Efficient Transcription and Replication of Simian Immunodeficiency Virus in the Absence of NF- κ B and Sp1 Binding Elements

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Ten mutants of the simian immunodeficiency virus (SIV) SIVmac239 bearing deletions (Δ) or substitutions (subst) in the NF- κ B and/or Sp1 binding elements were created, and the replicative capacities of the mutants were analyzed. All mutants, including one extensively mutagenized strain entirely missing the NF- κ B and four Sp1 binding elements, replicated with wild-type kinetics and to a wild-type level in peripheral blood mononuclear cell cultures in 50 to 100% of the experiments. One group of mutants replicated very similarly to SIVmac239 in kinetics and yield in CEMx174 cells ($2 \times \text{NF-}\kappa\text{B} \approx \text{SIVmac239} \approx \Delta \text{NF-}\kappa\text{B} \approx \Delta \text{Sp1234} \approx \text{substNF-}\kappa\text{B} \approx \text{substSp12} \approx \text{substSp23}$), while a second group replicated with delayed or slightly delayed kinetics in CEMx174 cells ($\text{SIVmac239} > \text{substSp34} > \Delta \text{NF-}\kappa\text{B} \Delta \text{Sp1234} \approx \Delta \text{NF-}\kappa\text{B} \Delta \text{Sp1} > \text{substSp1234}$). Reversions or additional mutations were not detected in the U3 and R regions of proviral DNA from CEMx174 cells infected with the SIVmac239 mutants. Similar results were obtained when mutants of SIVmacMER (a macrophage-competent derivative of SIVmac239) were tested in peripheral blood mononuclear cell and CEMx174 cultures. However, the growth of most mutated viruses was suppressed in primary rhesus monkey alveolar macrophages ($\text{SIVmacMER} \approx 2 \times \text{NF-}\kappa\text{B} \approx \text{substNF-}\kappa\text{B} > \Delta \text{NF-}\kappa\text{B} > \Delta \text{NF-}\kappa\text{B} \Delta \text{Sp1234} \approx \Delta \text{NF-}\kappa\text{B} \Delta \text{Sp1} > \Delta \text{Sp1234} \approx \text{substSp12} > \text{substSp23} \approx \text{substSp34} \approx \text{substSp1234} \approx \text{SIVmac239}$). Thus, changes in the Sp1 binding sites had the most dramatic effects on SIVmac replication in primary macrophage cultures. Analysis of long terminal repeat-driven secreted alkaline phosphatase activity in transient assays showed that, unlike human immunodeficiency virus type 1, the SIV long terminal repeat possesses an enhancer region just upstream of the NF- κ B element which maintains significant levels of basal transcription in the absence of NF- κ B and Sp1 sites. This region is responsive to transactivation by Tat. In addition, the SIV TATA box was shown to be stronger than that of human immunodeficiency virus type 1. Therefore, the surprisingly high replicative capacity of NF- κ B and Sp1 binding site mutants of SIVmac is due to unique features of the enhancer/promoter region.

The transcription of retroviruses is directed by regulatory sequences in the long terminal repeats (LTRs), which are located at the ends of proviral DNA. Each LTR consists of three regions: U3, R, and U5 (6). Major transcriptional control elements (TCEs) reside within the U3 region of retroviral LTRs. Efficient transcription of human immunodeficiency virus type 1 (HIV-1) requires the presence of the TATA box promoter, binding sites for transcription factors NF- κ B and Sp1, and the Tat-responsive region (TAR) (10, 11, 14, 18, 19, 21, 30, 38 [reviewed in references 8 and 12]). HIV-1 contains three Sp1 and two NF- κ B binding sites upstream of the TATA box. Their elimination results in essentially total inactivation of the provirus (11, 16, 23, 39). While a number of other transcription factors have been linked to various parts of the HIV-1 LTR (12, 33, 40), none of them can compensate for the loss of NF- κ B and Sp1 binding sites. The HIV-1 TCEs appear to possess a high degree of functional redundancy (32). Mutated HIV-1 missing one of the NF- κ B and all Sp1 sites is still capable of efficient replication in a number of cell types, including stimulated peripheral blood lymphocytes (23, 39). The latter findings are somewhat different from the results obtained with reporter gene constructs in transient assays in which the loss of one of the NF- κ B or Sp1 sites was readily detectable and in which the elimination of two or more sites had a profound effect on LTR-driven transcription (1, 3, 15, 25, 30, 35).

While numerous studies have focused on the relative importance of NF- κ B and Sp1 binding sites for HIV-1 replication in cell lines and primary activated lymphocytes, we are aware of only a single study which has addressed a role in primary macrophages. Moses et al. (29) used transfection assays to examine the effects of HIV-1 enhancer mutations on expression in primary monocytes/macrophages. Sp1 elements had the greatest effect in unstimulated monocytes/macrophages, but other elements became important in stimulated monocytes/macrophages (29). Similarly, very little has been done to address the effects of NF- κ B and Sp1 binding site mutations on the replication of the simian immunodeficiency virus (SIV) SIVmac and HIV-2 in T-cell lines, primary lymphocytes, and primary macrophages. Bellas et al. (2) studied an NF- κ B binding site mutant of SIVmac and found a much greater effect on replication in primary macrophages than in cell lines or primary lymphocytes.

The transcriptional control region of the pathogenic SIV clone SIVmac239 contains a TATA box, four putative Sp1 binding sites, and only one NF- κ B binding site (36). While similar to the TCE of HIV-1 (37, 42), the SIVmac structure is closer to that of HIV-2 (13, 41). Previous transfection studies have indicated that HIV-2 and SIVmac contain additional enhancer elements that are not found in HIV-1, that are located upstream of the NF- κ B binding site, and that are responsive to T-cell receptor and monocyte stimulation (5, 25, 26, 37).

In this report, we describe the characteristics of variant SIVmac239 strains with extensively mutagenized TCEs. Their growth capacities exhibited in primary macaque peripheral blood mononuclear cells (PBMCs), primary macrophages, and

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MUTATION	SEQUENCE	VIRUS
<div> <div>Stop-codon for nef gene</div> <div> <div>9866</div> <div>9880</div> <div>9890</div> <div>9900</div> <div>9910</div> <div>9920</div> <div>9930</div> <div>9940</div> <div>9950</div> <div>9962</div> </div> <div> <div>NF-κB</div> <div>Sp1</div> <div>Sp1</div> <div>Sp1</div> <div>Sp1</div> <div>TATA-box</div> </div> </div>		
None	TGAAACAGCAGGGACTTTCCACAAGGGGATGTTACGGGAGGTACTGGGGAGGAGCCGTCGGGAACGCCCACTTCTTGATGTATAAATATCACTG	SIVmac239
NF- κ B deletion		SIVANF κ B
Sp1 deletion	 AGACAGCAGGGACTTTCCACAA	SIVASp1234
Double NF- κ B		SIV/2xNF κ B
Point mutation of NF- κ B	CTC.....	SIVsubstNF κ B
NF- κ B and Sp1 complete deletion		SIVANF κ BASp1234
NF- κ B and 1st Sp1 deletion		SIVANF κ BASp1
Substitution of 1st and 2nd Sp1	TCAAGCACCTGACTATCTGCA.....	SIVsubstSp12
Substitution of 2nd and 3rd Sp1	ACTATCTGCACACTAGTCTAG.....	SIVsubstSp23
Substitution of 3rd and 4th Sp1	ACTAGTCTAGTACAGCACTCG.....	SIVsubstSp34
Substitution of all Sp1	TCAAGCACCTGACTATCTGCACACTAGTCTAGTACAGCACTTG.....	SIVsubstSp1234

FIG. 1. Mutations in SIVmac239 transcriptional control elements. The nature of the introduced mutations, their sequence, and the designations of respective mutant viruses are shown. Proviral SIVmac239 genome sequences can be found in an article by Regier and Desrosiers (36). Dots indicate identities in sequence, and dashes indicate deletions. The stop codon for the *nef* gene, NF- κ B and Sp1 binding sites, and the TATA box are underlined. Mutant SIV/2xNF κ B contains an insertion of the sequence shown between nucleotides 9889 and 9890, creating a duplication of the NF- κ B element.

CEMx174 cells, taken together with additional data on LTR-driven transcription, show that even the total loss of NF- κ B and Sp1 sites in SIVmac may be partially or completely compensated for by unique elements present in the SIVmac enhancer/promoter region.

MATERIALS AND METHODS

Construction of mutated viruses. Ten mutants of SIVmac239/*nef*-open bearing changes in the NF- κ B and Sp1 binding sites were created by means of site-specific mutagenesis (Fig. 1). The mutations were introduced into both proviral LTRs.

The wild-type SIVmac239 was previously cloned in two parts and is carried on plasmids p239Sp5' (5' clone) and p239SpE3' (3' clone), which contain the left and right halves of the genome, respectively (36). In this work, we have used a modified 3' clone of SIVmac239 (kindly provided by Z. Du, Harvard Medical School), in which an additional *Eco*RI site was introduced just after the viral sequence and from which the cellular DNA sequences were eliminated. The resulting 3,834-bp *Sph*I-*Eco*RI viral DNA fragment was cloned into plasmid pSp72. The use of this construct significantly simplified subcloning and recloning procedures. The viral sequence in this 3' clone is identical to that in the 3' clone used previously (36), except that the flanking cellular sequences have been removed and the stop codon in *nef* has been repaired to Glu.

The 854-bp *Sac*I-*Eco*RI fragment of the 3' clone was then subcloned in pSp72 and used for the introduction of site-specific mutations. We have employed PCR-directed mutagenesis (17) with one or two mutagenic primers and two terminal primers in each reaction mixture. Terminal primers spanned *Bsu* 36I and *Pf*MI sites, which are unique in the reconstructed 3' subclone. The resulting 528-bp fragment was digested with those enzymes and inserted into the *Sac*I-*Eco*RI subclone. In some instances, the mutation-containing PCR product was initially cloned into blunt-end cloning vector (PCRII; Invitrogen Corp., San Diego, Calif.) or pUC18/*Sma*I (Pharmacia, Piscataway, N.J.), and then, upon sequence verification, recombined back into the *Sac*I-*Eco*RI subclone; the *Sph*I-*Sac*I fragment of proviral DNA was then cloned into this mutated subclone, thus creating a full 3' clone of SIVmac239. The mutated clones were multiply sequenced through the TCE region and all restriction sites used in mutagenesis. The introduction of TCE mutations into the 5' clone was performed essentially as described earlier (18). The *Sph*I-*Sac*I fragment of the SIVmac macrophage-competent strain MER was inserted into the mutated subclones for the creation of TCE mutants of SIVmacMER. This strain differs from SIVmac239 only by

three amino acid changes in the *env* gene, which are located in the 3' part of the virus genome between the *Sph*I and *Sac*I sites (27, 28).

Molecular cloning. All routine cloning procedures were performed essentially as described earlier (18). Epicurian Coli XL1-Blue MRF' Supercompetent Cells (Stratagene Cloning Systems, La Jolla, Calif.) were used for the transformation and plasmid growth. Construction of mutated secreted engineered alkaline phosphatase (SEAP) expression plasmids was performed with wild-type HIV and SIV LTR-SEAP plasmids (kindly provided by R. Means, Harvard Medical School). These plasmids contained the SEAP gene under the control of viral TCEs (nucleotides 9806 to 10093 for SIV and 9405 to 9611 for HIV), which included all NF- κ B and Sp1 binding sites, the TATA box, and the full transactivation-responsive region (TAR). These fragments of the viral LTRs had been inserted into a SEAP-containing plasmid between unique *Mlu*I and *Hind*III sites (26a). These plasmids were used for PCR-directed mutagenesis for the creation of various HIV and SIV LTR-SEAP constructs. Each of the resulting plasmids was sequenced at least twice through the viral insert. PCR-amplified viral fragments from infected culture cell lines were treated with polynucleotide kinase and cloned into pUC18/*Sma*I-cut vector (Pharmacia) according to manufacturer's recommendations.

Primers. All nucleotide primers were custom-made by Amifof, Inc. (Boston, Mass.) or GIBCO BRL (Grand Island, N.Y.). The terminal primers used in PCR mutagenesis were 5'-GATTACACCTCAGGACCAGG-3' (9548 to 9567) and 5'-GCACCGGCCAAGTGCTGGTGA-3' (10089 to 10069). Primers 5'-GGG ATTTATTACAGTGCAAGAAGA-3' (6 to 29) and 5'-GGCTAGGAGAGAT GGGAACACACA-3' (724 to 701) were used for the amplification of SIVmac U3 and R regions from infected cells.

PCR amplification and DNA sequencing. PCR amplification and DNA sequencing were performed essentially as described earlier (18). A thermal cycler produced by MJ Research (Watertown, Mass.) was used for PCR amplification.

Nucleotide sequence analysis. Nucleotide sequences were analyzed and aligned by using MacVector version 4.1.4. software (International Biotechnologies, Inc., New Haven, Conn.). The sequence of SIVmac239 has been published previously (36). The sequence of HIV-1 NL4-3 was taken from GenBank (accession number M19921) (4).

Cells. Rhesus monkey PBMCs, CEMx174, and COS-1 cells were maintained as described earlier (18). Alveolar macrophages were obtained from bronchoalveolar lavage specimens, as described previously, from healthy, mature rhesus macaques that were serologically negative for SIV, type D retrovirus, and simian foamy virus (27). Macrophages were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum and 2.5% human type AB serum (GIBCO), penicillin

(100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 to 0.5 µg/ml) at a concentration of 5×10^5 cells per ml in 24- or 48-well flat-bottom plates. The culture medium was changed twice weekly.

Preparation of virus stocks. Pelleted CEMx174 cells were transfected with *Sph*I-digested 5'- and 3'-SIVmac plasmids by a DEAE-dextran procedure (31). For stock preparation, virus was harvested at or near the peak of virus production, filtered, and stored in aliquots at -80°C . Stocks were assayed for the concentration of SIV p27^{gag} antigen with a commercially available antigen capture kit (Coulter Corp., Hialeah, Fla.).

Virus replication in vitro. CEMx174 cells split 1:3 the previous day were either transfected as described above or infected with an aliquot of SIVmac containing 2 ng of viral p27 protein. Activated PBMCs were infected with the same amount of SIVmac upon stimulation with phytohemagglutinin (Sigma, St. Louis, Mo.) as described previously (18). Alveolar macrophages were infected 1 day after their isolation with SIVmac containing 10 ng of p27 per 10^6 cells. Cell supernatants were sampled, clarified, and assayed for SIV p27 by antigen capture.

Isolation of cellular DNA. Infected CEMx174 cells were pelleted, washed, and lysed in a small volume (50 to 100 µl). DNA was isolated in a single microcentrifuge tube for each sample with the HRI AmpPrep kit (HRI Research, Inc., Concord, Calif.) and subjected to the PCR amplification technique described above.

SEAP assay. COS-1 cells were transfected with various HIV or SIV LTR-SEAP plasmids (1 µg) in the presence of DEAE-dextran (7) 1 day after being split. SEAP in the culture supernatant was detected 16 to 18 h later with the Phospha-Light chemiluminescent reporter gene assay system (Tropix, Inc., Bedford, Mass.) according to the manufacturer's recommendations. Each assay was done in triplicate. Each experiment was performed three to six times separately.

RESULTS

Replication of SIVmac239 TCE mutants in vitro. TCE mutations were placed into both LTRs to prevent generation of parental sequences by recombinational or replicative events. All SIVmac239 TCE mutants replicated in CEMx174 cells after transfection of cloned DNA (Fig. 2A and B). While most mutants consistently replicated with kinetics similar to those of the wild type after transfection, the appearance of several mutants was delayed. The replication of mutant viruses $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1}$ and substSp34 was slightly delayed, and the replication of $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1234}$ and substSp1234 was substantially delayed (Fig. 2A and B). The latter two viruses are the most severely altered of all the mutants (Fig. 1). Virus stocks prepared from transfection were used to infect CEMx174 cells with amounts normalized to contain 2 ng of p27 CA antigen. The pattern of replication after infection was similar to that observed after transfection (Fig. 2C). The greatest delays in replication were observed with $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1234}$, $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1}$, and substSp1234. All mutant viruses ultimately reached similar levels of virus production in CEMx174 cells (Fig. 2C).

The replication of mutant viruses was also examined with phytohemagglutinin-stimulated PBMCs from rhesus monkeys in the presence of interleukin 2. Normalized amounts of virus containing 2 ng of p27 were again used for infection. The replication of mutant viruses in rhesus monkey PBMCs was not significantly delayed in most cases. In fact, the severely altered mutant $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1234}$ was not delayed in four of four experiments, and viruses $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1}$ and substSp1234 exhibited wild-type kinetics in two of four experiments. Representative results of the replication of SIVmac239 TCE mutants in rhesus PBMCs are shown in Fig. 3. A summary of the results of all experiments is presented in Table 1.

Sequence of the TCE region in SIVmac239-infected cells. Total cellular DNA from SIV-infected CEMx174 cell cultures was used for PCR amplification of the U3 and R regions (Fig. 4). Multiple clones were derived and sequenced through the TCE of each mutant. The clones derived from $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1234}$ (six clones) and substSp1234 (four clones) were sequenced through all of the U3 and R regions. No reversions in the TCE were detected, and except for an occasional sporadic mutation in a single clone, no other mutations that could possibly explain the

FIG. 2. Replication of SIVmac239 TCE mutants in CEMx174 cells. Results of transfection with SIV $\Delta\text{NF}\kappa\text{B}$, SIV ΔSp1234 , SIV/2xNF κB , SIV point mutant NF κB , SIV $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1234}$, and SIV $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1}$ (A) and with SIVsubstSp mutants (B) are shown. (C) Virus stocks derived from the transfections described above were used for infection of CEMx174 cells. Virus containing 2 ng of p27 was used for each infection. Symbols: A, $\blacksquare$, SIV $\Delta\text{NF}\kappa\text{B}$; $\bullet$, SIV ΔSp1234 ; $\blacktriangle$, SIV/2xNF κB ; $\blacklozenge$, SIVsubstNF κB ; $\square$, SIV $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1234}$; $\circ$, SIV $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1}$; $\triangle$, SIVmac239; B, $\blacksquare$, SIVmac239; $\bullet$, SIVsubstSp12; $\blacktriangle$, SIVsubstSp23; $\circ$, SIVsubstSp34; $\square$, SIVsubstSp1234; C, $\blacksquare$, SIV $\Delta\text{NF}\kappa\text{B}$; $\bullet$, SIV ΔSp1234 ; $\blacktriangle$, SIV/2xNF κB ; $\blacklozenge$, SIVsubstNF κB ; $\square$, SIV $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1234}$; $\circ$, SIV $\Delta\text{NF}\kappa\text{B}\Delta\text{Sp1}$; $\triangle$, SIVsubstSp12; $\diamond$, SIVsubstSp23; $\boxtimes$, SIVsubstSp34; $\boxtimes$, SIVsubstSp1234; $\odot$, SIVmac239.

surprisingly high replicative capacity of these mutant viruses were observed.

Replication of SIVmacMER TCE mutants in vitro. We also wanted to test the replication of mutant viruses in primary macrophage cultures. Since SIVmac239 replicates poorly in primary cultures of rhesus monkey macrophages (9, 28), we transferred the TCE mutations into a different genetic back-

FIG. 3. Replication of SIVmac239 TCE mutants in rhesus monkey PBMCs. Virus containing 2 ng of p27 was used for each infection. SIVmac239 was used in parallel. The results of two separate experiments with PBMCs from two different rhesus monkeys (A and B) are shown. Symbols: ■, SIV Δ NF κ B; ●, SIV Δ Sp1234; ▲, SIV/2xNF κ B; ◆, SIVsubstNF κ B; □, SIV Δ NF κ B Δ Sp1234; ○, SIV Δ NF κ B Δ Sp1; △, SIVsubstSp12; ◇, SIVsubstSp23; ▤, SIVsubstSp34; ▥, SIVsubstSp1234; ⊙, SIVmac239.

bone. SIVmacMER contains three mutations in the envelope relative to SIVmac239: 67V→M, 176K→E, and 382G→R (27, 28). These mutations confer the high replicative capacity of the virus for primary rhesus monkey macrophages (27). We

FIG. 4. PCR amplification of proviral U3 and R regions from the DNA of infected CEMx174 cells. DNAs from the cultures infected with SIV Δ NF κ B, SIV Δ Sp1234, SIV/2xNF κ B, SIVsubstNF κ B, SIV Δ NF κ B Δ Sp1234, and SIV Δ NF κ B Δ Sp1 (lanes 1 to 6, respectively) were used for amplification as described in Materials and Methods. The locations of molecular size markers (base pairs) are shown to the left.

thus exchanged the TCE mutations into the background of SIVmacMER and analyzed the growth properties of the mutant viruses.

The TCE mutants of the macrophage-competent strain SIVmacMER replicated in CEMx174 cells and primary rhesus PBMC cultures similar to the equivalent mutants of SIVmac239. SIVmacMER viruses Δ NF κ B Δ Sp1234, Δ NF κ B Δ Sp1, substSp1234, substSp34, and Δ NF κ B grew with a delay in CEMx174 cells in transfection and infection experiments (Fig. 5A). As with SIVmac239 TCE mutants, the delay in replication of these SIVmacMER mutant viruses was slightly longer after transfection than after infection. All viruses ultimately reached the wild-type level of production in CEMx174 cells. Only SIVmacMERsubstSp1234 exhibited a slight growth delay in stimulated rhesus monkey PBMCs (Fig. 5B and C), while all other viruses exhibited wild-type kinetics and levels of production.

In contrast to the results with CEMx174 and rhesus macaque PBMC cultures, most of the SIVmacMER TCE mutants showed both delayed and suppressed replication in primary rhesus macrophages (Fig. 6 and Table 2). The results varied slightly in macrophage cultures derived from different animals. However, it could be clearly seen that only 2xNF κ B and substNF κ B mutants showed a wild-type pattern of replication

TABLE 1. Replication of SIVmac239 TCE mutants in vitro

Virus	Replication capacity ^a		
	CEMx174 cells		PBMCs (infection)
	Transfection	Infection	
SIV Δ NF κ B	++++ (1)	++++ (3)	++++ (4) (slightly delayed once) ^b
SIV Δ Sp1234	++++ (1)	++++ (3) (slightly delayed once)	++++ (4) (slightly delayed once) ^b
SIV/2xNF κ B	+++++ (2)	At least +++++ (3)	++++ (4)
SIVsubstNF κ B	++++ (1)	++++ (3)	++++ (4) (delayed once) ^b
SIV Δ NF κ B Δ Sp1234	Delayed (2)	Delayed (2)	++++ (4)
SIV Δ NF κ B Δ Sp1	Delayed once (2)	Delayed (3)	Delayed twice (4) ^c
SIVsubstSp12	++++ (2)	++++ (3)	++++ (4)
SIVsubstSp23	++++ (2)	++++ (3)	++++ (4)
SIVsubstSp34	Delayed (1)	Delayed (1)	++++ (4) (slightly delayed twice)
SIVsubstSp1234	Substantially delayed (2)	Delayed (2)	Delayed twice (4)

^a +++++, replication reached wild-type level (no less than 50%) with no or an unsubstantial delay compared with SIVmac239; +++++, replication reached the peak level earlier than with wild-type SIVmac239; slightly delayed, replication reached wild-type levels 2 to 3 days later than SIVmac239; delayed, replication reached wild-type levels 4 to 7 days later than SIVmac239; substantially delayed, replication reached wild-type levels $\geq$ 7 days later than SIVmac239. The number of experiments for each mutant with each cell type is shown in parentheses.

^b In this case, replication of the mutant in PBMCs reached 30% of the wild-type level.

^c In these cases, replication of the mutants in PBMCs reached 10 and 30% of the wild-type level.

FIG. 5. Replication of SIVmacMER TCE mutants in CEMx174 cells (A) and rhesus monkey PBMCs (B and C). Virus containing 2 ng of p27 was used for each infection. The results of two separate experiments with PBMCs from two different rhesus monkeys are shown. Symbols: ■, SIVmacMER Δ NF κ B; ●, SIVmacMER Δ Sp1234; ▲, SIVmacMER/2xNF κ B; ◆, SIVmacMERsubstNF κ B; □, SIVmacMER Δ NF κ B Δ Sp1234; ○, SIVmacMER Δ NF κ B Δ Sp1; △, SIVmacMERsubstSp12; ◇, SIVmacMERsubstSp23; ✕, SIVmacMERsubstSp34; ▣, SIVmacMERsubstSp1234; ○, SIVmacMER; □, SIVmac239.

in all experiments. The ability of SIVmacMER to replicate in primary macrophages was impaired to various degrees by all other TCE mutations. Mutant Δ NF κ B was partially affected, mutant Δ NF κ B Δ Sp1 was even more suppressed, mutants Δ NF κ B Δ Sp1234 and substSp12 grew to a significant level in only one experiment out of three, and the other mutants (Δ Sp1234, substSp23, substSp34, and substSp1234) never exhibited substantial growth in primary rhesus macrophages. A summary of the replicative capacities of SIVmacMER TCE mutants in vitro is shown in Table 2.

Different transcriptional activities of SIV and HIV LTR sequences with mutations in the TCE. In an attempt to explain the significant replicative capacities of SIVmac strains with severely altered TCEs, we constructed a number of reporter plasmids in which the gene for SEAP was expressed under the control of HIV or SIV LTR sequences (Fig. 7). The elimination of all NF- κ B and Sp1 binding sites from the HIV LTR reduced SEAP activity in transfected COS-1 cells to background levels (Fig. 8A). This represents a reduction of greater than 200-fold. Coexpression of Tat was able to increase SEAP expression driven by the nonmutated HIV LTR approximately 14-fold, but it was not able to increase expression from the NF- κ B- and Sp1-deleted form over background levels (Fig. 8B and E). In contrast, deletion of NF- κ B and Sp1 elements from the SIV LTR did not abolish SEAP expression from the corresponding plasmid (Fig. 8C). In fact, the SIV-SEAP plasmid missing the NF- κ B and Sp1 binding sites retained approximately 10% of its activity (Fig. 8C). Sequences upstream of the NF- κ B and Sp1 binding sites in the SIV LTR were principally responsible for this residual activity, since their removal reduced the residual expression from 10% to 2% (Fig. 8C). Enhanced transcription caused by these upstream sequences was also responsive to Tat transactivation (Fig. 8D and E). In addition to these upstream enhancer elements, the SIV LTR appeared to contain a slightly more efficient TATA box than the HIV-1 LTR (Fig. 8A to D).

Since the HIV LTR reporter plasmid used for the data in Fig. 8 contained only 20 bp upstream of the NF- κ B and Sp1

FIG. 6. Replication of SIVmacMER TCE mutants in primary rhesus monkey macrophages. Virus containing 10 ng of p27 was used to infect 10^6 cells. The results of two separate experiments with macrophages from two different rhesus monkeys are shown. Symbols are the same as those in Fig. 5.

TABLE 2. Replication of SIVmacMER TCE mutants in vitro

Virus	Replication capacity ^a		
	CEMx174 cells	PBMCs	Primary macrophages
SIVmacMER Δ NF κ B	Delayed once (2)	++++ (2)	+++ (3)
SIVmacMER Δ Sp1234	++++ (2)	++++ (2)	++ (3)
SIVmacMER/2xNF κ B	++++ (2)	++++ (2)	++++ (3)
SIVmacMERsubstNF κ B	++++ (2)	++++ (2)	++++ (3)
SIVmacMER Δ NF κ B Δ Sp1234	Delayed (3)	++++ (2)	++ (3) ^b
SIVmacMER Δ NF κ B Δ Sp1	Delayed (3)	++++ (2)	++ (3)
SIVmacMERsubstSp12	++++ (2)	++++ (2)	++ (3)
SIVmacMERsubstSp23	++++ (2)	++++ (2)	++ (3)
SIVmacMERsubstSp34	Delayed (2)	++++ (2)	++ (3)
SIVmacMERsubstSp1234	Delayed in 3 of 4 (4) ^c	Slightly delayed (2)	++ (3)

^a +++++, replication reached the wild-type level (no less than 50%) with no delay or an unsubstantial delay compared with SIVmac239 in all experiments; slightly delayed, replication reached wild-type levels ≥ 4 days later than SIVmac239; delayed, replication reached wild-type levels ≥ 4 days later than SIVmac239; +, replication reached the wild-type level in two of three experiments; ++, replication reached the wild-type level in one of three experiments; +, replication has not reached the wild-type level in any of three experiments. The number of experiments for each mutant with each cell type is shown in parentheses.

^b The level of replication of this mutant in macrophages may be graded as between ++ and +++.

^c One separate stock of this mutant has exhibited wild-type replication in CEMx174 cells. PCR amplification of DNA from infected cells and sequencing of five separate clones through the U3 and R regions have not shown any consistent reversions and differences from "normally" delayed SIVmacMERsubstSp1234 in this part of the proviral sequence. This mutant may have compensatory mutations elsewhere in the genome.

elements, we constructed an equivalent HIV LTR reporter plasmid with 65 bp of upstream sequences. Deletion of the NF- κ B and Sp1 binding sites from this plasmid also reduced SEAP expression to background levels (Fig. 9). Thus the enhancer element present upstream of the NF- κ B and Sp1 binding sites in the SIV LTR contains no equivalent element in the corresponding region of the HIV-1 LTR.

We also examined the effects of LTR mutations on the SEAP reporter plasmid activity in CEMx174 cells (Fig. 10). The *tat* gene was cotransfected in all of these assays. As in COS-1 cells, elimination of the NF- κ B and Sp1 sites from the HIV-1 LTR reduced SEAP activity from transfected cells to background levels. In contrast, the SIV-SEAP plasmid missing the NF- κ B and Sp1 binding sites retained 19% of residual activity.

DISCUSSION

The transcriptional control region of HIV-1 contains two binding sites for NF- κ B and three sites for Sp1 (12). These sequences quite clearly account for the vast majority, if not the entirety, of enhancer activity in the HIV-1 LTR. While mutation of each of these elements individually has differential effects on HIV-1 replication on different cell substrates, elimination of all NF- κ B and Sp1 binding sites reduces HIV-1 replication to below detectable levels in all cell types tested (23, 39). Thus, no enhancer elements that can functionally compensate for the loss of NF- κ B and Sp1 binding sites are present in the HIV-1 LTR.

The transcriptional control regions of HIV-2 and SIVmac

FIG. 7. Schematic representation of HIV and SIV LTR sequences introduced into SEAP reporter constructs. Nucleotide numbers corresponding to the inserted and deleted proviral sequences are shown. US stands for upstream U3 sequences. The HIV Δ NF κ B Δ Sp123 plasmids have had nucleotides 9425 to 9483 deleted. The HIV (SIV TATA-box) plasmids have had nucleotides 9500 to 9509 changed to nucleotides 9948 to 9957 of SIVmac239. The viral sequence in plasmid HIV Δ US Δ NF κ B Δ Sp123 starts with nucleotide 9484. Plasmid SIVmac Δ NF κ B Δ Sp1234 has had nucleotides 9876 to 9927 deleted, plasmid SIVmac (HIV-1 TATA-box) has had nucleotides 9948 to 9957 changed to nucleotides 9500 to 9509 of HIV NL-43, and the viral sequence in plasmid SIV Δ US Δ NF κ B Δ Sp1234 starts with nucleotide 9928. The designation of each LTR-SEAP construct is shown. Nucleotide numbers are derived from reference 36 for SIV and from GenBank (4) for HIV-1 NL-43 (accession number M19921).

FIG. 8. Transcriptional activity of HIV and SIV wild-type and mutant LTR-SEAP constructs. COS-1 cells were transfected with HIV LTR-SEAP constructs without (A) or with (B) Tat, and SEAP activity in culture supernatants was measured 16 to 18 h later. Similar experiments were also performed with SIV LTR-SEAP constructs without (C) and with (D) Tat. The activity in mutant-transfected cells is expressed as a percentage of that of the wild type. (E) Tat responsiveness in HIV and SIV LTR-SEAP constructs. The results are summaries of three to six independent experiments.

contain one binding site for NF- κ B and three or four putative binding sites for Sp1 (13, 36). The relative importance of these elements for the replication of HIV-2 and SIVmac has not to our knowledge been previously investigated. However, transient expression assays have demonstrated the presence of enhancer elements upstream of the single NF- κ B binding element in HIV-2 and SIVmac and that these additional enhancer elements are responsive to cell activation signals (5, 25, 26, 37).

The main objective of our present study was to examine the relative importance of the NF- κ B and Sp1 binding elements for replication of SIVmac in primary PBMCs and primary

macrophages of rhesus monkeys. A collection of 10 SIVmac mutants bearing changes in the NF- κ B and Sp1 elements was created for this purpose. Since interaction and cooperation between transcription factors may greatly influence the level of transcriptional activity (24, 34, 35), both deletion and substitution mutations were employed.

Unexpectedly, all mutated SIVs replicated with kinetics and to levels similar to those of parental wild-type virus in rhesus monkey PBMC cultures. Even mutant virus missing all NF- κ B and Sp1 sites replicated similarly to wild-type virus in rhesus monkey PBMC cultures. At least three viruses, Δ NF κ B Δ Sp1234, substSp1234, and Δ NF κ B Δ Sp1, showed a consistent delay in CEMx174 cells.

The terminally differentiated macrophage represents a system in which gene expression is principally dependent on constitutively produced transcription factors. One might expect, therefore, that lentiviral transcription in these cells might be more sensitive to specific changes in individual elements. Indeed, we found that most changes in Sp1 binding sites had a dramatic effect on SIV replication in macrophages. In particular, Sp1 substitution mutations which had no effect on SIV replication in CEMx174 and PBMC cultures severely impaired virus replication in primary rhesus monkey macrophages. In contrast, the NF- κ B site had little impact on SIV replication in

FIG. 9. Transcriptional activity of HIV/largeUS LTR-SEAP constructs. The plasmids HIV/largeUS and HIVΔNFκBΔSp123/largeUS were created by the insertion of HIV NL-43 nucleotides 9362 to 9404 into plasmids HIV NL-43 and HIVΔNFκBΔSp123 of HIV LTR-SEAP.

macrophages. The SIVmacMER mutant with point mutations in the NF- κ B site did not show any growth retardation in primary rhesus monkey macrophages, and the NF- κ B deletion mutant was only marginally delayed in these cultures. Our results are in direct disagreement with those reported by Bellas et al. (2), who found profound effects of the NF- κ B site for replication of SIVmac in primary rhesus monkey alveolar macrophages. Our results indicate a cooperative relationship among Sp1 sites or between Sp1 sites and other enhancer elements. This cooperation appears to be essential for high-level SIV replication in unstimulated alveolar macrophages. The relative effects of NF- κ B and Sp1 binding elements will not necessarily apply to other sources of monocytes/macrophages.

The results of transient expression assays suggest that the 70 bp upstream of the NF- κ B element in SIVmac are principally responsible for the surprisingly high replicative capacity of ΔNFκBΔSp1234 in primary rhesus monkey PBMC cultures. Our results with transient transfection assays are consistent with previous reports of enhancer elements immediately upstream of the NF- κ B binding sites in SIVmac and HIV-2 (5, 25, 26, 37). We additionally report that this enhancer element is responsive to Tat transactivation. Thus, this novel enhancer element, not present in HIV-1, can apparently allow a significant level of SIV transcription and replication in the complete absence of NF- κ B and Sp1 binding sites.

FIG. 10. Transcriptional activity of LTR-SEAP constructs in CEMx174 cells. Five micrograms of SEAP plasmid and 2 μ g of Tat plasmid were used for each transfection. The results shown are the average of duplicate measurements.

In rhesus monkeys infected with a SIV mutant with a 182-bp deletion in unique *nef* sequences, additional *nef* sequences are lost over time, including *nef* sequences which overlap U3 (22). However, particular *cis*-acting sequences known to be important for viral replication are specifically retained. These include the polypurine tract, known to be important for reverse transcription, and U3 terminal sequences, known to be important for viral DNA integration. It is worth noting that the 50 bp of sequences immediately upstream of the NF- κ B element, which correspond to the novel enhancer element, are also uniformly retained (22). While multiple mutations over a 300-bp stretch of upstream U3 sequences (US) were previously shown not to affect SIVmac239 replication in vitro or in vivo (18), these mutations did not involve the 50 bp of sequence immediately upstream of the NF- κ B site containing this novel enhancer element. The novel enhancer element is overlapped and contained within the *nef* reading frame.

In addition to the novel enhancer element, our results indicate that the SIVmac TATA box (TATAAATAT) is more efficient than the HIV-1 TATA box (ATATAA) and that this contributes to the residual transcriptional activity in the absence of NF- κ B and Sp1 binding elements. Previous studies have also shown that enlargement of the HIV-1 TATA box may compensate for other mutations in the TCE (20, 39). However, this increased activity of the SIVmac TATA sequences appears to contribute less than the enhancer elements upstream of NF- κ B.

The relative importance of these transcriptional control elements in SIVmac can be evaluated under even more relevant conditions in whole animals. The use of the SIVmac239 clone for these constructions will allow assessment of the effects of these TCE mutations on viral replication and pathogenic potential in vivo. Furthermore, the differential effects of these mutations in macrophages versus lymphocytes in culture will allow us to draw correlations regarding which is more predictive of the effects in vivo. Such experiments are in progress.

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