Simian Immunodeficiency Virus Promoter Exchange Results in a Highly Attenuated Strain That Protects against Uncloned Challenge Virus

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Among the many simian immunodeficiency virus (SIV) immunogens, only live attenuated viral vaccines have afforded strong protection to a natural pathogenic isolate. Since the promoter is crucial to the tempo of viral replication in general, it was reasoned that promoter exchange might confer a novel means of attenuating SIV. The core enhancer and promoter sequences of the SIV macaque 239nefstop strain (NF- κ B/Sp1 region from –114 bp to mRNA start) have been exchanged for those of the human cytomegalovirus immediate-early promoter (CMV-IE; from –525 bp to mRNA start). During culture of the resulting virus, referred to as SIVmegalo, on CEMx174 or rhesus macaque peripheral blood mononuclear cells, deletions arose in distal regions of the CMV-IE sequences that stabilized after 1 or 2 months of culture. However, when the undeleted form of SIVmegalo was inoculated into rhesus macaques, animals showed highly controlled viremia during primary and persistent infection. Compared to parental virus infection in macaques, primary viremia was reduced by >1,000-fold to undetectable levels, with little sign of an increase of cycling cells in lymph nodes, CD4⁺ depletion, or altered T-cell activation markers in peripheral blood. Moreover, in contrast to wild-type infection in most infected animals, the nef stop mutation did not revert to the wild-type codon, indicating yet again that replication was dramatically curtailed. Despite such drastic attenuation, antibody titers and enzyme-linked immunospot reactivity to SIV peptides, although slower to appear, were comparable to those seen in a parental virus infection. When animals were challenged intravenously at 4 or 6 months with the uncloned pathogenic SIVmac251 strain, viremia was curtailed by ~1,000-fold at peak height without any sign of hyperactivation in CD4⁺- or CD8⁺-T-cell compartment or increase in lymph node cell cycling. To date, there has been a general inverse correlation between attenuation and protection; however, these findings show that promoter exchange constitutes a novel means to highly attenuate SIV while retaining the capacity to protect against challenge virus.

Simian immunodeficiency virus (SIV) infection of rhesus macaques is widely used to explore the immunopathology of AIDS and to evaluate potential candidate vaccines in the face of challenge virus, whether they be SIVs or simian/human immunodeficiency virus (SHIV) chimeras (17). The model is essential when initially evaluating immunogens presented by way of viral vectors (29, 31, 36, 38, 39, 42). To date subunit vaccines, DNA vaccination, or various combinations have not proved too effective at controlling challenge virus (2, 4, 10, 14, 22). In contrast, the greatest degree of protection has been achieved with live attenuated strains of the macaque virus (SIVmac) harboring gene deletions. They can elicit strong protection even against challenge with an uncloned pathogenic isolate (12, 18, 46).

By associating deletions in the vpx, vpr, and nef genes the recombinant SIVs became more and more attenuated. However, this resulted in an inverse correlation between the level of

attenuation and the degree of protection against homologous challenge (18). Safety is the overriding problem for a live attenuated vaccine of any sort and, in the case of SIVmac, all attempts have failed to demonstrate a safety level commensurate with use in humans. The SIVmac Δ nef strain which confers strongest protection can both induce AIDS in neonates (3, 37) and, over time, revert to a pathogenic phenotype (1, 8, 37, 45).

In contrast to the attenuation resulting from the introduction of deletions into SIV genes, deletions engineered into the SIV promoter, or redesigning the long terminal repeat (LTR), attenuated little SIV in vivo (16, 30). An alternative to modifying the SIV promoter is to exchange it for that of a DNA virus. Given that the promoter would have been optimized in a totally different context, exchanging promoters may alter far more profoundly the replication of the resulting SIV chimera. The human cytomegalovirus major immediate-early (CMV-IE) promoter is widely used in molecular biology for driving high gene expression in transfection assays. However, in a more biological context, that of a recombinant adenovirus vector with a reporter gene under the control of the CMV-IE promoter, expression is far more restricted than anticipated from transfection assays (41).

Given such restriction of the CMV-IE promoter in vivo, it

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FIG. 1. Structure of the SIVmegalogo promoter chimeras. The LTR structure of parental and SIVmegalogo chimera is presented. The positions of transcription factor binding motifs (for a review, see reference 28) and TAR sequences are shown. The CMV-IE promoter and SIV sequences are fused such that the transcription start site of CMV coincides with that of SIV.

was reasoned that exchanging the core enhancer/promoter sequences of SIV by those of CMV-IE might be strong enough to drive the expression of viral mRNA but might at the same time attenuate the resulting chimera. Not only was replication of the chimera, called SIVmegalogo, in rhesus macaques highly attenuated but the infection also conferred strong protection to challenge virus. This finding shows that promoter exchange may indeed constitute a novel way of attenuating SIV.

MATERIALS AND METHODS

Constructs. The wild-type SIVmac239 was available as two half plasmids: p239SpSp5' and p239SpE3' (19, 35). The 3' plasmid contains the nef stop codon. The 524-bp CMV-IE promoter fragment corresponds to bases 622 to 1145 for HEHCMVP1 (accession number X03922). To generate SIVmegalogo, both half plasmids were modified. The chimera was made by first deleting SIV U3 promoter sequences between the nef stop codon and the SIV transcription start (positions -114 to +1) introducing the *NotI* site instead in the two halves (named p239SpSp5'*NotI* and p239SpE3'*NotI*). Two PCR fragments were generated by using, respectively, the following primers: 5'-TAAGAATGCGGCCGCTTACATAACTTACGG with 5'-CTGACGGTTCACTAAACGAGCTCTGCTTATATA and 5'-TTTAGTGAACCGTCAGTCGCTCTGCGGAGAGGCTG with 5'-ACGCGAATTCAGTGTGCTCTGCAATATCTGA (the *NotI* and *EcoRI* sites are underlined). PCR products were purified and annealed in PCR mix without primer for five cycles. External primers were then added for 30 more cycles. Annealed PCR products were cloned and double digested with *NotI* and *NarI* (or *EcoRI*), and the resulting fragments were gel purified and introduced into the p239SpSp5'*NotI* (or p239SpE3'*NotI*) plasmid at the *NotI* and *NarI* (or *EcoRI*) sites.

Virus production and isolation. Half plasmids were double digested with *EcoRI* and *SphI* and ligated. Stocks of SIVmegalogo or SIVmac239nefstop were prepared by electroporation of CEMx174 cells (960 μ F, 250 V). Viruses were harvested at 10 and 20 days posttransfection, respectively, for SIVmac239 and SIVmegalogo, filtered (0.2- μ m pore size), divided into aliquots, and stored at -80°C. Titration of infectivity was performed by calculation of the 50% tissue culture infectious dose(s) (TCID₅₀) by the Kärber method. CEMx174 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin (100 U/ml), and streptomycin (100 μ g/ml). Pe-

ripheral blood mononuclear cells from rhesus macaques (rPBMC) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin-streptomycin, and 5 μ g of phytohemagglutinin (PHA)/ml for the first 2 days, after which 20 U of human recombinant interleukin-2/ml was added for the remainder of the experiment. Virus was isolated by coculture of CEMx174 with rPBMC after 2 days of PHA activation, maintained at least 2 months, and regularly tested for reverse transcriptase (RT) activity. RT activity was determined on 10 μ l of centrifuge supernatant as recommended (Innova-gen).

Sequence analyses. Chimeric or wild-type LTR and nef DNA sequences were amplified by nested PCR under standard conditions with flanking primers, i.e., 5'-CTAACCAGCAAGAGGCCTTCTTAACATG and 5'-GGAGTCACTCTGCCAGCACCGGCCCA and then 5'-GGCTGACAGAAGGAAACTCGCTA and 5'-GGAGTCACTCTGCCAGCACCGGCCAAG. Products were cloned by using the Topo-2.1 TA (Invitrogen) and sequenced.

CAT constructs and assays. The HIV type 1 (HIV-1) Tat- and Rev-dependent chloramphenicol acetyltransferase (CAT) reporter construct has been previously described (32), as have the pSV2/Tat and Rev expression plasmids encoding the HIV-1 Tat and Rev genes (27, 32). Wild-type and modified promoter fragments were subcloned upstream of the bacterial CAT gene via *NotI* and *NarI* sites. For each assay, 4×10^6 CEMx174 cells were transfected with 8 μ g of CAT plasmid and 3 μ g of pSV2/Rev HIV with or without pSV2/Tat expression plasmids by using DEAE-dextran. Note that when pSV2/Tat was not added plasmid expression DNA were adjusted to 6 μ g with pSV2gpt in order to have equal promoter concentration. After 4 days, total protein lysate concentrations were determined by a commercial dye-binding method (Bio-Rad), and equal amounts of protein from each lysate were used in the standard CAT assays. All experiments were performed at least twice. Chromatograms were quantified by storage phosphor system (Molecular Dynamics, Inc.). Relative conversion values were determined by normalizing the amounts of radioactivity in C14 chloramphenicol-acetylated forms of mutant constructions to those of wild-type promoter constructions under tat control and multiplying those values by 100.

SIV inoculation and quantitation. Rhesus monkeys (*Macaca mulatta*) of Chinese origin were serologically negative for SIV, type D retrovirus, and simian foamy virus. Animals were inoculated intravenously with 200 TCID₅₀ of SIVmegalogo and SIVmac239 (SIVmegalogo stock, $\sim 2 \times 10^5$ TCID₅₀/ml titrated on CEMx174 cells, $\sim 8 \times 10^8$ RNA copies/ml; SIVmac239nefstop stock, $\sim 5 \times 10^4$ TCID₅₀/ml, $\sim 4.6 \times 10^8$ RNA copies/ml). Macaques were challenged with 200 TCID₅₀ of SIVmac239 (see above) or with 10 50% animal infectious doses of

FIG. 2. Replication kinetics and evolution of SIVmegalo promoter during replication on CEMx174 cells (left panels A, C, and E) and rPBMC (right panels B, D, and F). Five million cells were infected by 1 ng of RT activity of SIVmac239 or SIVmegalo. RT activity released in the supernatant was measured after infection of CEMx174 cells (A) or rPBMC (B). SIVmac239neftop or SIVmegalo growth curves on CEMx174 cells are represented as the average of three separate experiments (vertical bars representing the standard deviation [SD]), whereas the growth curves on rPBMC were obtained from one donor (macaque 93035); comparable results were obtained from four other donors. (C and D) Genomic DNA was extracted from different time points, and a PCR was performed with primers spanning the recombinant LTR region. The PCR product size was visualized under UV. The SIVmegalo amplicon was 750 bp, while that of SIVmac239 was 260 bp. (E and F) Nucleotide sequences obtained from PCR product 15 days after infection are indicated as horizontal bars relative to repeat sequences described in pCMV-IE. The frequencies of sequences are reported on the right.

FIG. 3. Detail of the recombined LTR sequences obtained from in vitro CEMx174 or rPBMC infected cells or from in vivo lymph node infected cells (macaque 93035). CEMx174 or rPBMC (macaque 93035) were infected with SIVmegalo, the sequence of which is reported at the top of each lane. At 2 months after infection a unique viral sequence was obtained on CEMx174 cells. At 1 month after infection the two major viral sequences obtained from PBMC were also reported. At 100 days after infection of macaque 93035 with SIVmegalo, LNMC were collected and submitted to PCR amplification of the promoter region. All 10 sequences out of 10 showed the same deletion of 190 bp.

Immune responses. Enzyme-linked immunospot assay (ELISPOT) assays were performed on fresh rPBMC as described previously (2). Cells were seeded in duplicate at 5×10^5 cells per well. Peptide pools were added to each well to a final concentration of $2 \mu\text{g}$ of each peptide/ml in $100 \mu\text{l}$ of complete medium. PHA ($5 \mu\text{g/ml}$; Sigma) was used as a positive control, and RPMI alone was used

as a negative control. Spots were counted under a dissecting microscope. Only ELISPOT duplicates within 20% were considered significant. Gag pool peptides were composed of 15-mers as follows: 36-50, WAANELDRFGLAESL; 57-71, CQKILSVLAPLVPTG; 64-78, LAPLVPTGSENLSKSL; 71-85, GSENLSKSLYIE TVCVI; 134-148, NYPQGQIGGNYVHPL; 148-162, PLSPTLNAWVKVLE; 169-183, EVVPGFQALESGETP; 176-190, ALSEGETPYDINQML; 216-230, LQHPQPAPOQGQLRE; 244-258, DEQIQWMYRQONPIP; 251-265, YRQONPIPVGNIYRR; 258-272, PVGNIYRRWIQLGLQ; 271-285, LQCV RMYNPTNILD; 292-306, EPFQSYVDRFYKSLR; 356-370, GPGQKARML AEALKE; and 427-441, CPDRQAGFLRGLPGW. Nef pool peptides was composed of 15-mers as follows: 15-29, DLRLLRLRARETGY; 22-36, RARGE

FIG. 4. Activity of deleted chimeric LTRs in CAT and virus assays. (A) Structure of the HIV-1 Tat- and Rev-dependent CAT reporter construct. (B) CAT activity of deleted LTR compared to SIVmac239 and SIVmegalo reference clones. Δclone61 was derived from the CEMx174 culture at 60 days (Fig. 3). (C) Growth curve of Δclone61 virus compared to SIVmac239 and SIVmegalo controls.

TYGRLLGEVE; 29-43, GRLLGEVEDGYSQSL; 43-57, PGGLDKGLSSLSCEG; 57-71, GQKYNQGYMNTWPWR; 63-77, GQYMNTWPWRNPAAER, 70-84, WRNPAEEREKLAIRK; 105-119, RVPLRTMSYKLAIVDM; 119-133, MSHFIKEKGGLGEGY; 147-161, EKEEGIIIPDWQDYTS; 154-168, PDWQDY TSGPGIRYP; 161-175, SGPGIRYPKTFGLW; 175-189, WKLVVPNVSDAE QED; 201-215, SQWDDPWGEVLAWKF; 208-222, GEVLAWKFDPTLAYT; 215-229, FDPTLAYTYEAYVRY; 222-236, TYEAYVRYPEEFGSK; 243-257, EVRRRLTARGLLNMA; and 249-263, TARGLLNMADKKETR. Heat-inactivated HIV-2 antigen was prepared as previously described (21). CD4 or CD8 depleted rPBMC population was obtained by negative selection by using MACS beads (Miltenyi Biotec) as recommended by the manufacturer. The T-cell population depletion was >90%. Sera were tested for neutralizing antibodies to SIVmac239 as described previously (44). Antibody titers were determined by limiting dilution with HIV-2 enzyme-linked immunosorbent assay kit (Elaviva-II; Sanofi-Pasteur).

Flow cytometry. EDTA-treated blood was incubated for 15 min with antibodies to CD4 (Becton Dickinson), CD8 (Leu-2a; Becton Dickinson), and CD69 (Becton Dickinson) added at a 1:20 dilution. Erythrocytes were lysed with Lyse&Fix reagents (Immunotech). Samples were washed three times in phosphate-buffered saline and fixed in 1% paraformaldehyde-phosphate-buffered saline. All samples were analyzed on a three-color FACScan cytometer (Becton Dickinson).

Statistics. Statistical analyses were performed with StatView software by using Mann-Whitney U signed-rank nonparametric test.

RESULTS

Replication of chimeric SIV-CMV promoter constructs ex vivo. The SIV promoter/enhancer sequences 3' to nef and up to the transcription start site were exchanged for the 524-bp fragment encoding the CMV-IE promoter widely used in mo-

lecular biology. In this chimera, the transcription start site coincides exactly with that initiated by the CMV-IE promoter (Fig. 1). The resulting construct has two NF- κ B and three SP1 proximal promoter sites, which is very reminiscent of the SIV and HIV promoter-proximal sequences. CEMx174 cells were transfected with ligated inserts derived from half plasmids. Supernatants were harvested regularly, and viral stocks were made when the RT activity was maximal (i.e., 10 days posttransfection for SIVmac239nefstop and 20 days posttransfection for SIVmegalo). For replication studies, five million CEMx174 cells were infected with 1 ng of RT activity, a value which corresponds to ~ 1 TCID₅₀ per 10^3 cells. The resulting virus, SIVmegalo, grew on the human CEMx174 cell line and rPBMC, albeit with a delay of several days compared to parental virus (Fig. 2A and B). No difference could be observed compared to wild-type virus in terms of virus cytopathogenicity or the morphology of viral particles as seen by electron microscopy.

In order to understand the delayed peak viremia for SIVmegalo, the promoter region was analyzed to verify its stability. Primers spanning the cloning sites were used to amplify the promoter region from total cellular DNA from SIVmegalo-infected cells. Of three independent cultures, a typical analysis is shown in Fig. 2C and D. Deletions in the promoter were apparent as early as day 6, whereas by day 15 most amplicons harbored deletions. Most samples collected 15 days

FIG. 5. Viral load after infection of Chinese rhesus macaques (*Macaca mulatta*) with SIVmac239nefstop or SIVmegalo. Individual serum viremia profiles determined by bDNA assay (Chiron) are shown on the top panel for 15 monkeys infected with SIVmegalo (A) and for five SIVmac239nefstop-infected monkeys (B). Given the large number of points and wide range, the bottom panel (C) shows median values $\pm$ the SD. The serum viremia cutoff was 10³ RNA copies/ml.

after culture on CEMx174 or rPBMC showed promoter-distal deletions in the region from -420 to -130 bp (Fig. 2E and F and 3). Many involved deletions between the numerous 17-, 18-, 19-, and 21-bp repeat sequences in the CMV-IE promoter, although there were deletions elsewhere. By days 30 to 60, one promoter form dominated the CEMx174 or rPBMC culture. It

resulted from a 269-bp deletion between the second and the fourth 19-bp repeats which harbor CRE sites (Fig. 3). A minor form obtained from rPBMC after 1 month culture showed deletion between the first and the third 21-bp repeats (Fig. 3). A few point mutations were observed in the promoter or TAR sequences, although they were never fixed. PCR amplification and subsequent sequencing revealed no changes in tat.

The most promoter proximal deletion identified in these analyses was the 268-bp deletion present after 60 days of culture on CEMx174 cells. In order to ascertain whether this deleted CMV promoter was functionally competent or not, this chimeric LTR (Δ clone61) was subcloned into a HIV-1 (tat+rev)-dependent CAT expression plasmid (Fig. 4A) (32). After transfection of CEMx174 cells by this plasmid, SIVmegalo Δ clone61, it was evident that its activity in a transient assay was certainly greater than that of SIVmegalo and even of wild-type SIVmac239 (Fig. 4B). When the Δ clone61 was cloned into the SIVmegalo backbone, the virus grew as well as wild-type SIVmac239, indicating that deletions in the chimeric LTR did not inactivate the virus (Fig. 4C).

Primary infection in rhesus macaques. Fifteen Chinese rhesus macaques (*M. mulatta*) were inoculated intravenously by a 200 TCID₅₀ of SIVmegalo stock with <5% deletions in the chimeric LTR, along with five control animals with parental SIVmac239nefstop virus. For 11 of 15 animals infected with SIVmegalo, viremia was below the detection threshold of 1,000 RNA copies/ml as determined by Chiron bDNA 3.0 assay (Fig. 5A). One macaque scored $\geq 1,500$ copies/ml for just one time point, while the remaining three animals showed discrete peaks delayed by 7 to 26 days compared to parental virus. By 2 months postinfection SIVmegalo viremia was undetectable in all animals. Compared to primary infection for the parental virus (Fig. 5B), there was a $>10^3$ -fold reduction in viremia (Fig. 5C). No decrease in the peripheral blood CD4⁺-cell count was noted for up to 15 months postinfection, nor was virus detected in lymph node biopsies by in situ hybridization at 4 days (one animal), 14 days (two animals), 21 days (two animals), or 2 months (five animals) postinfection in contrast to parental virus infection (6). All attempts to recover SIVmegalo during primary infection by coculture of up to 10⁷ PBMC failed. Virus could be isolated only from 10 million LNCM after depletion of the CD8⁺ T cells. Such a dramatic reduction in viremia during primary or chronic infection suggests a highly attenuated phenotype for SIVmegalo (24, 43).

The proportion of CD4⁺ or CD8⁺ cells bearing the CD69 early activation marker was not above baseline throughout primary infection (Fig. 6) in sharp contrast to parental virus infection, again suggesting that SIVmegalo replication was highly restricted. To ensure that there was not a small ephemeral peak, the CD69 marker was followed even more closely in one animal (macaque 96R0258) over the initial 18-day period. Although no lymphocyte activation comparable to wild-type was found, there was a small peak centered around day 8, indicative of some immune activation for one SIVmegalo-infected macaque (Fig. 6, insets). Lymph node biopsies showed no significant sign of hyperplasia, as measured by the surface area of germinal centers. The numbers of Ki67⁺ cells/mm² in SIVmegalo-infected monkeys at 2 months versus those observed in naive animals were very slightly but not significantly elevated (see below).

FIG. 6. Dynamics of activated CD69⁺ CD4⁺ and CD69⁺ CD8⁺ cells in peripheral blood during primary SIVmegalo infection. CD69 markers were monitored in CD4⁺- and CD8⁺-T-cell subpopulations for seven SIVmegalo-infected animals and two SIVmac239nefstop-infected animals for 2 months. Insets show the profiles for samples taken almost daily for a single animal (96R0258) between days 4 and 11.

A very sensitive marker of ongoing SIV replication is the reversion of an in-phase stop codon. For example, for SIVmac239nefstop reversion is found in all animals by 15 days postinfection (20). With this in mind, SIVmegalo was designed with an in-phase nef stop codon so that the tempo of ongoing replication and mutation could be addressed. Accordingly, this region of the gene was amplified from PBMC or LNMC DNA. For 9 of 12 animals tested there was no reversion whatsoever out to 2 months; 2 of the 12 animals showed a mixture of TAA/CAA (stop/Gln), while for the remaining animal all sequences encoded CAA (Gln). In this latter case (animal 97R0084), however, viremia remained below the detection threshold. In contrast, for the two control animals tested, i.e., infected by SIVmac239nefstop, the nef stop codon had reverted to wild-type GAA (Glu) codon by day 15, a finding in keeping with previous reports (20). It appears that SIVmegalo replication was so highly restricted that the mutant spectrum generated in 2 months was too small to allow selection of wild-type nef.

Persistence of SIVmegalo and immune responses. As might be expected for a retroviral infection, SIVmegalo persisted, albeit at very low titers. For example, viral DNA could be amplified intermittently from PBMC DNA out to at least 9 to

15 months postinfection (detection threshold one copy/200,000 cells), although virus could not be isolated from 10^7 rPBMC. Moreover, SIVmegalo was successfully recovered by coculture from $\geq 10^7$ CD8-depleted LNMC biopsied at 2 months postinfection or later. For 12 of 15 macaques, antibody titers came up to levels at the low end of the range for parental virus infections, albeit with somewhat delayed kinetics (Fig. 7). Thereafter titers were maintained, as expected of a persistent infection. For the remaining three animals, two showed antibody titers below the threshold of 1/100. Western blotting of these sera by using strips specific for HIV-2 either showed nothing or a weak reaction to p27gag (not shown). However, all three had been infected since viral DNA could be detected in PBMC DNA. Despite these very low antibody titers, the viral loads for these animals did not differ substantially from titers for antibody-positive SIVmegalo-infected animals (Fig. 5). No neutralizing antibodies were detected in any of the numerous sera tested.

ELISPOT assays with pooled SIV Gag or Nef peptides, as well as heat-inactivated HIV-2 virions were performed on PBMC (Fig. 8A, B, and C, respectively). The number of gamma interferon (IFN- γ)-producing cells increased progressively during the first 4 to 6 months showing that SIVmegalo

FIG. 7. Serum antibody responses to SIVmegalo infection. (A) Reciprocal dilution titers for the 15 SIVmegalo-infected (A) and five SIVmac239nefstop (B) control macaques. The cutoff was a 100-fold dilution.

also stimulated cellular immunity. The extent of the response was similar to the one generated during the parental infection. Depletion of CD4⁺ or CD8⁺ cells from PBMC showed that anti-Gag ELISPOT activity was present in both CD4⁺- and CD8⁺-T-cell lymphocyte compartments (Fig. 9).

To address the question of the stability of the SIVmegalo promoter, the region was amplified from DNA extracted from a lymph node from an SIVmegalo-infected monkey (animal 93035) taken at day 100. Viral DNA in the lymph node sample all had the same 190-bp deletion in the 5' enhancer region, which corresponds to a deletion noted in in vitro-infected PBMC from the same animal (Fig. 3). Given that a larger deletion did not abolish viral replication (Fig. 4), it must be presumed that genomes harboring this deletion were replication competent.

Challenge with pathogenic SIVs. Given that robust immune responses were obtained from a highly attenuated SIVmegalo infection, an initial homologous challenge study was performed on two animals (macaques 93029 and 93035) at 6 and 15 months, respectively, postinfection. The monkeys were challenged intravenously with 200 TCID₅₀ of the isogenic SIVmac239nefstop virus. Viremia was systematically negative postchallenge, whereas the kinetics of viremia in two control animals were as expected for Chinese subspecies monkeys (25), indicating that challenge virus was contained by ~3.5 logs at peak height (Fig. 10). No increase in the number of CD69⁺ CD4⁺ or CD69⁺ CD8⁺ cells in peripheral blood was noted. Postchallenge, nested PCR of PBMC DNA was intermittently positive for either SIVmac239nefstop or SIVmegalo LTR.

Given such a degree of protection, a challenge study was performed with uncloned SIVmac251, which can diverge by up

to 5% in the surface envelope protein compared to SIVmac239. Two groups of four macaques infected by SIVmegalo were randomly selected and challenged intravenously by 10 50% animal infectious doses of the uncloned pathogenic SIVmac251 isolate at 4 months (macaques 96R096, 97R0012, 96R0276, and 96R0202) and 6 months (macaques 960954, 960938, 970364, and 970222). Viremia was observed in all eight animals between days 15 and 25 postchallenge; viremia returned to below baseline levels by 30 days in seven of the eight animals (Fig. 11A and B). Compared to control animals (Fig. 11C), viremia was contained by ~3 logs at peak and thereafter out to 150 days. This level of protection obtained against challenge virus is comparable to the results achieved with SIVmac239Δnefstop-vaccinated animals (11). When represented as median values, there was a small difference between challenge at 4 and 6 months (Fig. 11D, total virus load [area under the curve] of 3.8×10^5 versus 1.9×10^4 RNA copies, respectively) although, given the small sample sizes, the difference was not significant ($P = 0.15$). The only lymph node biopsy slightly positive (0.5 cells/mm²) at 2 months postchallenge for SIV of these eight challenged animals by in situ hybridization was from one of the animals challenged at 4 months after SIVmegalo infection. Perhaps not surprisingly, this was the challenged animal of the eight that showed the highest viremia after challenge (96R0276).

Peripheral blood CD4 cell counts were stable postchallenge (mean slopes of -0.03 and -0.02% of CD4⁺ cells per day) for vaccinated animals challenged at 4 and 6 months, respectively, whereas they declined significantly for control animals (mean slope of -0.31% of CD4⁺ cells per day), indicating progression to disease. For some animals, generally for those with low

FIG. 8. IFN- γ ELISPOT responses against pooled Gag (A) or Nef (B) peptides or heat-inactivated HIV-2 virions (C) in PBMC drawn from SIVmac239nefstop-infected macaques during primary infection, along with two SIVmac239nefstop-infected animals as controls. Spot frequencies are given as the signal minus the background. A cocktail of 16 SIV Gag or 19 Nef 15-mer peptides was used. Note that the animal (96R0202) that failed to produce an antibody response to SIVmac239nefstop showed ELISPOT responses comparable to some of the antibody-positive animals.

FIG. 9. Anti-Gag peptide responses in CD4⁺ and CD8⁺ T-cell compartment. Three months after infection with SIVmac239nefstop, rPBMC from four SIVmac239nefstop-infected animals were submitted to a CD4⁺ or CD8⁺ T-cell depletion (>90% depletion was achieved) and used for IFN- γ ELISPOT assay against pooled Gag peptides. Spot frequencies are given as the signal minus the background.

initial antibody titers, there was an anamnestic antibody response (Fig. 12). However, this did not correlate in any way with the height of viremia upon challenge. PCR amplification of a segment spanning the LTR showed that viremia was due to SIVmac251 and not reactivation of SIVmac239nefstop. Once again, no increase in the number of CD69⁺ CD4⁺ or CD69⁺ CD8⁺ cells in peripheral blood was noted. The modest degree of viremia was reflected in the mean number of Ki67⁺ cells per mm² in lymph node biopsies taken at 60 to 70 days postchallenge (Fig. 13). Importantly, the values were significantly less than the SIVmac251 challenged animals at 2 months. Altogether, these data indicate that challenge was accompanied by little replication and tissue inflammation.

FIG. 10. Serum viremia of a pilot challenge study with homologous virus. At 9 and 15 months after SIVmac239nefstop inoculation, two macaques were challenged with 200 TCID₅₀ of homologous virus (SIVmac239nefstop). Two naive control animals were inoculated with the same dose of virus on the same day. The serum viremia cutoff was 10³ RNA copies/ml.

FIG. 11. SIVmegalo infection protects against uncloned pathogenic SIVmac251 challenge virus. (A and B) Serum viremia for animals challenged at 4 and 6 months, respectively. (C) The data for six naive macaques infected by the same SIVmac251 challenge stock are also given. Panel D shows the median values $\pm$ the SD. The serum viremia cutoff was 10^3 RNA copies/ml. The difference between the combined challenge groups ($n = 8$) and naive controls ($n = 6$) is statistically significant ($P = 0.0019$, Mann-Whitney test).

DISCUSSION

As previously shown, efficient transcription and replication of SIV can be achieved in the absence of NF- κ B and Sp1 binding elements *ex vivo* (15) and can induce AIDS in rhesus monkeys *in vivo* (16). This unexpected result was due to a regulatory element located immediately upstream of NF- κ B binding site that allows efficient viral replication in absence of the entire core-enhancer region (34). By replacing the SIV enhancer promoter region by that of CMV-IE, a very similar replication profile on CEMx174 or rPBMC was obtained (15). However, this virus was highly attenuated in macaques compared to Δ NF- κ B Δ Sp1234 constructs (16). This virus retained the capacity to replicate in its host as proven by deletion analysis and to establish a chronic infection. First, these data show that the CMV-IE promoter is able to overcome the upstream regulatory element defined by Pohlmann et al. (34), and sec-

ond, these data show that variation in the pattern of protein expression by promoter can lead to drastic physiopathological changes. A generally similar construct has been described, although only *ex vivo* data were reported (13). Replication kinetics were comparable to those of SIVmegalo.

In fact the CMV-IE promoter was not well adapted to the SIV scaffold, for it grew initially slowly. When replication took off, it was accompanied by deletions in the promoter distal regulatory region between positions -450 and -200 bp. Once this region was deleted *in vitro*, the mutant virus acquired kinetics similar to those of wild-type virus on the CEMx174 cell line and on rPBMC (Fig. 4C). The deletions presumably resulted in enhanced transcription and replication (burst size), resulting in their outgrowing other variants, which was confirmed by CAT assay (Fig. 4B). The SIVmegalo promoter after 2 months infection harbored a deletion resulting in the loss of

FIG. 12. Anamnestic antibody response in SIVmegalogo-challenged animals. Four animals were challenged at 4 (A) and 6 (B) months after SIVmegalogo infection with a pathogenic SIVmac251 strain. The numbers under datum points indicate the numbers of coincident points.

FIG. 13. SIVmegalogo infection protects against uncloned pathogenic SIVmac251 challenge virus. Mean number of Ki67⁺ cells per mm² in lymph node biopsies taken at 60 to 70 days postinfection or postchallenge. Sample sizes are indicated by "n," while the SD, maximum, and minimum values are also shown. The SIVmegalogo-infected animals were macaques 960938, 960954, 970222, 970364, 97R0012, and 97R0276; those infected by SIVmegalogo and challenged by SIVmac251 were macaques 97R0012, 96R0276, 970222, and 970364; the SIVmac251-infected animals were macaques 970034, 960976, 264, 9025, 92418, and 92428. All differences between SIVmac251 and the other categories are statistically significant ($P < 0.01$).

the four 16-bp repeats, two 19-bp repeats, two 18-bp repeats, and one 21-bp repeat which encode eight transcription factors motifs in total. Analogous deletions in the CMV-IE promoter have been made experimentally and have been shown to augment transcription in transfection assays, so there is general concordance (40). The transcriptional improvement is probably due to the juxtaposition of regulatory elements, which act as an enhancer. Similarly, clones derived from lymph nodes of SIVmegalogo-infected monkey are deleted in a manner that does not affect enhancer/promoter activity (40). Thus, it seems that maximal CMV-IE activity is essential for viral replication. Since the HIV/SIV RT is very prone to making deletions, especially between homologous sequences (9, 26, 33), the rapidity with which they may be detected ex vivo or in vivo is understandable, particularly if there is a selective advantage.

When inoculated into rhesus macaques, SIVmegalogo grew very poorly, so much so that there were only six positive serum RNA samples among the 15 animals infected. Despite this, the SIVmegalogo infection established itself since virus could be occasionally detected in PBMC or isolated from LNMC. The poor replication of SIVmegalogo was reflected in the slow kinetics of antibody titers (Fig. 7A). CMV promoter readily accumulated deletions during ex vivo culture on PBMC of macaque 93035 (Fig. 2), one form was identical to the major viral form obtained in LNMC of macaque 93035 after 3 months of SIVmegalogo infection (Fig. 3). The structure of promoter obtained in this animal at 100 days was almost identical to a

construct, denoted *d/NdeI*, which functioned as well as the undeleted promoter in transient-transfection assays (40).

The SIVmegalogo-infected animals were challenged with the isogenic SIVmac239nefstop or the uncloned SIVmac251. All animals infected with SIVmegalogo prior to challenge showed a remarkable control of the challenge virus with at least a three-fold log decrease in the peak high compared to naive animals (Fig. 11). As already shown (11, 46), when a longer period of time elapses after vaccination, it seems that the efficiency of the control of challenge virus is slightly increased between 4 and 6 months. This slight improvement in control was associated with a better ELISPOT response against whole heat-inactivated HIV-2 (mean, 62 versus 124 spots/ 10^6 PBMC [ELISPOT assay]), pooled Gag (mean, 52 versus 96), or Nef peptides (mean, 50 versus 150). It was also associated with slightly higher antibody levels before challenge, from a median titer of 5,500 after 4 months to $\sim 10,000$ after 6 months. It is possible that better protection could have been obtained if the challenge had occurred later after vaccination. Since it is easier for a live attenuated SIV vaccine to protect against challenge via the vaginal mucosa as opposed to the intravenous route (18), it is possible that SIVmegalogo might protect even better against mucosal challenge.

The present study was performed with the Chinese subspecies of rhesus macaque as opposed to the more widely used Indian macaques. Generally, peak viremia is ~ 10 -fold less in the Chinese macaque (25). Bearing this in mind, it appeared that SIVmegalogo was still very attenuated when we compared

the viral load generated in SIVmegaloinfected animals to SIVmac239nefstop- or SIVmac251-infected monkeys in the Chinese subspecies. Moreover, after SIVmegaloinfection these animals are able to control remarkably well the pathogenic inoculum compared to naive animals. Attenuation might be related to the delayed replication kinetics (Fig. 2A), which should prove favorable to immune control (5). Another possibility might be that although SIVmegaloinfection targets CD4⁺ cells, including T lymphocytes, replication is more confined to other CD4⁺ cells, thus sparing the helper T lymphocyte and allowing good control of the virus.

In conclusion, we have shown that SIVmegaloinfection is a highly attenuated virus that still retains the capacity to protect strongly against a pathogenic uncloned slightly heterologous strain (3 logs at peak viremia compared to naive animals). Consequently, promoter exchange can constitute a novel means to attenuate SIV. The present results show that it is possible to produce robust anti-SIV immune responses with surprisingly little replication. Unravelling this phenomenon should contribute to our understanding of one of the scientific challenges facing HIV vaccine research (23).

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