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A Block to Efficient Replication of Simian Immunodeficiency Virus in C8166 Cells Can Be Overcome by Duplication of the NF-κB Binding Site

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Abstract
Sequence analysis of the acutely lethal pbj14 strain of simian immunodeficiency virus (SIVpbj14) clone revealed among other differences from its less pathogenic counterparts a duplication of its binding site for nuclear factor kappa B (NF-κB) in its long terminal repeats (LTR). We have investigated whether introducing a similar duplication into the pathogenic molecular clone SIVmac239 would alter its biological properties. We compared an SIV which possessed 2 NF-κB sites to the wild type, a single NF-κB site virus, with respect to its ability to replicate in vitro in established CD4+ T cell lines, primary peripheral blood mononuclear cells (PBMCs), and primary alveolar macrophages. The virus containing 2 NF-κB sites exhibited no apparent difference from wild type in established cell lines 174xCEM, MT-2 and MT-4, or in primary PBMC or tissue macrophage cultures. However, the 2 κB virus replicated well in the established cell line C8166, while the wild type, 1 κB virus replicated very poorly in this cell type, suggesting that duplication of the NF-κB site is capable of overcoming a block to efficient replication of SIVmac239 in C8166 cells. Interestingly, Em*, a macrophage tropic SIVmac that differs from SIVmac239 by 9 amino acids in the envelope region yet possesses only one NF-κB binding site, also replicates well in C8166. The data suggest that the replication of wild type SIVmac239 is restricted in C8166 cells, but that this restriction can be overcome either by changes in the LTR or by changes in the envelope region.

Simian immunodeficiency virus (SIV) is a primate lentivirus closely related to the human immunodeficiency virus (HIV) [2, 6] with respect to sequence, genomic organization, tropism for CD4+ T cells and cells of monocyte/macrophage lineage, capacity to establish persistent infections of the host, and for some viruses the ability to induce an immunodeficiency disease, very similar to AIDS in humans, in susceptible host. Rhesus macaques experimentally inoculated with the molecularly cloned SIVmac239 developed within 0.5–2 years a syndrome characterized by AIDS-like features including immunosuppression, opportunistic infections, CD4+ T cell depletion, emaciation, and encephalopathy [3, 10, 18]. SIV is the best animal model available to date for HIV and AIDS pathogenesis studies.

Isolates of HIV and SIV possess either two or one copy of a binding site for nuclear factor kappa B (NF-κB) in the retroviral long terminal repeat (LTR). Despite the high degree of conservation of this site, HIVs and SIVs deleted or mutated in their NF-κB site are replication competent, and have been shown to replicate well in primary mononuclear cells [1, 11, 16]. Interestingly, however, mutation of the κB site in SIV seems to prevent efficient replication in primary alveolar macrophages [1].

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It is not known whether the biological properties of viruses possessing 1 κB site differ from those with 2 κB sites. However, of the five subgroups of primate lentiviruses, HIV-1/SIVcpz contain 2 κB binding sites in their LTRs whereas HIV-2/SIVsm/SIVmac contain only single κB binding site in their LTRs. It is generally believed that HIV-1 is more pathogenic than HIV-2. Interestingly enough, a molecular clone of an acutely lethal SIV, termed SIVpbj14, contained 2 κB binding sites [4]. A recent report mapped certain biological properties of SIVpbj14 to the Nef protein RQ to YE mutation [4a].

We reported the NF-κB sequence of the SIVpbj14 in the molecular clone SIVmac239 and investigated its ability to replicate in tissue culture. Addition of a second κB site has no dramatic effect on the growth of SIVmac239 in macaque peripheral blood mononuclear cells (PBMC) or in the established cell lines MT-4 or 174xCEM. However, we found that in C8166 cells, the κB site duplication can dramatically increase the replicative capabilities of the virus. Moreover, we and others found changes located in the envelope gene are also capable of allowing efficient replication of SIVmac239 in this cell type.

Materials and Methods

Cell Culture

MT-2, MT-4 cells, C8166 and 174xCEM cells were obtained from NIH AIDS Research and Reference Reagent Program and were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 m*M* glutamine, and 50 units of penicillin and 50 μg of streptomycin/ml. Cells were maintained by twice weekly dilution to 5×10^5 cells/ml. Rhesus macaque *(Macaca mulatta)* PBMCs were obtained by banding over sodium diatrizoate-Ficoll as described [1, 10]. PBMCs were treated for 48 h with 1 μg of phytohemagglutinin A (PHA)/ml of the medium used for the established cell lines. After 48 h, PBMCs were washed and resuspended in culture medium containing 10% interleukin-2 (IL-2; Pharmacia) at a concentration of 10^6 cells/ml. Cultures of alveolar macrophages were obtained by bronchoalveolar lavage from rhesus macaques as described [1, 3] employing RPMI 1640 medium supplemented with glutamine and antibiotics as above and 10% HIV-negative human serum (Gibco) at 5×10^5 cells/ml in 24- or 48-well plates (Falcon).

Generation of Virus

Plasmid DNA for the 5′ and 3′ halves of the SIVmac239 genome were digested with the restriction enzyme *Sph*I. These two halves were then ligated with T4 DNA ligase and transfected using DEAE-dextran as described [10] onto 174xCEM cells. Virus-containing supernatants were collected, clarified by low-speed centrifugation (500 *g*) and filtered through a 0.45-μm membrane, aliquoted, and stored at −80° C. The virus was quantitated by p27 Gag antigen capture ELISA-type assay employing a commercially available ELISA kit (Coulter, Hialeah, Fla., USA).

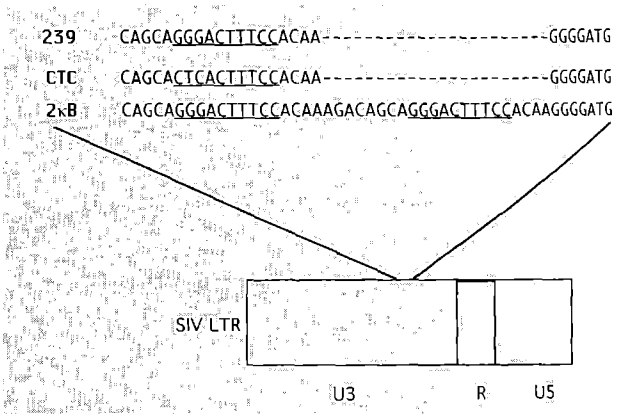

Fig. 1. Sequences of the relevant portion of the SIV LTR.

Site-Specific Mutagenesis

Generation of site-specific mutation of both the 5′ and 3′ LTR of SIVmac239 *nef*-open was achieved using a nested four-primer, polymerase chain reaction (PCR) protocol as described [1, 9]. The final product of the second round PCR reaction was digested with *Hin*cII and *Sau*3A and ligated into a *Bam*HI, *Hin*cII-digested bluescript pBS KS-plasmid (Stratagene). One resulting LTR subclone was then used to regenerate the 5′ half by subcloning a *Nde*I-*Hin*cII fragment into a *Nde*I-*Hin*cII-digested parental genomic 5′ half; the 3′ half was generated by subcloning an *Nde*I-*Hin*cII, *Eco*RI linker fragment into a *Nde*I (partially digested)-*Eco*RI 3′ end of SIVmac239.

Oligonucleotide Primers

For the external primers, the sense primer was starting at position 221 in the U3 of SIVmac239 provirus [18], upstream of the *Nde*I site with an added *Hin*dIII site at its 5′ end. Its sequence was 5′-CTC-*AAGCTT*GTGGGATGACCCTTGGGG-3′. The antisense primer was starting at position 779 in the U5 with an added *Eco*RI site at its 5′ end. Its sequence was 5′-GT*GAATTC*GGGTCCTAACAGACC-AGGG-3′. The internal sense primer was 5′-CAGCAGGGAC-TTTCCACAAAGACAGCAGGGACTTTCCACAAGGG-3′. The antisense primer was 5′-TTGTGGAAAGTCCCTGCTGTCTT-TGTGGAAAGTCCCTGCTGTTTCAGCG-3′. The underlined sequence was the NF-κB binding site.

Virus Infections

All infections employed 4 ng equivalents of virus stock that were prepared simultaneously for any given infection under conditions optimum for virus infectivity. 174xCEM cells were transfected with proviral DNA and passaged every 3 days. At time of maximum cytopathic effects the cells were washed and resuspended in fresh medium. Twenty-four hours later the supernatant fluid was harvested. Under these conditions, the infectivity of the stocks is 50 $TCID_{50}$ infectious doses/ng p27 Gag protein as titered on 174xCEM cells. For the established cell lines, 4 ng of p27 core antigen equivalents was adsorbed to 2×10^6 cells in 1 ml of tissue culture medium (RPMI 1640 plus 10% heat-inactivated fetal bovine serum) for 2 h. Cells were then washed 3 times with phosphate-buffered saline to remove unabsorbed virus and cultured. Cells were split 2 or 3 times weekly to a density of 5×10^5 cells/ml. Virus in the cell-free supema-

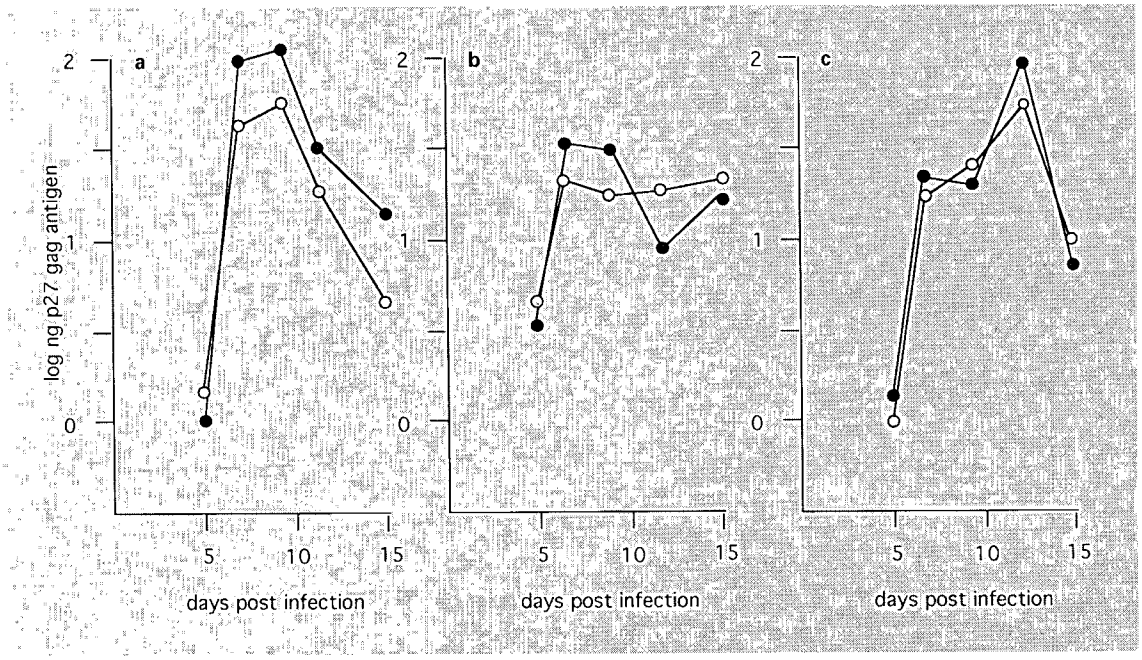

Fig. 2. Duplication of the κB site has no effect on growth of SIV in IL-2-stimulated rhesus macaque PEMC. **a–c** Growth curves of PBMCs from 3 individual macaques. Wild-type SIVmac239 [1 κB (○); 2 κB (●)].

tant was assayed by antigen capture ELISA (Coulter). For infection of PBMCs, rhesus macaque PBMCs were isolated by banding over sodium diatrizoate-Ficoll as described [1, 10], stimulated for 48 h with 1 μg phytohemagglutinin/ml, washed, and infected with 4 ng p27 equivalents of virus for 2 h. Cells were then washed 3 times and cultured in the presence of purified human IL-2 (10%; Pharmacia). Cells were split to 10^6/ml twice weekly. Macrophage cultures were infected by incubation of the cells with 4 ng p27 equivalents of virus overnight, followed by washing with phosphate-buffered saline and cultured in the medium described under Cell Culture.

Results and Discussion

We sought to determine whether the NF-κB binding site duplication seen in SIVpbj14 would affect the replication potential of SIVmac239. We used a four-oligonucleotide primer PCR-based mutagenesis procedure to introduce the same duplication into SIVmac239. Figure 1 shows the sequences of the relevant portion of the LTRs of the wild type, CTC, and 2 κB viruses. We compared the ability of the 2 κB virus with the wild-type (1 κB) to grow in primary rhesus macaque PBMCs. PBMCs were obtained from 3 individual macaques, stimulated with PHA for 48 h, infected with either the wild-type or 2 κB virus, and cultured in the presence of IL-2. At different times after infection, cell culture supernatants were collected and assayed for p27 Gag antigen production. Figure 2 shows that in PBMCs from 3 different animals, the duplication of the κB site has a modest or no effect on the peak levels of p27 Gag antigen or in the kinetics of virus growth. Therefore, duplication of the κB site does not appear to alter the ability of SIVmac239 to grow in PHA- and IL-2-stimulated PBMCs.

Recently we demonstrated that the κB site plays an important role for growth of SIV in primary tissue macrophages. We introduced the κB duplication into Em*, a macrophage-tropic SIVmac, to determine whether a second κB site would markedly affect replication in primary alveolar macrophages. Alveolar macrophages were obtained from a rhesus macaque and cultured and infected as previously described [1]. Figure 3 shows that in primary macrophage cultures, the virus with the κB site duplication grows with kinetics and peak levels of virus production similar to that of the wild-type. These data suggest that while mutations that eliminate κB binding affect SIV growth in primary macrophages, duplication of the κB site does not seem to alter virus growth characteristics in these cells.

We then studied the properties of the 2 κB virus in CD4+ T cell lines. We infected 174xCEM, MT-2, MT-4,

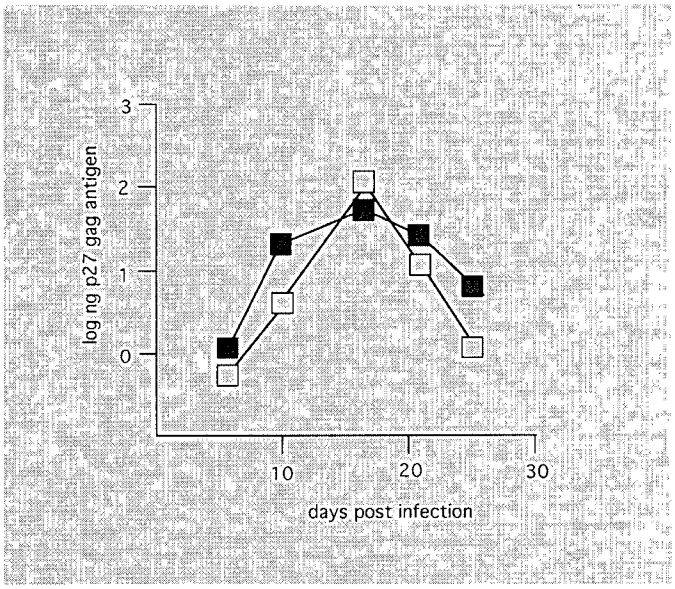

Fig. 3. Duplication of the κB site does not enhance viral replication in primary rhesus macaque primary alveolar macrophages. □ = SIVmacEm* (1 κB site); ■ = SIVmacEm*-2 κB.

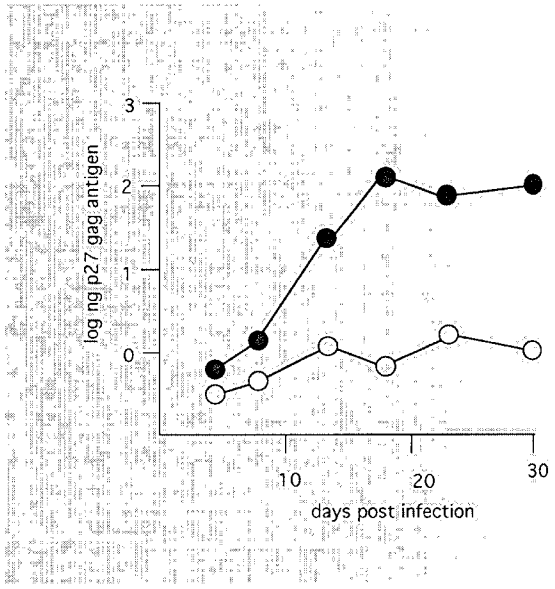

Fig. 5. Duplication of the κB site greatly increases the ability of SIVmac239 to replicate in the established CD4+ cell line C8166. Virus in the cell-free supernatant was assayed by antigen capture ELISA (Coulter). ○ = Wild-type SIVmac239 (1 κB); ● = 239-2 κB.

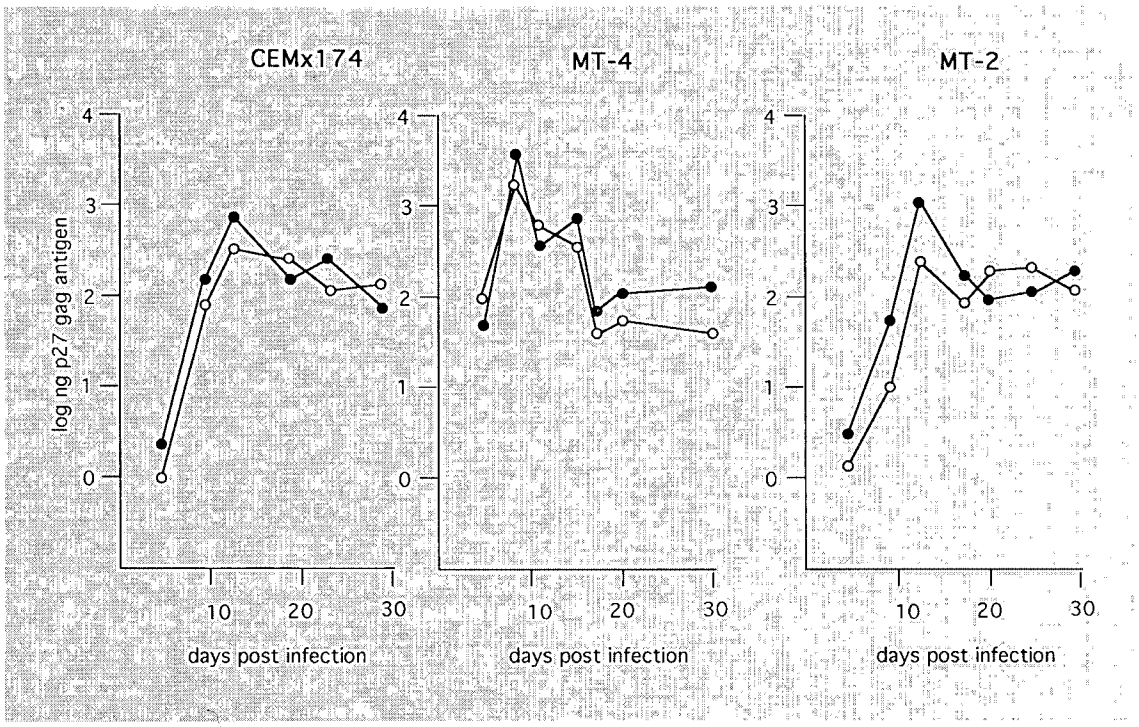

Fig. 4. Duplication of the κB site has modest effects on growth of SIV in three established CD4+ cell lines. Virus in the cell-free supernatant was assayed by antigen capture ELISA (Coulter). ○ = Wild-type SIVmac239 (1 κB); ● = 239-2 κB.

Table 1. The ability of a κB duplication to overcome a block to efficient replication is specific to C8166 cells

Cells	Viruses			
	SIVmac239	SIVmac239-2κB	SIVmacEM*	SIVmacEM*-2κB
Macaque PBMCs	++	++	++	++
Chimpanzee PBMCs	–	–	ND	ND
Macaque macrophages	–	–	++	++
A3.01	–	–	–	–
U937	–	–	–	–
MT-2	++	++	–	–
MT-4	+++	+++	+++	+++
174xCEM	+++	+++	+++	+++
C8166	+	++	++	++

Cells indicated were infected with the indicated viruses, and at different times after infection, culture supernatants were analyzed for p27 antigen by ELISA. The peak amount of virus for each infection was compared as follows: – = no p27 antigen detected (level of sensitivity is 0.05 ng/ml); + = between 0.05 and 10 ng/ml; ++ = between 10 and 100 ng/ml; +++ = greater than 100 ng/ml. ND = Not done.

and C8166 cells with the two viruses. Figure 4 shows that in 174xCEM, MT-2, and MT-4 cells, the two viruses grow equally well. However, in C8166 cells, only the 2 κB virus could replicate efficiently. The C8166 cell is a human T cell leukemia virus type I (HTLV-I)-immortalized, nonproducer line developed by cocultivation of human umbilical cord blood lymphocytes with T cells derived from human T cell leukemia patients [21]. C8166 cells exhibit morphological, cytochemical, and other phenotypic characteristics similar to those of other HTLV-I-infected cells but contain only low amounts of viral proteins. These cells contain at least one copy of HTLV-I provirus/cell and transcribe viral RNA similar in size to virus-producing cells. Virus expression in these cultures was not enhanced by IUdR treatment [21]. C8166 cells supported several strains of HIV/SIV replication in culture. Figure 5 shows that the 2 κB virus would achieve peak levels of p27 antigen production 50-fold higher than that of wild-type virus. The data suggest that in at least one cell line, duplication of the κB site can alter the biological properties of the virus.

To ensure that the 22-nucleotide duplication did not generate novel nuclear factor binding sites, we performed electrophoretic mobility shift assays using the 2 κB oligo as the probe. In this experiment, the single NF-κB nucleotides were used as the competitor. We observed no additional band shift after the cold single NF-κB oligo competition suggesting that no additional transcriptional factor binding sites were generated in the duplication of the NF-κB binding sites [data not shown].

We then sought to determine whether the κB duplication would increase the replication of virus in other cell systems in which we had observed poor replication of SIVmac. However, table 1 demonstrates that the κB duplication did not overcome blocks to efficient replication of SIVmac239 in U937, A3.01, chimpanzee PBMCs, or primary macaque macrophages. In addition, κB site duplication did not overcome blocks to efficient replication of Em* in MT-2 cells. It appears, then, that the effect observed in C8166 cells is not general.

It appeared that there was some block to efficient replication of SIVmac239 in C8166 cells that could be overcome by changes in the enhancer region of the virus, suggesting perhaps that SIVmac239 replication was restricted at a transcriptional level. Because the addition of tumor promoter phorbol myristate acetate (PMA) to certain latently HIV-infected cell lines can lead to activation of latent virus [5], we treated C8166 cells that had been infected 20 days previously with either SIVmac239 or SIVmac239(CTC) (a κB deficient mutant SIV) with 50 ng/ml PMA. 48 h later we assayed culture supernatants for p27. Table 2 demonstrates that PMA induction of SIVmac239, but not SIVmac239(CTC)-infected cultures, leads to a 6-fold increase in virus production. These data are consistent with the notion that SIVmac239-infected cells are transcriptionally restricted for virus replication, and that PMA stimulation is effective only in the presence of an intact κB site.

The data suggested that infection of C8166 cells with SIVmac239 leads to restricted infection, and might serve

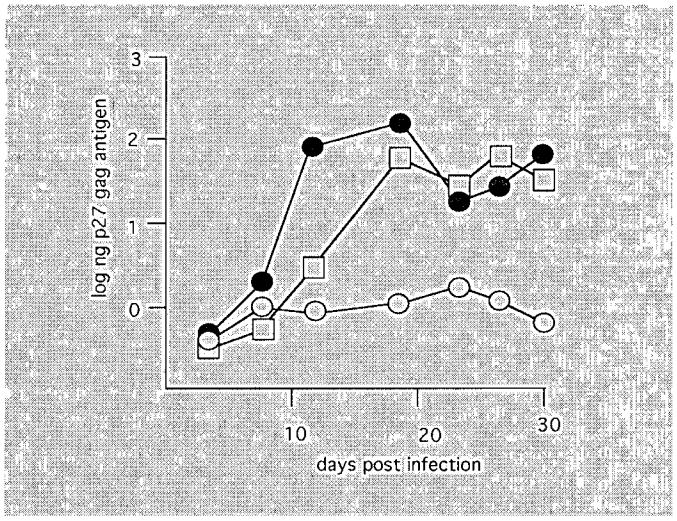

Fig. 6. The block to efficient replication of SIVmac239 in C8166 cells can also be overcome by changes in the envelope region. Virus in the cell-free supernatant was assayed by antigen capture ELISA (Coulter). ○ = Wild-type SIVmac239 (1 κB); ● = SIVmac-239-2 κB; □ = Em* (1 κB).

Table 2. PMA treatment of C8166 cells increases the amount of p27 antigen (ng/ml) produced by cells infected with SIV mac239 but less so by cells infected with SIVmac239CTC

Viruses	Experiment 1		Experiment 2	
	–PMA	+PMA	–PMA	+PMA
SIVmac239	3.5	24.2	5.8	31.7
SIVmac239CTC	5.1	8.9	3.9	7.2

Cells were infected with SIVmac239 or SIVmac239CTC, and then cultured for 20 days. PMA was added at 50 ng/ml, and the cells were cultured for an additional 48 h, at which time the supernatant was assayed for p27 antigen.

as a novel model system for analysis of other viral sequences that affect restricted replication. Specifically, we thought that there might be second site mutations in other regions of the genome that might overcome the restriction of virus growth. Figure 6 demonstrates that Em*, a virus with the same LTR as SIVmac239, and in fact differing from SIVmac239 by 9 amino acid residues in the envelope region [14], can replicate well in C8166 cells. This result suggests that the block to efficient replication of SIVmac239 could be overcome by defined changes in either the LTR or in the envelope region.

The data presented here suggest that for most cell types analyzed and for primary lymphocytes or macrophages, duplication of the NF-κB site in SIVmac239 does not affect its ability to replicate. However, in C8166 cells, a virus with two κB sites grows well while the wild-type virus grows extremely poorly. Em*, a virus possessing a single κB binding site in LTR and differing by 9 amino acid residues in envelope, also replicates well in C8166 cells. There are at least two possible explanations: either Em* Env enables the virus to get into the cells much more efficiently or the Em* gp120 is able to modulate NF-κB binding activity. Recent data suggested that the latter was more likely [20].

The fact that duplication of the κB site can alter the biological properties of the virus is interesting; the inability to observe this effect in primary cells urges caution, however, in the interpretation as to its physiologic relevance. We observed no effects of the second κB site on the SIVmac239 replication in either primary macrophages or PBMCs. Indeed, a recent report demonstrated that a chimeric virus expressing the pbj14 genome under the control of an LTR harboring only 1 κB site induced death soon after inoculation of a pig-tailed macaque, suggesting that the κB duplication in the pbj14 molecular clone is not necessary for the ability to induce acute disease [16].

Our results also support the notion that transcriptional control elements in SIV can play a powerful role in viral tropism. Deletion of the κB site in SIVmac hinders replication in primary macrophages, while κB duplication overcomes a block to efficient replication in C8166 cells. In addition, our results suggest that infection of C8166 cells with wild-type SIV leads to a state similar to latency; activation of infected cells with the tumor promoter PMA increased virus production, similar to results seen in other latency models. For example, the latently HIV-1 infected U1 monocytic cell line responds to an NF-κB inducer like PMA by greatly increasing viral production [5, 7]. Here we demonstrate that this activation was not seen, however, when an SIV lacking a κB site was used to infect the cells, further implicating the κB site in activation from latency.

One intriguing result of our experiments is that they suggest that the block to replication of SIVmac239 in C8166 cell is at a transcriptional level, and that changes in envelope can overcome this block. That a block to efficient replication of a virus can be overcome by changes either in the envelope region or by changes in a transcriptional control region may suggest unexpected interactions between these regions. While viral envelope plays an

important role in virus entry into a cell, there is data supporting the notion that it may have other effects on the cell. HIV envelope has been reported to be mitogenic to T cells, or lead to induction of cytokines. For example, treatment of monocytes with purified HIV gp120 induces production of IL-1β and TNF-α; this induction can be blocked by soluble CD4 [13]. In addition, recent data suggest that gp120 can modulate NF-κB binding activity [20]. Other data support the notion that retroviral envelopes may affect cells. Spleen focus-forming virus gp52 envelope can interact with the erythropoietin receptor and lead to erythropoietin-independent proliferation of cells [12].

It has been demonstrated that SIVmac239 and SIVmacEm* viruses do not differ dramatically in their ability to enter C8166 cells and that the inability of SIVmac239 to replicate in macrophages, while SIVmacEm* replicates well in macrophages, is not at the level of entry [14]. These data suggest that two viruses whose only sequence differences are in the envelope region differ in ways independent of virus entry into cells. Further studies on the nature of the block to efficient SIVmac239 replication in C8166 cells should provide insigth into the manner by which either envelope sequences or LTR sequences can overcome this block.

Acknowledgments

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