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The NF-κB Binding Site Is Necessary for Efficient Replication of Simian Immunodeficiency Virus of Macaques in Primary Macrophages but Not in T Cells In Vitro

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We demonstrate here that the nuclear factor-κB (NF-κB) binding site in the simian immunodeficiency virus (SIVmac) long terminal repeat is essential for efficient virus replication in primary alveolar macrophages but dispensable for efficient replication in primary T cells. Mutation of the NF-κB site does not seriously impair replication of a T-cell-tropic SIVmac239 or a macrophagetropic SIVmacEm* in peripheral blood lymphocytes or established CD4$^+$ cell lines; however, mutation of the NF-κB site prevents efficient SIVmacEm* replication in primary alveolar macrophages. These data suggest that efficient replication in primary macrophages requires both envelope and long terminal repeat determinants.

Simian immunodeficiency virus (SIV) is a primate lentivirus closely related to the human immunodeficiency virus (HIV) (3, 7) with respect to sequence, genomic organization, tropism for CD4$^+$ cells, capacity to establish persistent infections of the host, and, for some viruses, ability to induce an immunodeficiency disease. Rhesus macaques experimentally inoculated with the molecularly cloned SIVmac239 develop within 0.5 to 2 years a syndrome characterized by AIDS-like features, including immunosuppression, opportunistic infection, CD4$^+$ T-cell depletion, emaciation, and encephalopathy (4, 14, 27). SIV in the rhesus monkey is quite possibly the best animal model for AIDS research.

Isolates of SIV and HIV vary dramatically in their abilities to infect primary macrophages (2, 4, 8, 15, 28). Because infection of macrophages may be important in allowing these viruses to establish long-term persistent infections of the host and also may be involved in certain disease manifestations, in particular in central nervous system disorders (for a review, see reference 9), there has been interest in identifying sequences that affect replication in macrophages. Recent studies have demonstrated that envelope sequences play an important role in macrophage tropism (12, 24, 31); however, no evidence has suggested that long terminal repeat (LTR) sequences can differentially affect replication in macrophages versus T cells.

All isolates of HIV and SIV of which we are aware possess in their LTRs at least one perfectly conserved copy of a binding site for the nuclear factor-κB (NF-κB). Many experiments have demonstrated that the NF-κB site is important for inducible expression of HIV type 1 (HIV-1) LTR-reporter constructs in transient-expression assays in T cells (22, 25). However, Ross et al. (29) and Leonard et al. (16) found that deletion of the NF-κB sites had a modest or no effect on HIV-1 replication in peripheral blood lymphocytes (PBLs). This result is rather surprising in light of the high degree of conservation of the NF-κB binding site. To determine the role of the NF-κB site in SIV replication in both T cells and macrophages, we constructed an SIV possessing LTRs lacking the SIV NF-κB site and examined its growth properties.

MATERIALS AND METHODS

Cell culture. MT-2, MT-4, C8166, and CEMx174 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 50 U of penicillin and 50 μg of streptomycin per ml. The cells were maintained by twice weekly dilution to 5×10^5 cells per ml. Rhesus macaque (*Macaca mulatta*) PBLs were obtained by banding in sodium diatrizoate-Ficoll medium as described elsewhere (14). PBLs were treated for 48 h with 1 μg of phytohemagglutinin per ml of the medium used for the established cell lines. After 48 h, the PBLs were washed and resuspended in culture medium containing 10% interleukin-2 (Pharmacia) at a concentration of 10^6 cells per ml. Cultures of alveolar macrophages were obtained by bronchoalveolar lavage from rhesus macaques as described elsewhere (4) with RPMI 1640 medium supplemented with glutamine and antibiotics as described above and 10% HIV-negative human serum (GIBCO) at 5×10^5 cells per ml in 24- or 48-well plates (Falcon).

Generation of virus. Plasmid DNAs for the 5′ and 3′ halves of the SIVmac genome were digested with the restriction enzyme *Sph*I. These two halves were then ligated with T4 DNA ligase and transfected with DEAE-dextran as described elsewhere (14) onto CEMx174 cells. Virus-containing supernatants were collected, clarified by low-speed centrifugation (500 × *g*), filtered through 0.45-μm-pore-size membranes, aliquoted, and stored at −80°C. Virus was quantitated by a $p27^{gag}$ antigen capture assay with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Coulter, Hialeah, Fla.).

Site-specific mutagenesis. Generation of site-specific mutation of both the 5′ and 3′ LTRs of SIVmac239 *nef* open (239) and SIVmacEm* *nef* open (Em*) was achieved by using a four-primer polymerase chain reaction (PCR) protocol as described elsewhere (12). The final product of the second-

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round PCR was digested with *Hin*cII and *Sau*3A and ligated into a *Bam*HI- and *Hin*cII-digested Bluescript pBS KS$^-$ plasmid (Stratagene). One resulting LTR subclone was then used to regenerate the 5′ half by subcloning an *Nde*I-*Hin*cII fragment into an *Nde*I-*Hin*cII-digested parental genomic 5′ half; the 3′ half was generated by subcloning an *Nde*I-*Hin*cII and *Eco*RI fragment in the polylinker into an *Nde*I (partially digested)-*Eco*RI 3′ end of 239. To incorporate this LTR into the 3′ half of Em*, the *Sac*I-*Eco*RI fragment of the 239CTC was ligated into an *Sac*I-*Eco*RI-digested Em*.

Preparation of nuclear extracts. Nuclear extracts were prepared by a modification of a procedure developed by Christine Jamieson (13a). A total of 5×10^7 cells were pelleted by low-speed centrifugation ($500 \times g$) and washed three times in ice-cold phosphate-buffered saline. The cellular pellet was then gently resuspended in 0.5 ml of 0.5% Nonidet P-40–10 mM Tris hydrochloride (pH 7.6)–1 mM EDTA, and the suspension was held at 4°C for 5 min. The nuclei were collected by centrifugation ($500 \times g$) and resuspended in 100 μl of buffer C (17). The nuclei were then vortexed vigorously for 2 min. The extracts were then centrifuged at $12,000 \times g$ for 5 min at 4°C. The supernatants were then aliquoted and stored for no longer than 3 weeks at −80°C.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays were performed essentially as described by Manley et al. (17) with Tris-borate as the buffering system. Complementary oligonucleotides were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, annealed, and separated from unincorporated label by polyacrylamide gel electrophoresis. Binding reactions (25-μl final volume) employed 10,000 cpm of probe, 5 μg of nuclear extract, 100 ng of poly(dI)-poly(dC), and 100 ng of sonicated, denatured salmon sperm DNA. Unlabeled competitor oligonucleotides were present at 100-fold molar excess. The complexes formed were visualized by electrophoresis through nondenaturing polyacrylamide gels and autoradiography of the dried gels. The sequences of the sense strand of the oligonucleotides used were as follows: NF-κB, GCAGGGACTT TCCACAA; CTC, GCACTCACTTTCCACAA; AA, GCAG GGACTTTAAACAA; and Sp1, ACAAGGGGATGTTACG GGGAGGTACGGGGAGGTACGGGGAGGAGCC.

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. CEMx174 cells were transfected with proviral DNA and passaged every 3 days. At the time of maximum cytopathic effect, 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, independent of the NF-κB mutation, is 50 50% tissue culture infectious doses per ng of p27^{gag} protein as titrated on CEMx174 cells. For the established cell lines, 4 ng of p27^{gag} 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. The cells were then washed three times with phosphate-buffered saline to remove unadsorbed virus and were cultured. The cells were split two or three times weekly to a density of 5×10^5 cells per ml. The amount of virus in the cell-free supernatant was determined by antigen-capture ELISA (Coulter). For infection of PBLs, rhesus macaque (*M. mulatta*) PBLs were isolated by banding in sodium diatrizoate-Ficoll medium as described previously (14), stimulated for 48 h with 1 μg of phytohemagglutinin per ml, washed, and infected with 4 ng of p27 equivalents of virus for 2 h. The cells were then washed three times and cultured in the presence of purified human interleukin-2 (10%) (Pharmacia). The cells were split to 10^6 cells per ml twice weekly. Macrophage cultures were infected by incubation of the cells with 4-ng equivalents of virus overnight, followed by washing with phosphate-buffered saline and culturing in the medium as described above for cell culture.

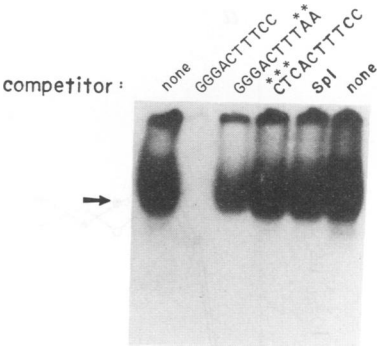

FIG. 1. A GGG-to-CTC mutation lacks the ability to compete for nuclear factors that normally bind the 239 NF-κB site. An electrophoretic mobility shift assay employing a double-stranded oligonucleotide corresponding to the SIV NF-κB site as probe (GGGACTT TCC) to detect factors found in T cells (C8166 extracts) was performed as described in Materials and Methods. The indicated unlabeled oligonucleotide competitors were used at 100 molar excess. Asterisks indicate positions at which the oligonucleotide differs from the wild-type 239 NF-κB site. Sp1, an oligonucleotide corresponding to the SIV Sp1 binding sites. The arrow indicates the position of migration of the protein-probe complex. Free, uncomplexed probe is not shown.

RESULTS

We employed a four-primer PCR-based in vitro mutagenesis procedure to generate an LTR subclone which contained a GGGACTTTCC-to-CTCACTTTCC (CTC) triple-point mutation in the NF-κB binding site. This mutation has been shown to abrogate binding of NF-κB-related proteins to the HIV-1 LTR (6) and showed no ability at 100-fold molar excess to compete for factors found in T cells that normally bind the wild-type SIV NF-κB site (Fig. 1). After DNA sequencing of the entire region amplified by PCR confirmed that no undesired changes had occurred during the mutagenesis procedure, virus was generated as previously described (14) into CEMx174 cells to generate the T-cell-tropic 239 lacking an NF-κB site (239CTC) or the macrophagetropic virus lacking this site, Em*CTC.

We compared the 239CTC mutant with 239 (wild-type) virus with respect to their abilities to grow in three CD4$^+$ cell lines known to support replication of 239. Figure 2 demonstrates that 239 and the 239CTC mutant show no dramatic differences in peak virus production in CEMx174, MT-2, or MT-4 cell lines; 239CTC virus production lags slightly behind that of 239 in MT-2 cells but not in CEMx174 or MT-4 cells. This lag was observed in three separate infections and is consistent with results observed by Ross et al. (29) that in certain T-cell types the HIV NF-κB site may play a role in replication. 239CTC virus, like the wild-type 239, leads to a lytic infection characterized by syncytium and giant cell formation (data not shown). Therefore, the NF-κB site of the

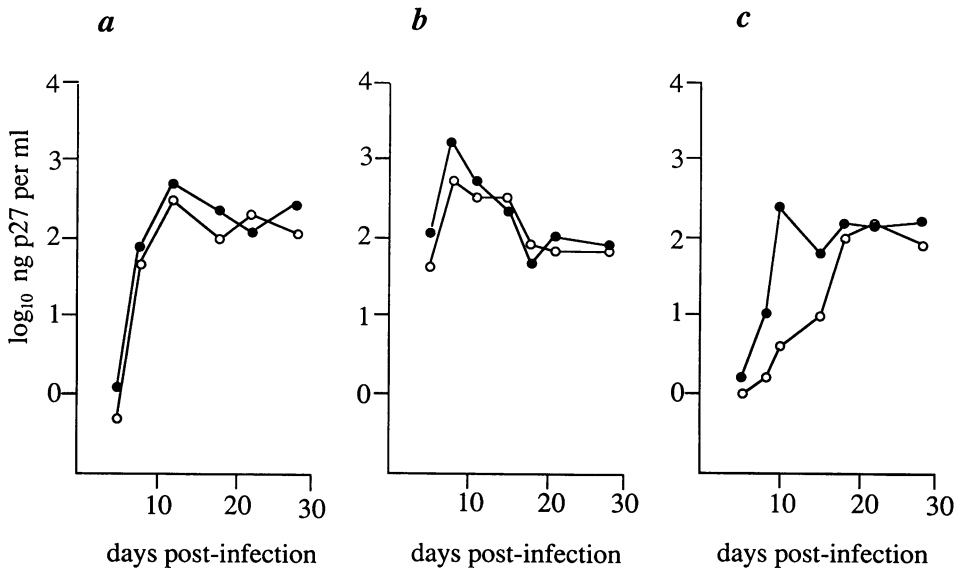

FIG. 2. The NF-κB site has modest effects on growth of SIV in three established CD4+ cell lines. Virus in the cell-free supernatant was assayed by antigen-capture ELISA (Coulter). (a) CEMx174; (b) MT-4; (c) MT-2. Open circles, 239CTC (no NF-κB site); closed circles, 239 (one NF-κB site [wild type]).

T-cell-tropic 239 appears to play a modest (if any) role in viral replication in these established cell lines.

We determined the effect of the SIV NF-κB site in primary cultures of rhesus macaque PBLs (Fig. 3). PBLs from three different rhesus macaques were stimulated for 2 days with phytohemagglutinin, infected with either the 239CTC or the 239 virus, and then cultured in the presence of interleukin-2. No significant difference between 239 and 239CTC regarding their growth in PBLs was seen. Therefore, in agreement with the results seen for HIV-1 deleted of its NF-κB sites (16, 29), an SIV lacking an NF-κB site replicates quite well in primary CD4+ T cells.

We considered the possibility that the lack of an effect of the NF-κB site in certain T cells is due to rapid reversion of the sequence to the wild type. However, this seems highly unlikely because (i) we used a triple-point mutation, which might be expected to be somewhat difficult to revert easily; (ii) the phenotypes of the virus as shown in the present paper are highly reproducible and independent of the particular DNA stock or virus stock used; and (iii) the lack of difference between the wild-type and mutants was seen independently of multiplicity of infection. In experiments not shown, we have observed no differences in growth kinetics between the wild-type and NF-κB mutant virus in CEMx174 cells

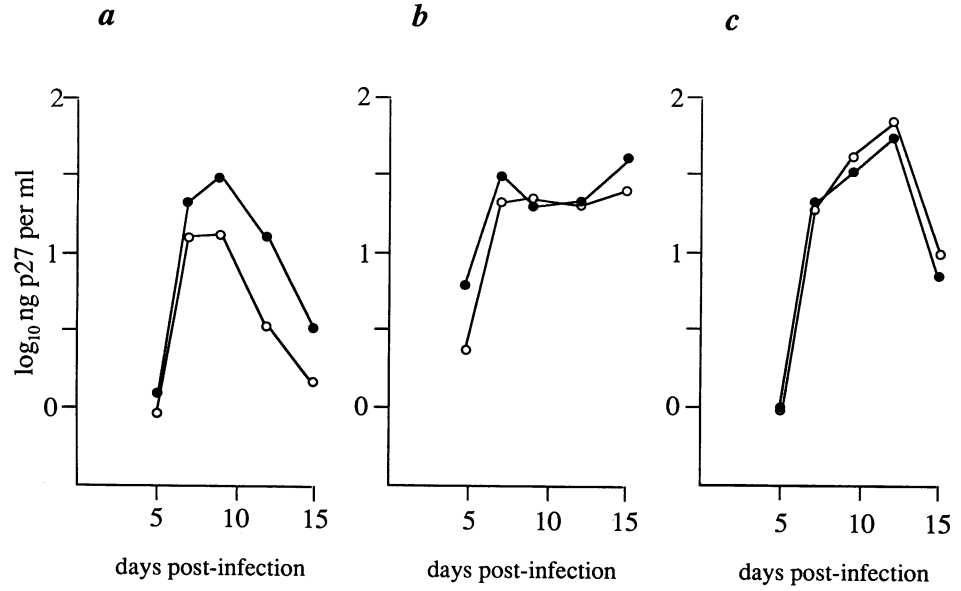

FIG. 3. The NF-κB site has no effect on growth of SIV in interleukin-2-stimulated rhesus macaque PBLs. (a, b, and c) Growth curves on PBLs from individual macaques. Open circles, 239CTC (no NF-κB site); closed circles, 239 (one NF-κB site [wild type]).

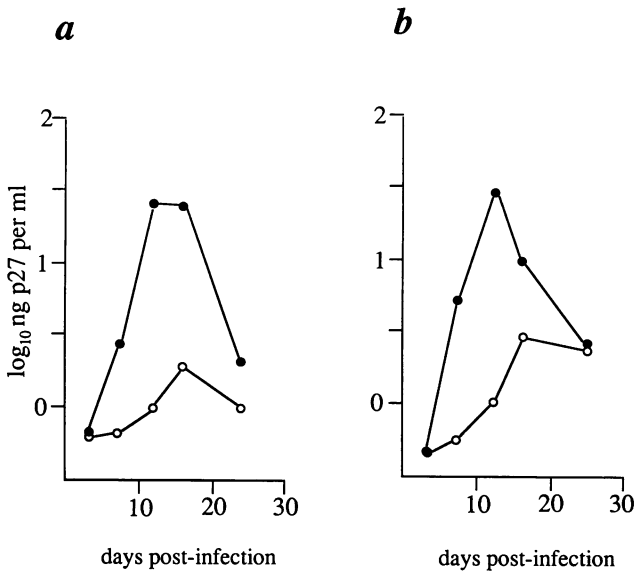

FIG. 4. The NF-κB site is important for growth of Em* in primary rhesus macaque primary alveolar macrophages. (a and b) Growth curves on macrophages from individual macaques. Open circles, Em*CTC (no NF-κB site); closed circles, Em* (one NF-κB site [wild type]).

when infections were initiated with 400, 40, 4, or 0.4 ng of $p27^{gag}$. If the mutants were reverting, one would perhaps expect that at a low multiplicity of infection one might see a longer lag before the mutant started to grow well; however, we never observed any differences, suggesting that the lack of the NF-κB mutation in T cells seems to be almost certainly a reflection of the input genotype.

Recent evidence suggests that cells of the monocyte-macrophage lineage may serve as a reservoir for virus and play an important role in allowing the virus to persist in vivo (11, 22, 30). Mori et al. recently showed that Em*, a virus which differs from 239 by only nine amino acid changes in the envelope gene, will efficiently replicate in primary macrophages (21). We introduced the CTC mutant LTR into this macrophagetropic Em*, creating Em*CTC, and we compared Em* and Em*CTC with respect to growth in primary rhesus macaque alveolar macrophages and T cells. As a control, we also tested the growth of both viruses in T cells, since Em* grows in these as well. Em*CTC replicates extremely poorly in macrophages, with peak levels from 5 to 10% of those of Em* (Fig. 4). In an experiment involving macrophages plated at a higher density, Em*CTC core antigen levels were less than 1% of that produced by Em* (data not shown).

There are some conditions under which HIV can replicate without being released into the medium (32). In this case, however, there is cell death and syncytium formation. Figure 5 documents that in addition to the lack of virus production by $p27^{gag}$ assay, an independent criterion, i.e., cytopathic effects induced by the virus, seems to suggest that the virus without NF-κB replicates less. Em* leads to giant cell formation and cell lysis, while Em*CTC has no detectable cytopathic effect in macrophages (Fig. 5). This Em*CTC virus is fully replication competent, because comparison of its growth with Em* in CEMx174 or MT-4 cells (data not shown) or primary PBLs (Fig. 6) reveals no or modest differences in growth capacity. Therefore, the SIV

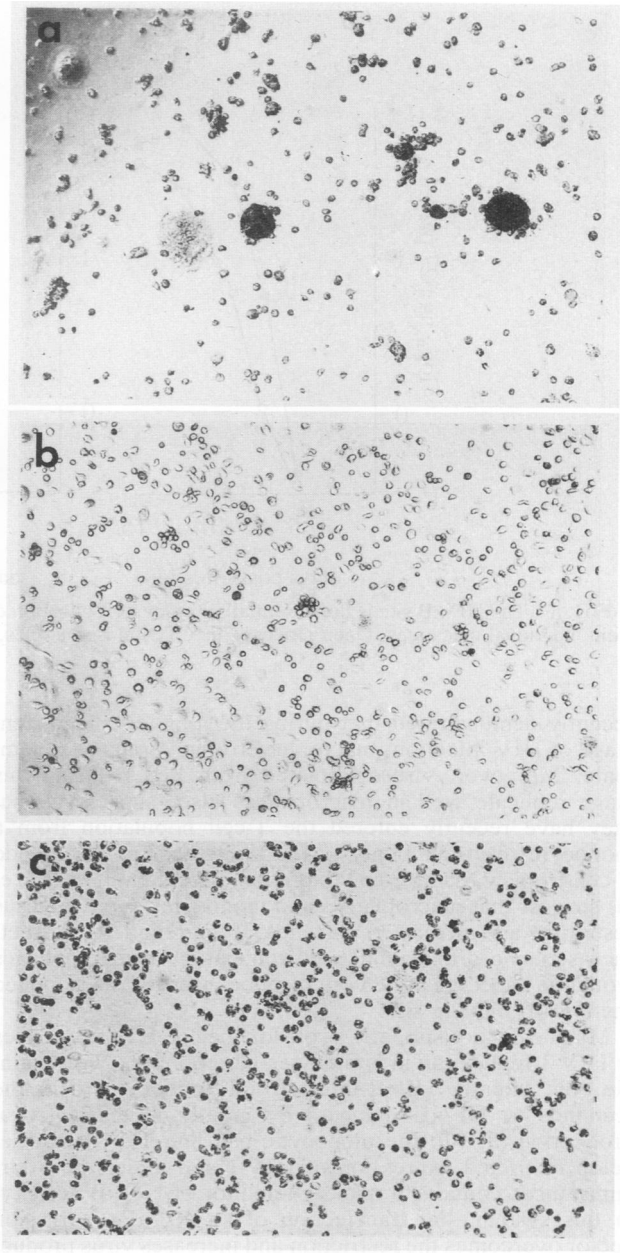

FIG. 5. Em*CTC is less cytopathic than Em* in macrophage cultures. Cultures were photographed with a low-power objective 15 days after infection with Em* (a), Em*CTC (b), or no virus (c).

NF-κB site appears to be required for efficient replication in primary macrophages in vitro.

DISCUSSION

This is the first demonstration of a potent effect of the NF-κB site for viral replication in primary cells. That this effect is in macrophages is intriguing in the light of recent findings. First of all, for nonprimate lentiviruses, the ability to infect and replicate well in macrophages is considered crucial to the ability to establish a long-term persistent infection (11, 23). In addition, Schuitemaker et al. (30) have

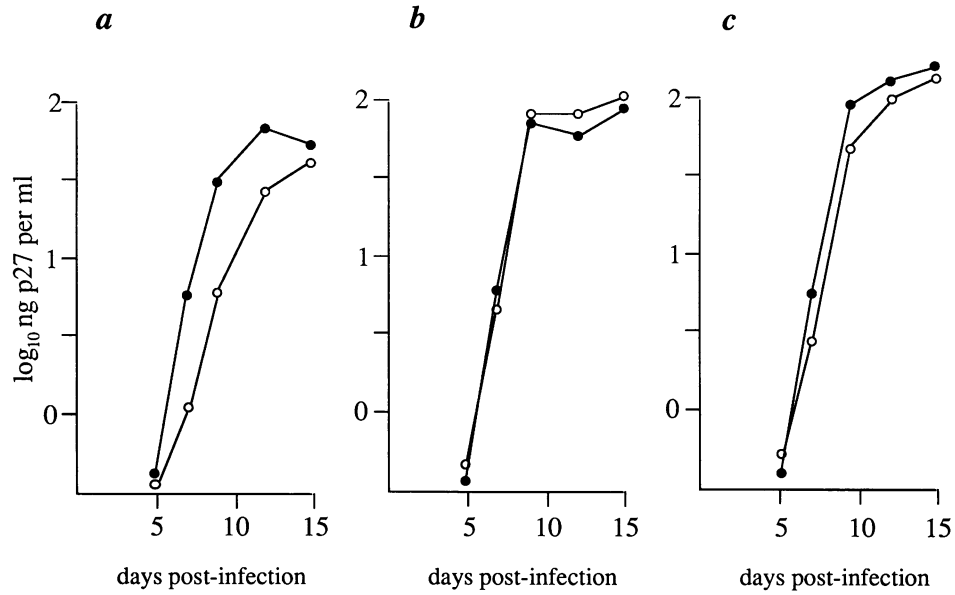

FIG. 6. The NF-κB site is not essential for efficient replication of Em* in PBLs. (a, b, and c) Growth curves of Em* or Em*CTC in PBLs from individual macaques. Open circles, Em*CTC; closed circles, Em*.

recently demonstrated that during the long-term persistent stage of HIV infection, macrophagetropic variants predominate. Moreover, viruses isolated from PBLs during this stage replicate well in macrophages also, suggesting that they have recently entered the T-cell population from a monocyte reservoir. Only in the late stages of disease do T-cell-tropic variants come to predominate. Schuitemaker et al. suggest that macrophages and monocytes serve as virus reservoirs and mediate persistence. If their model is correct, then sequences that influenced replication in macrophages would be expected to be very highly conserved, as is indeed the NF-κB binding site.

Much evidence supports a role for specific LTR sequences in HIV-1 replication in monocytes-macrophages. For example, the latently HIV-1-infected U1 monocytic cell line responds to NF-κB inducers by greatly increasing viral production (5, 10); the monocytic cell line THP-1 can develop restricted expression of HIV-1 and under these circumstances contains a nuclear inhibitor of NF-κB activity; in this system, the transfection of an NF-κB expression vector overcomes the restriction and increases virus production (19); and lipopolysaccharide, an NF-κB inducer, increases HIV replication in monocytes (26). In addition, HIV-1 infection of U937 monocytic cells leads to an increase in NF-κB activity, perhaps by action of the viral protease on an inactive NF-κB precursor (1). Finally, although conflicting reports exist, the HIV envelope protein may be able to stimulate monocytic cells to produce tumor necrosis factor alpha, which is known to activate NF-κB (18, 20).

The results of studies mapping determinants of macrophage tropism (13, 24, 31) have led to the suggestion that envelope sequences are the primary determinant and are sufficient for efficient replication in macrophages. Moreover, no evidence has been seen for a role of LTR sequences in macrophage tropism. However, in the studies described above, the NF-κB site in both the T-cell-tropic and the macrophagetropic parents was wild type; thus, a contribution of the NF-κB site to macrophage tropism would not have been detected in these experiments. In addition, the experiments described by Leonard et al. (16) and by Ross et al. (29) studying the effect of the NF-κB site on HIV-1 growth used a clone (NL-43) that does not replicate in primary macrophages; therefore, these investigators were not able to determine the contribution of the NF-κB site to HIV-1 growth in primary macrophages. In light of data suggesting an important role for macrophagetropic viruses in HIV and SIV persistence, it is tempting to speculate that in vivo selective pressure for maintenance of the NF-κB site may be a reflection of its importance for replication in macrophages.

It is quite possible, however, that in addition the NF-κB site does play a role in replication in T cells in vivo and that the in vitro conditions employed here do not reflect that role. The state of activation and growth factors present, etc., may be very different between PBLs in culture and in vivo; in addition, the way in which virus spreads and grows in in vitro culture may be very dissimilar to what happens in vivo. Indeed, the virus lacking NF-κB did show a reproducible lag in kinetics compared with that of the wild-type virus in MT-2 cells. This result is consistent with results obtained by Ross et al. (29), who noticed that with respect to virus growth kinetics, the HIV-1 NF-κB site had different effects, depending on the particular T-cell lines employed. One other possibility is that the NF-κB site is important in reactivation of latent virus in T cells, and growth curves as performed in the present study and by others (16, 29) do not reflect that activity.

However, the fact that we have employed the pathogenic molecular clone 239 in the present studies will allow us to experimentally infect rhesus macaques to determine the role of NF-κB in viral persistence and pathogenicity. One prediction of the results reported here would be that in rhesus macaques infected with the 239CTC NF-κB mutant virus, SIV will replicate only in the T-cell compartment; an inability to efficiently replicate in macrophages might prevent viral persistence. Careful monitoring of virus and viral DNA present in PBLs and macrophages at different times after infection would allow testing of this prediction.

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