. Schematic representation of modifications introduced into the SIVmac239 genome to eliminate the *nef*-LTR overlap. The 239wt clone (top) contains a 517-bp U3 region that overlaps the 792-bp *nef* ORF by 407 bp (79%) (34). The TPI region in *nef* was mutated, and an intact TPI element was inserted upstream of the single NF-κB site. Furthermore, 384 bp of upstream U3 sequences were deleted from the 5′ LTR to prevent recombination with the modified 3′ LTR region. The nefMTPIΔ5 provirus (bottom) contains a U3 region of 133 bp and a *nef* gene that neither overlaps the 3′ LTR nor contains essential *cis*-regulatory elements. The black bar indicates the position of the deletion, and the arrows indicate the positions of the mutated and functional TPI regions. Abbreviations: x, *vpx*; r, *vpr*.

These previous studies showed that large parts of the US region serve mainly as *nef* coding sequence and do not contain important transcriptional elements. Therefore, it is unclear why the U3 regions of HIV and SIV are considerably longer than those of other lentiviruses (about 450 to 560 bp in length) and why they overlap the *nef* ORF by about 70%. To address these questions, we mutated the *cis*-acting elements in the SIVmac239 *nef* gene and introduced an intact TPI element downstream of the mutated *nef* ORF. The TPI region was either inserted just 14 bp upstream of the 5′ end of the single NF-κB site or 50 bp further upstream, because previous studies suggested that this US region might contain important enhancer elements (21, 26, 27, 33). These modifications had several consequences: (i) the *nef* gene of these proviral constructs does not overlap the LTR region; (ii) the *nef* ORF does not contain essential *cis*-acting elements; and (iii) these SIVmac variants possess short U3-LTR sequences. We found that SIVmac does not require long U3 regions for efficient replication in vitro and in vivo in rhesus macaques. Furthermore, we established a system to study *nef* function using infectious viruses without the limitations of overlapping *cis*-regulatory and LTR elements.

MATERIALS AND METHODS

Mutant construction. Site-directed mutagenesis to generate the SIVmac239 TPI variants was performed by spliced overlap extension PCR as described previously (28). Briefly, the *env-nef* region of SIVmac239 (23, 34) was amplified using primer pNheI (5′-GTACAAATGCTAGCTAAG-3′) and pPPT5 (5′-CTA AACCACCTTTCTCCTTAATGAAGTGAGACATGTCTATTGC-3′). The *nef*-LTR region was amplified using primer pPPT3 (5′-AAGGAGAAAGGTGGTT TAGAGGGTATCTATTACAGTGCAAGAAG-3′) and p3nefSmaI (5′-TCCC CCCGGGGGAAAGTCCCTGCTGTT-3′). Mutated positions are underlined. The left- and right-half PCR products were gel purified, mixed in equimolar amounts, and subjected to a second PCR with primers pNhe1 and p3nefSmaI. To generate 239nefMTPIΔ5, the PCR product was cloned into the SIVmac 239ΔUS384 construct (33) by using the unique *Nhe*I and *Sma*I sites in the *env* gene and just upstream of the TPI region. An overview of the mutants analyzed is given in Fig. 1 and 2. To generate 239TPImut, the TPI region of wild-type SIVmac239 was replaced with the mutated sequence using the flanking *Bgl*II and *Nde*I sites in *nef*. Conversely, the mutated region in the 239nefMTPIΔ5 construct was replaced with the corresponding 239wt and *nef** sequences to generate the 239nef+TPIΔ5 and 239nef*TPIΔ5 variants. SIVmac239 *nef** contains a premature in-frame TAA stop signal at the 93rd codon of *nef* (23). Three additional variants (239nefMTPI, 239nef+TPI, and 239nef*TPI) were generated the same way except that the mutated *nef* alleles were inserted into the proviral 239ΔNU clone, in which 65 bp upstream of the core enhancer elements are maintained (17). The U3 deletions were present in both LTRs to prevent recombination. All PCR-derived inserts were sequenced to confirmed that only the intended changes were present.

Virus stocks, cells, and infectivity assays. For virus production, 293T cells were transfected by the calcium phosphate method, and virus production was quantitated as described previously (4, 5). 293T, CEMx174 cells, the herpesvirus saimiri-transformed T-cell line 221 (1), and rhesus peripheral blood mononuclear cells (rPBMC) were isolated and cultured as described previously (4, 5). The cells were infected, and virus replication was measured by reverse transcriptase assay as described (5). SIVmac infectivity was determined using sMAGI cells as described previously (7) and quantitated using the Galacto-Light Plus chemiluminescence reporter assay kit (Tropix, Bedford, Mass.), as recommended by the manufacturer.

Animal studies. Three juvenile rhesus macaques of Indian origin were infected by intravenous inoculation of SIVmac239nefMTPIΔ5 containing 5 ng of p27 produced by transfected 293T cells. The animals were healthy and seronegative for SIV, type D retroviruses, and simian T-cell lymphotropic virus type 1 at the time of infection. Blood was collected at regular intervals, and serological, virological, and immunological analysis was performed as described previously (5, 28, 38).

PCR analysis. SIV sequences spanning the entire *nef*-U3 region were amplified from rPBMC DNA with a nested PCR approach or from DNA isolated from positive PBMC-CEMx174 bulk cocultivation or infected CEMx174 cells by one round of amplification essentially as described (5, 28). Viral plasma RNA was isolated with the QIAamp RNA kit (Diagen, Basel, Switzerland), reverse transcribed with Superscript RT (Gibco-BRL, Eggenstein, Germany), and subjected to a standard nested PCR approach. PCR fragments were sequenced directly or following subcloning into the pCRII vector (Invitrogen Corp., San Diego, Calif.). Sequencing was performed as described previously (28). The following primers were used to analyze the proviral LTR sequences: pP1 (5′-GATCCAACTCTG GCCTACAC-3′; 260 to 278 and 9722 to 9741); pP2 (5′-CCGTCGTGGTTGG

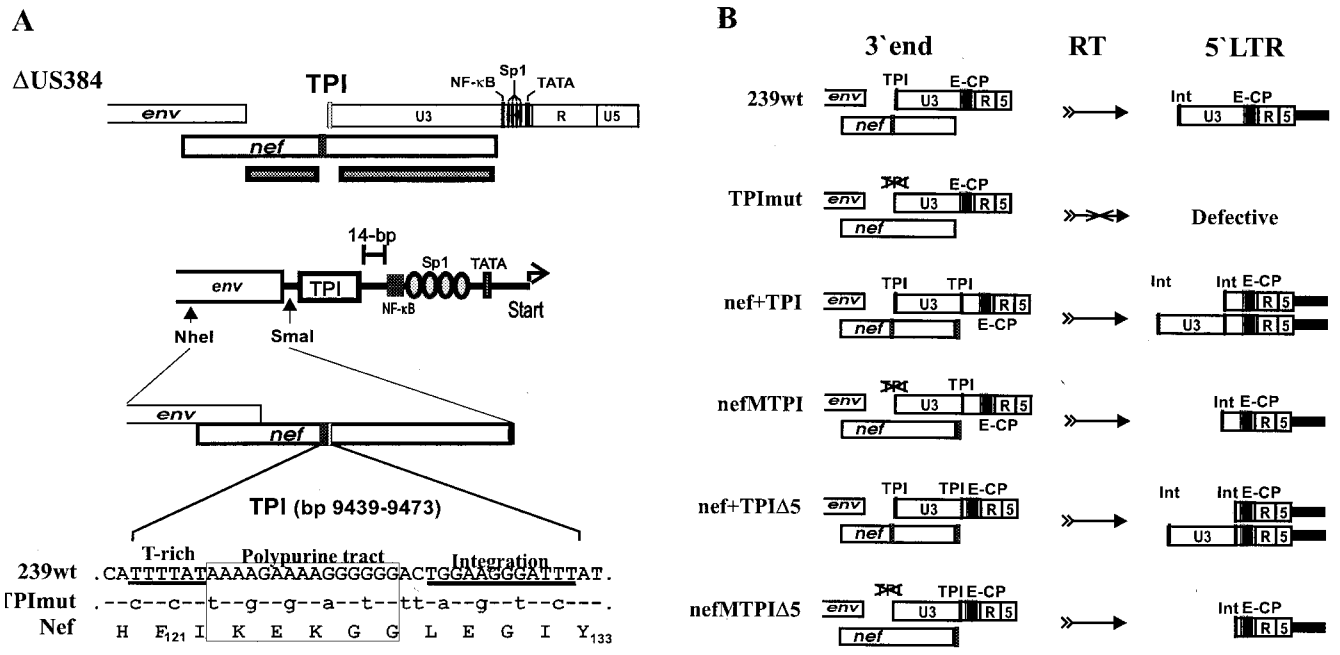

FIG. 2. Mutant construction and SIVmac239 *nef*-LTR variants analyzed. (A) The ΔUS384 construct, which contains a deletion of 182 bp in the *nef* unique region and 384 bp in the U3 region (33), was used to generate nefMTPIΔ5. As indicated below, a PCR restriction fragment containing 13 silent point mutations in the TPI region was cloned into ΔUS384 to generate nefMTPIΔ5. (B) Schematic presentation of the SIVmac constructs analyzed (left) and the deduced 5′ LTR promoter region (right). TPImut differs from 239wt only by the specific point mutations in the TPI region; nef+TPI and nef+TPIΔ5 contain wild-type *nef* genes; nefMTPI and nefMTPIΔ5 contain TPI-mutated *nef* alleles. Otherwise isogenic forms with a premature stop signal at the 93rd codon of the *nef* ORF were also generated. The TPIΔ5 forms contain 14 bp and the TPI forms contain 65 bp of US sequences. Abbreviations: E-CP, enhancer-core promoter; Int, U3-terminal sequences required for integration.

TTCCTGCC-3′; 891 to 912); pP3 (5′-TCGCTGAAACAGCAGGGACT-3; 400 to 420 and 9862 to 9882); and pP4 (5′-GATTTTCCTGCTTCGGTTTCCC-3′; 790 to 808 and 10252 to 10270). Numbers refer to positions in the proviral SIVmac 239wt sequence (34).

Western blot analysis. CEMx174 cells were infected with virus containing 10 ng of p27 core antigen derived from transfected 293 T cells. When cytopathic effects were observed, cells were pelleted, and lysates were generated as described previously (5). Expression of Nef proteins in whole cellular lysates was analyzed by immunoblot using a rabbit anti-Nef serum (13). For detection of p27 core protein, an anti-Gag monoclonal antibody derived from SIVmac p27 hybridoma cells (55-2F12) was used (18). For enhanced chemiluminescent detection, horseradish peroxidase-conjugated secondary antibodies were used as described by the manufacturer of the ECL detection system (Amersham, Chicago, Ill.).

RESULTS

Construction of SIV TPI mutants. The 792-bp SIVmac239 *nef* ORF overlaps about 80% (407 bp) of the 517-bp U3 region of the LTR and contains several essential *cis*-acting elements (named the TPI region in this study): a T-rich region (bp 363 to 368); the polypurine tract (bp 369 to 383); and sequences required for integration (bp 389 to 397) (numbers refer to the positions in the 239wt *nef* ORF). The TPI region in *nef* was inactivated, and intact *cis*-regulatory elements were inserted upstream of the single NF-κB site in the U3 region of the SIVmac239 LTR to eliminate the *nef*-LTR overlap (Fig. 1). A total of 13 point mutations were introduced to render the TPI region dysfunctional (Fig. 2A). These nucleotide substitutions did not alter the predicted Nef amino acid sequence. The following SIVmac239 variants were generated: (i) 239-TPImut is isogenic to 239wt except for the specific changes in the TPI region shown in Fig. 2A; (ii) 239-nef+TPI contains an insertion encompassing the TPI region and 65 bp of upstream U3 sequences, downstream of the *nef* gene; (iii) in 239-nefMTPI, the TPI region in the *nef* ORF of 239-nef+TPI is mutated; (iv) 239-nef+TPIΔ5 contains an insertion encompassing the TPI region just downstream of the *nef* gene and 14 bp upstream of the NF-κB binding site; and (v) in 239-nefMTPIΔ5, the TPI region in the *nef* ORF of 239-nef+TPIΔ5 is mutated (Fig. 2B). The right panel of Fig. 2B shows the 5′ LTR regions predicted after RT. The TPImut mutant is predicted to be inactive because it lacks sequences required for RT and integration. In contrast, the SIVmac239 nef+TPI and nef+TPIΔ5 variants contain two copies of these *cis*-regulatory elements. Therefore, RT and integration can result in two different forms of the proviral 5′ LTR. In comparison, the nefMTPI and nefMTPIΔ5 proviruses are predicted to contain only short U3 regions at the 5′ end of the genome. In addition to the mutants shown in Fig. 2B, *nef*-defective variants of the 239-nefMTPI and 239-nefMTPIΔ5 clones, containing a stop signal at the 93rd codon of the *nef* ORF, were also generated (239-nef*TPI and 239-nef*TPIΔ5, respectively).

SIV variants containing TPI elements downstream of *nef* are replication competent and express functional Nef. CEMx174 cells were infected with virus stocks derived from transiently transfected 293T cells to investigate the replicative potential of the SIVmac TPI variants. No RT activities above background levels were measured after infection with the control 239-TPImut virus (Fig. 3A). This variant, which does not contain

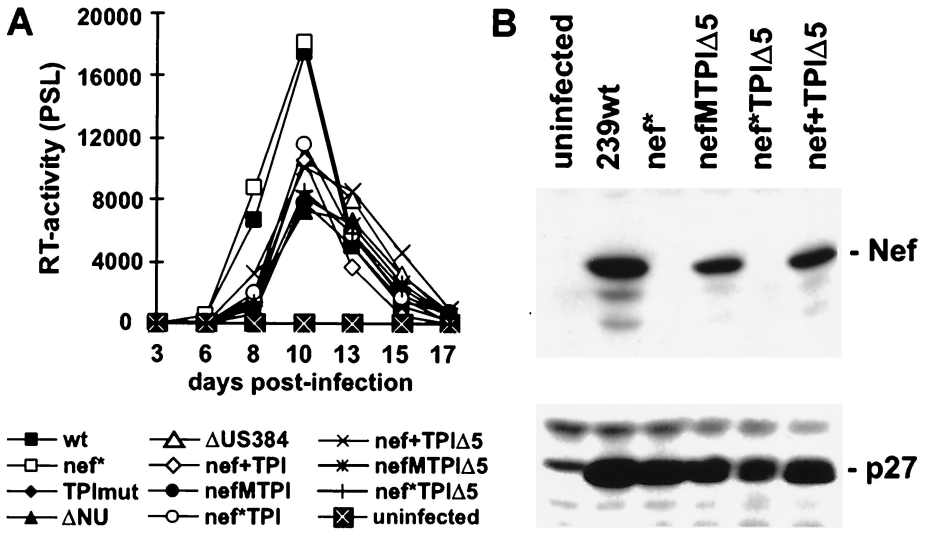

FIG. 3. SIVmac239 TPI variants are replication competent and express Nef. (A) Replication of SIVmac239 variants in CEMx174 cells. Virus containing 10 ng of p27 was used for infection. RT activity was determined using a phosphorimager. PSL, photon-stimulated light emission. (B) Nef and p27 antigen expression in infected CEMx174 cells was verified by immunoblot using rabbit anti-Nef antiserum or a monoclonal anti-p27 antibody as described previously (5). Similar results were obtained in two independent experiments.

intact TPI sequences at the 3′ end of the proviral genome, also did not replicate when very high doses of virus (up to 100 ng of p27 antigen) were used for infection (data not shown). In agreement with previous studies (24, 28), SIVmac 239wt and 239nef* replicated with comparable efficiency in CEMx174 cells. With the exception of 239-TPImut, all other SIVmac239 TPI variants replicated only slightly less efficiently than the parental 239wt clone (Fig. 3A). Thus, the presence of an additional TPI region or truncation of US sequences did not impair SIVmac replication in CEMx174 cells. PCR amplification and sequence analysis of *nef*-LTR and LTR-*gag* sequences at the end of culture demonstrated that all proviral sequences contained the predicted U3 sequences at the 5′ end of the viral genome (Fig. 2B) and showed that no reversions in the mutated *nef* alleles were selected during in vitro culture (data not shown). Western blot analysis revealed that SIVmac forms containing the 239wt or the TPI-mutated *nef* genes, but not the nef* variants, expressed the Nef protein (Fig. 3B and data not shown).

Next, we investigated if the TPI-mutated *nef* alleles are able to enhance SIVmac infectivity. As shown in Fig. 4, SIVmac 239wt infected sMAGI with about eightfold higher efficiency than the *nef*-defective 239nef* variant. Insertion of TPI-US65 (nef+TPI) or the TPI region (nef+TPIΔ5) downstream of the *nef* ORF did not reduce viral infectivity. Mutation of the TPI sequences in *nef* in the presence of a second downstream TPI region (nefMTPI and nefMTPIΔ5) resulted in slightly enhanced infectivity compared to the corresponding constructs with duplicated TPI regions (nef+TPI and nef+TPIΔ5). A premature stop codon reduced the infectivity of the nefMTPI and nefMTPIΔ5 variants to a level comparable to that of 239nef* (see nef*TPI and nef*TPIΔ5, Fig. 4). These results show that the TPI-mutated *nef* alleles are functional in enhancing viral infectivity.

It has been shown previously that a functional *nef* gene enhances SIVmac replication in the rhesus macaque T-lymphoid cell line 221, particularly in the absence of interleukin-2 (IL-2) (1). We investigated the replicative capacity of the SIVmac TPI variants in 221 cells to clarify if the mutated *nef* alleles are able to cause lymphoid cell activation. With the exception of the TPImut form, all variants replicated in 221 cells in the presence of IL-2 (Fig. 5A). However, intact *nef* genes resulted in faster growth kinetics and increased replication relative to the forms containing disrupted *nef* genes. In the absence of IL-2, only forms containing wild-type or TPI-mutated *nef* alleles showed marked levels of replication (Fig. 5B). The nef+TPIΔ5 and nefMTPIΔ5 variants, in which essentially the entire U3 region between the sequences required for integration and the core enhancer elements are deleted, were more active than the forms containing the 65 bp upstream of the NF-κB binding sites (Fig. 5). Thus, the TPI-mutated *nef* alleles

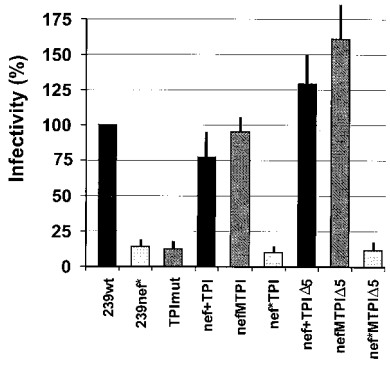

FIG. 4. SIV *nef* alleles containing changes in the TPI region enhance viral infectivity and replication. sMAGI cells were infected with the indicated SIVmac239 TPI-*nef* variants containing 50 ng of p27 antigen. Infections were performed in triplicate with three different virus stocks.

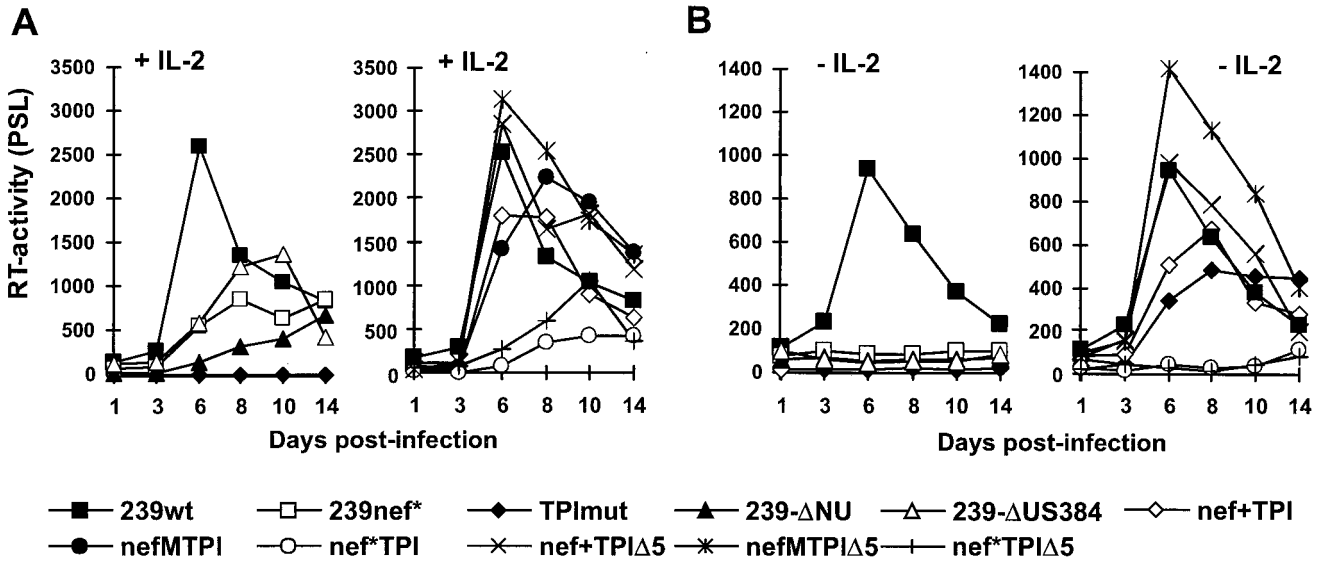

FIG. 5. SIVmac239 variants without overlapping *nef*-U3 sequences express functional Nef and replicate efficiently in 221 cells. Replication in of the indicated 239 mutants in 221 cells was tested in the presence (A) and absence (B) of IL-2. Similar results were obtained in two independent experiments using different virus stocks.

stimulated SIVmac replication with an efficiency comparable to that of 239wt *nef,* and short U3 regions accelerated rather than reduced viral replication in 221 cells. In agreement with the results obtained using 221 cells, the 239nef+TPI, 239nefMTPI, 239nef+TPIΔ5, and 239nefMTPIΔ5 variants replicated efficiently in rPBMC which were infected immediately after isolation and stimulated with phytohemagglutinin (PHA) 3 days later (Fig. 6A). The *nef*-defective SIVmac variants 239nef*, 239ΔNU, 239ΔUS384, 239nef*TPI, and 239nef*TPIΔ5 were inactive under these experimental conditions. In comparison, only the TPImut variant did not show appreciable levels of replication in stimulated PBMC, although the forms expressing functional Nef showed a higher replicative capacity than the nef* or *nef*-deleted forms (Fig. 6B).

These results demonstrate that the TPI-mutated *nef* alleles are expressed in infected cells and enhance SIVmac infectivity and replication with an efficiency comparable to that of the 239wt *nef* allele. In agreement with previous studies (21, 26, 33), the U3 region upstream of the core enhancer element was dispensable for efficient viral replication.

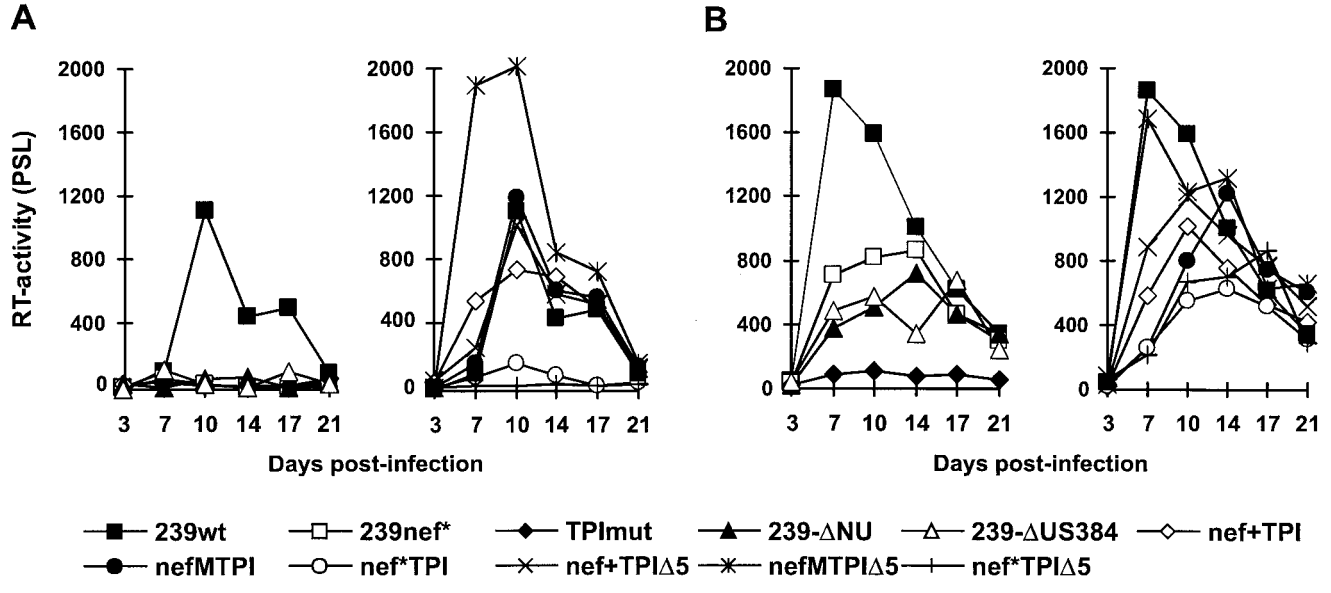

FIG. 6. Replication of SIVmac TPI-*nef* variants in rPBMC. (A) Unstimulated rPBMC were infected immediately after isolation and stimulated with PHA 3 days postinfection. (B) rPBMC were PHA stimulated for 3 days prior to infection. The results shown were derived from a single experiment using rPBMC derived from the same animal. Similar results were obtained with rPBMC from two different rhesus macaques.

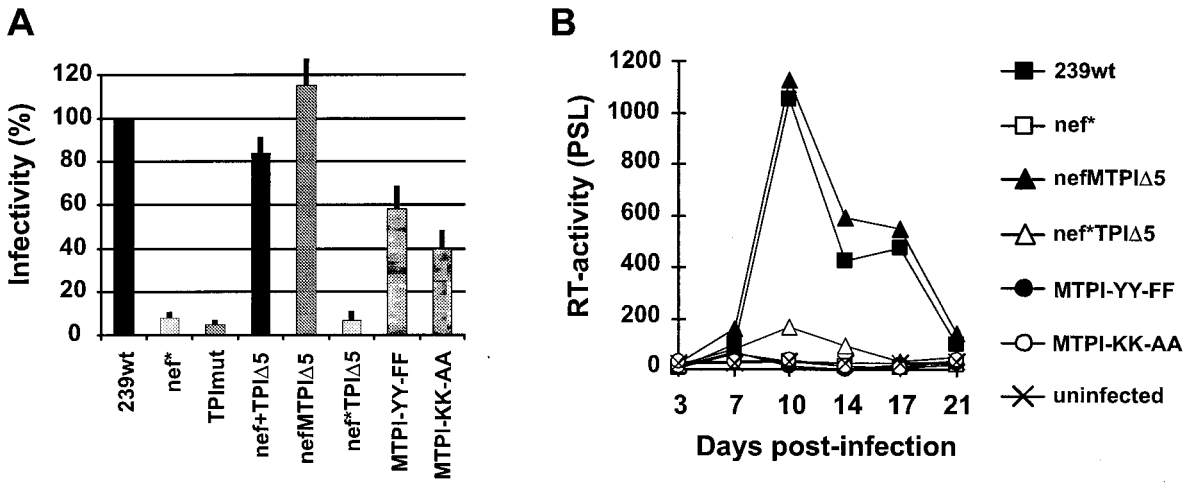

FIG. 7. *cis*-Regulatory elements in *nef* encode amino acids residues important for Nef function. (A) sMAGI cells were infected in triplicate with 293T cell-derived virus stocks containing 50 ng of p27. Infectivity is shown relative to that of 239wt virus. (B) rPBMC were infected immediately after isolation and stimulated with PHA at day 3 postinfection. Similar results were obtained in an independent experiment.

Amino acid residues encoded by TPI region are important for Nef function. One rationale for the construction of the SIVmac TPI mutants was to establish a system that allows investigation of Nef function without the complications of essential overlapping *cis*-regulatory sequences. We introduced mutations in the *nef* gene of the nefMTPIΔ5 variant, predicting changes of two lysines, $K_{124}/K_{127} \rightarrow A_{124}/A_{127}$ (MTPI-KK-AA), and two tyrosine residues, $Y_{133}/Y_{134} \rightarrow F_{133}/F_{134}$ (MTPI-YY-FF). Some of the nucleotide substitutions are present at positions corresponding to important *cis*-regulatory elements, like the polypurine tract in 239wt (Fig. 2A). Changes in both the lysine and tyrosine residues reduced the ability of SIV-Nef to increase virion infectivity for sMAGI cells (Fig. 7A). Furthermore, the mutated *nef* alleles did not stimulate SIVmac replication in PBMC culture (Fig. 7B). Our results demonstrate that these amino acid residues in Nef are not only highly conserved, because they are encoded by essential *cis*-regulatory RNA elements, but are also important for Nef function.

SIVmac239 nefMTPIΔ5 variant replicates efficiently in rhesus macaques. Three rhesus macaques were experimentally infected with the nefMTPIΔ5 variant to investigate whether SIVmac variants without overlapping *nef*-LTR sequences and with short U3 LTR sequences replicate efficiently in vivo. The nefMTPIΔ5 mutant was selected for in vivo analysis because it contained the shortest U3 region (133 bp) in conjunction with an intact *nef* gene and showed a phenotype similar to that of 239wt in in vitro infectivity and replication assays. The replicative capacity of the nefMTPIΔ5 mutant was compared with that of 239wt and ΔNU, which have been extensively analyzed in vivo (5, 17, 24). As shown in Fig. 8A, the p27 levels observed during the acute phase of infection by nefMTPIΔ5 (1,721 ± 1,017 pg/ml) were only 2-fold lower than those observed for 239wt infection (3,428 ± 2,648 pg/ml, $n = 15$) and about 25-fold higher than those observed in animals infected with the *nef*-deleted SIVmac ΔNU variant (67 ± 39 pg/ml, $n = 4$). In agreement with the high levels of plasma viremia, the levels of viral RNA ($7.1 \times 10^6 \pm 6.2 \times 10^6$ copies/ml) were also more similar to infection with pathogenic *nef*-open forms of SIVmac239 ($1.1 \times 10^7 \pm 7.5 \times 10^6$, $n = 11$), than to ΔNU infection ($1.8 \times 10^5 \pm 1.2 \times 10^5$, $n = 4$) (Fig. 8B). The initial peak levels (days 11 to 15) of urinary neopterin, a marker of immune activation, were high (15.9 [± 2.0] times baseline), even compared to those measured in 239wt-infected animals (10.5 ± 3.1, $n = 10$) (Fig. 8C). All three animals that received the nefMTPIΔ5 variant became chronically infected and maintained high cell-associated viral loads throughout the course of infection, comparable to 239wt-infected macaques (Fig. 9A). Similarly, in monkeys Mm10032 and Mm10033, the RNA levels were comparable to those observed in 239wt infection (Fig. 9B). The remaining animal, Mm10034, showed RNA loads intermediate between those after 239wt and ΔNU infection. All animals showed a marked reduction in $CD4^+$ cells during acute infection, which was most apparent for the $CD4^+$ $CD29^+$ memory T-cell subset (day 0, 199 ± 65/μl; 2 weeks postinfection [wpi], 101± 19/μl; 4 wpi, 35 ± 48/μl). Mm10032 and Mm10033 showed partial recovery and maintained relatively stable $CD4^+$ cell counts (≥500/μl at 44 and 52 weeks of follow-up, respectively). The overall $CD4^+$ T-cell count measured during chronic infection corresponded to about 50% of the preinfection values. Similarly, the number of $CD4^+$ $CD29^+$ cells remained at about 40% of the preinfection value (data not shown). Unexpectedly, Mm10034, which developed the lowest viral load (Fig. 9), had the greatest reduction in the number of circulating $CD4^+$ T cells. The absolute number of $CD4^+$ T cells dropped from 580/μl to 32/μl, and the $CD4^+$ $CD29^+$ cell count fell from 108/μl to 5/μl by 4 wpi. Thereafter, the $CD4^+$ T-cell count of Mm10034 partially recovered (164/μl by 52 wpi), whereas the number of $CD4^+$ $CD29^+$ cells remained very low (<10/μl; data not shown). One animal, Mm10032, had to be euthanized at 44 wpi because of severe diarrhea. Hemolytic *Escherichia coli, Klebsiella* sp., *Giardia* sp., and *Entamoeba* sp. were isolated from the intestine, indicating a severe immunodeficiency associated with opportunistic infections. The remaining two animals are still alive 1 year postinfection. However, all animals infected with the nefMTPIΔ5 variant exhibited signs of immunodeficiency, indicated by either $CD4^+$

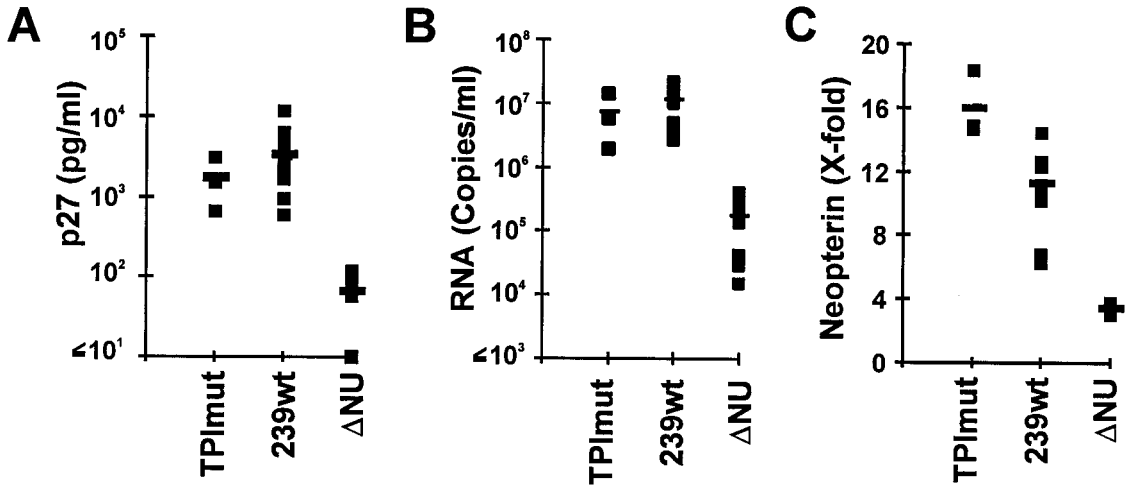

FIG. 8. nefMTPIΔ5 variant behaves similar to 239wt in acutely infected rhesus macaques. Maximum levels of p27 plasma antigenemia (A), viral RNA (B), and urinary neopterin (C) in three animals infected with the nefMTPIΔ5 variant. For comparison, values obtained from macaques infected with ΔNU or 239wt are also indicated. Peak levels of p27 plasma antigenemia and of viral RNA were always observed at 2 wpi. The neopterin/creatinine ratio is expressed for each animal as the fold increase over the mean ratios determined prior to infection as described previously (20).

cell loss or overt AIDS-like symptoms. The levels of replication and characteristics of infection were similar to those observed in *nef*-open SIVmac239 infection.

HIV and SIV are highly variable and point mutations in *nef* that attenuate viral replication can revert rapidly in vivo (24). Therefore, we investigated whether changes in the mutated *nef*-LTR region were selected in the animals infected with the nefMTPIΔ5 mutant. Sequence analysis of *nef*-LTR PCR fragments amplified directly from plasma RNA, PBMC, or virus-positive bulk cocultures revealed that no reversions in the mutated TPI region in the *nef* gene were detectable after 40 weeks of follow-up (data not shown). Furthermore, the TPI region downstream of *nef* and upstream of the NF-κB site was always maintained. We found that the TPImut variant did not show significant levels of replication (Fig. 5 and 6), suggesting that the mutated elements were nonfunctional. Next, we performed PCR analysis of *nef*-LTR sequences of virus reisolated from animals infected with SIVmac239 nefMTPIΔ5 to further

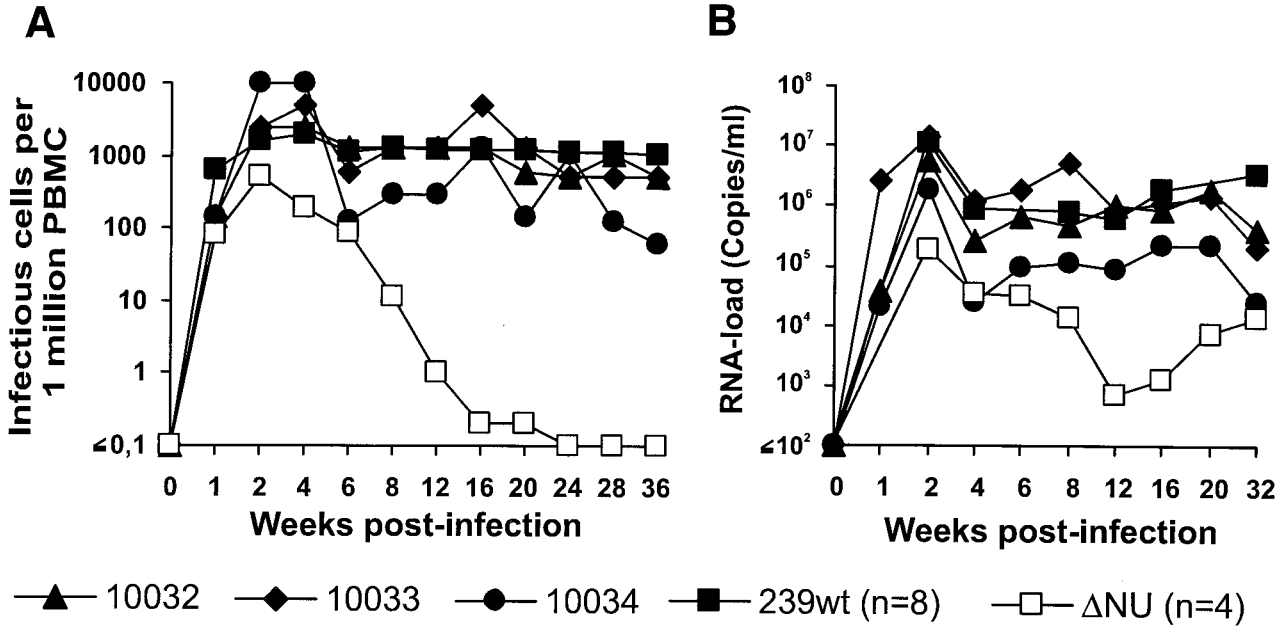

FIG. 9. Replication of SIVmac239 nefMTPIΔ5 variant in vivo. (A) Number of infectious cells per 10^6 PBMC. (B) Viral RNA load. The detection limit for viral RNA is approximately 40 copies/ml (38). For comparison, curves for the average values obtained from rhesus macaques infected with 239wt and ΔNU are indicated. Standard deviations are not shown for clarity. Parameters were determined as described in Materials and Methods.

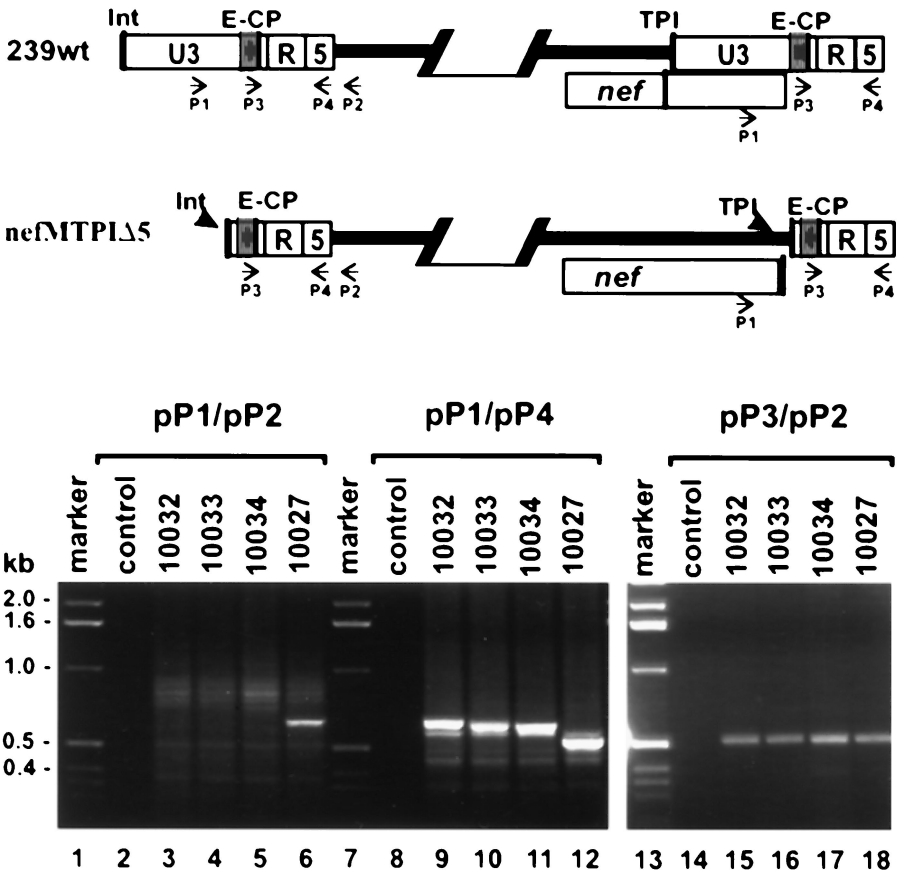

FIG. 10. Analysis of *nef*-LTR sequences derived from nefMTPIΔ5-infected rhesus monkeys. (A) Schematic representation of the predicted 5′ and 3′ ends of the 239wt and nefMTPIΔ5 proviruses. Arrows indicate the positions of primers used for PCR amplification. The abbreviations are described in the legend to Fig. 2. (B) SIV LTR and *nef*-LTR sequences were amplified from positive PBMC-CEMx174 bulk cocultures. PBMC were derived at 40 wpi from three macaques infected with nefMTPIΔ5 (Mm10032, Mm10033, and Mm10034) and one animal that received 239wt (Mm10027). PCR products were separated by electrophoresis through 1.5% agarose gels.

confirm that the mutated TPI region was not used for reverse transcription and integration and that function was not restored in infected macaques. Amplification with primers pP1 and pP2, which bind to the US region and the noncoding region flanking the 5′ LTR, yielded a 632-bp fragment from DNA prepared from positive bulk cocultures derived from 239wt-infected animals (Fig. 10, lane 6, and data not shown). In contrast, no specific product was obtained with virus reisolated from the three animals infected with the nefMTPIΔ5 mutant (Fig. 10, lanes 3 to 5). This result confirms that the U3 region of the 5′ LTR is truncated and does not contain the binding site for primer pP1. In comparison, PCRs performed with primers p1 and p4 yielded products for both 239wt- and nefMTPIΔ5-infected animals (Fig. 10, lanes 9 to 12). The fragments obtained for the mutant form were larger than the products obtained for 239wt. This was expected because these primers span the region at the 3′ end of the viral genome that contains the inserted TPI region in the nefMTPIΔ5 genome (Fig. 10, upper panel). Finally, PCRs with primers pP3 and pP2, which bind to the 3′ end of the U3 region, which is maintained at the 5′ LTR of both the 239wt and the nefMTPIΔ5 proviruses, yielded products of 513 bp for DNA from all bulk cocultures (Fig. 10, lanes 15 to 18). Thus, the results of both the sequence and PCR analyses consistently show that the mutations in the *nef*-LTR region of nefMTPIΔ5 did not revert in infected animals and that the mutant virus maintained a truncated U3 region.

DISCUSSION

We designed SIVmac239 mutants containing an intact *nef* gene which does not overlap the U3 region of the LTR and does not include essential *cis*-regulatory elements. The TPI-mutated *nef* alleles were functional in enhancing SIVmac infectivity and replication. Elimination of the U3 sequences upstream of the core enhancer elements had little if any effect on replicative capacity in cell culture or in rhesus macaques. Our observation that the upstream U3 sequences of SIVmac serve primarily or exclusively as *nef* coding sequence is consistent with previous studies. It has been shown that the US region is selectively deleted in vivo in the absence of an intact *nef* gene (26) and can be mutagenized extensively without loss of virulence (21).

We generated two sets of SIVmac239 TPI variants. One contained a U3 region of 183 bp, in which 65 bp of US sequences were maintained, and the other contained a U3 region

of only 117 bp, in which essentially all US sequences are deleted. The 65 bp upstream of the single NF-κB binding site were always preserved in macaques infected with *nef*-deleted SIVmac239 (26) and were not altered in a previous study on the functional role of the US region (21). This region of the SIVmac LTR contains binding sites for Ets family transcription factors, which allow efficient viral replication in the absence of the entire core enhancer element (33). We found that the nefMTPIΔ5 variant, which does not contain the US65 region, was more active in infectivity and replication assays than the nefMTPI variant. Our results extend previous studies (21, 26, 33) and demonstrate that the 3′ end of the U3 region, encompassing the NF-κB and Sp1 binding sites and the TATA box is sufficient for efficient replication of SIVmac both in vitro and in vivo in rhesus macaques. It remains to be clarified why the genome of primate lentiviruses is always organized so that the *nef* gene overlaps the 3′ LTR. Certainly we cannot exclude that this genomic organization has some subtle advantage in certain cell types or tissues. Indeed, although the characteristics of infection with the nefMTPIΔ5 variant were much more similar to those of 239wt than to those of 239Δ*nef*, it seemed that this mutant virus was relatively well controlled by the antiviral immune responses. Studies in large numbers of infected animals would be required to clarify if these minor differences are significant. A number of factors could explain how the TPI mutations affect viral replication in infected macaques. Nef expression levels might be slightly reduced, the mutations could affect the stability of the viral RNA, or the US sequences might have some effect on transcriptional activity in primary cells. However, the wild-type-like phenotype of the mutant virus in vivo and in vitro and the lack of reversions in infected macaques indicate that these attenuating effects may be very subtle.

Previous results on the functional relevance of the upstream U3 region were derived from infections with *nef*-deleted viruses (26, 27) or from SIVmac mutants that still contained the overlap of the *nef* gene and the LTR (21). In contrast, the nefMTPIΔ5 variant contains an intact *nef* gene that neither overlaps the LTR nor contains any essential *cis*-regulatory elements. We demonstrate that the TPI-mutated *nef* allele increases SIVmac infectivity and replication with an efficiency indistinguishable from that of 239wt *nef*. Usually, about 60% of the *nef* gene is overlapped by the U3 region of the LTR or by important *cis*-regulatory elements. Detailed structure-function analysis of Nef is complicated because mutations in the central region or the 3′ half of the *nef* gene not only might alter Nef function but also could have unexpected effects on transcriptional activity, integration, or RT. The constructs generated in the present study extend the experimental possibilities for the analysis of *nef* function in infected cells. Such systems are obligatory to study the effect of Nef on viral replication and pathogenesis. However, it remains important to investigate other *nef* functions in primary infected cells because (i) effects of Nef on cellular signal transduction or activation are often not observed in immortalized tumor cell lines, (ii) cellular kinases or other factors might only be expressed in relevant primary cells and binding to Nef might be affected by the presence of other viral proteins, (iii) the influence of Nef on the cell surface expression of various molecules might depend on the coexpression of other viral factors, e.g., both Vpu and Env also reduce CD4 surface expression levels (10, 40), and (iv) essentially all activities of Nef strongly depend on protein expression levels. Thus, overexpression of Nef by expression constructs in transfected cells may generate misleading data. The constructs generated in this study should be useful for delineating the molecular mechanisms that underlie the various Nef functions and investigating their relevance to viral replication in vivo. We show that lysine and tyrosine residues, which are encoded by important *cis*-regulatory elements in the 239wt *nef* ORF, are important for the ability of Nef to increase infectivity and replication. Our results suggest that this region in the *nef* gene is highly conserved not only because it contains important regulatory elements, but also because it encodes residues that are important for Nef function.

We demonstrate that the conserved genomic organization of the 3′ end of the genome of primate lentiviruses, specifically the large overlap between the *nef* gene and the LTR, is not obligatory for efficient replication of SIVmac. We are currently investigating if analogous HIV-1 TPI variants can be generated. Our initial focus was on SIV rather than on HIV-1 in this study because SIVmac239 allows studies of viral pathogenicity in a well-characterized animal model. Pathogenic HIV-SIV hybrid Nefs have recently been described (2, 25). However, the current forms are of limited value for pathogenesis studies because they only induced disease in a subset of infected macaques. The SIV constructs described in this work should prove useful for the detailed structure-function analysis of Nef in infected cells.

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