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The human immunodeficiency virus type 2 *vpr* gene is essential for productive infection of human macrophages

(*nef* gene/macrophage infection)

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ABSTRACT **The human immunodeficiency virus (HIV) genetic determinant(s) responsible for tropism in human T cells or macrophages are not well defined. We studied the role of the HIV type 2 (HIV-2) *nef* and *vpr* genes in viral tropism. HIV-2 mutants, lacking either *vpr* or *nef* genes, or both *vpr* and *nef*, were obtained by site-specific mutagenesis of a biologically active HIV-2 proviral clone (HIV-2$_{sbl/isy}$), which is infectious in both human T cells and macrophages. Viral progeny carrying mutations of *nef*, *vpr*, or of both *nef* and *vpr* genes replicated more efficiently than the parental virus in primary human peripheral blood cells and in the human Hut 78 T-cell line. In contrast, the HIV-2 *nef*$^-$ mutant infected human macrophages as efficiently as the parental virus, whereas viruses lacking the *vpr* gene either alone or in conjunction with the lack of the *nef* gene did not replicate in macrophages. Thus, some lack of *nef* in HIV-2 enhances viral replication in T cells and does not interfere with viral replication in primary macrophages, whereas *vpr* is essential for replication of HIV-2 in human macrophages. Because the parental HIV-2$_{sbl/isy}$ cloned virus also infects rhesus macaques, the use in animal studies of these HIV-2 mutants with differences in cell tropism and rates of replication will be highly useful in understanding the mechanism of viral infectivity and possibly pathogenicity *in vivo*.**

AIDS is associated with at least two retroviruses, human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) (1, 2). HIV-1 infection is prevalent in AIDS patients worldwide, while HIV-2 is prevalent in some West African countries (3). HIV-2 is genetically and structurally related to HIV-1 (4–6) but appears far less pathogenic than HIV-1 (7). In addition to the structural genes (*gag, env*) and the *pol* gene, HIV-1 and HIV-2 have similar accessory genes (*nef, vif, vpr, tat*, and *rev*). One distinctive difference between the two viruses is the presence of the *vpu* gene in HIV-1 (8) and the *vpx* gene in HIV-2 (9–11).

Various monkey species are naturally infected with lentiviruses related to HIV-1 and HIV-2 (12–20). Simian immunodeficiency virus (SIV) of rhesus macaque (SIV$_{mac}$) and of sooty mangabey (SIV$_{sm}$) are more closely related structurally and genetically to HIV-2 than to HIV-1 (21–24). SIV of African green monkey (SIV$_{agm}$), evolutionarily equally distant from HIV-1 and HIV-2 (18, 19), differs structurally from both HIVs because it lacks *vpr*, although it carries *vpx*. SIV in the mandrill (SIV$_{mnd}$), on the other hand, lacks *vpx* but carries *vpr* (20). Interestingly, the *nef* gene is maintained in all HIVs and SIVs. The biological implication of these genetic differences among the known primate immunodeficiency viruses could play a key role in viral tropism, virus–host interaction, and disease development, but at the current time this role is not known. We have used a replication-competent HIV-2 molecular clone (HIV-2$_{sbl/isy}$) (6) that is infectious in primary peripheral blood human T cells and macrophages *in vitro* and infects rhesus macaques *in vivo* (25) to study the function of *vpr* and *nef* in viral tropism, infectivity, and cytopathogenicity *in vitro*.

MATERIALS AND METHODS

Cloning, Mutagenesis, and DNA Analysis. To reconstitute a replication-competent provirus in plasmids we used DNA fragments from a recombinant λ DNA clone containing a complete provirus of HIV-2$_{sbl/isy}$. The EGP plasmid was obtained in two steps by subcloning a 4-kilobase (kb) *Eco*RI fragment and a 2.3-kb *Eco*RI–*Sac* I fragment in Bluescript (Stratagene). The KF-3 plasmid was obtained by cloning a 6-kb *Xba* I fragment at the *Kpn* I–*Sac* I site of Bluescript after treating both the vector and the insert with T4 polymerase to generate blunt ends (26). The two plasmids were linearized by using *Sac* I. Linearized KF-3 was treated with bacterial alkaline phosphatase, and the DNAs of both plasmids were ligated. The ligated DNA was purified by phenol extraction and transfected into Hut 78 cell line. To mutagenize *nef*, we linearized the KF-3 plasmid with *Eco*RV and introduced a *Cla* I linker (5′-GATCGATC-3′), which generated an in-frame termination codon and truncated the *nef* open reading frame at amino acid 59. The *vpr* gene open reading frame was mutated (27) by using the mutagenic primer (5′-CTATAGACTTAAGCACCAGC-3′) that changed the AUG codon to the TAG termination codon and introduced another in-frame termination codon, TAA, three bases downstream. This mutation was introduced in an M13 subclone containing a viral *Bgl* II–*Sac* I fragment of 900 base pairs (bp). The mutated *Bgl* II–*Sac* I fragment was substituted in the EGP plasmid. The plasmids containing the mutated *vpr* and *nef* genes were ligated together as described above and transfected into Hut 78 cells.

Southern blot analysis of the genomic DNA was performed as described (28). Polymerase chain reaction analysis (29) of genomic DNA (1 μg) was done by using the described primers (60 ng each), a denaturation step at 94°C for 20 min followed by hybridizing at 55°C for 20 min, and extension at 72°C for 30 min for a total of 30 cycles.

Production of Viral Stocks. The plasmids containing the isogenic and mutated proviral DNAs were transfected into the Hut 78 cell line, as described. Transfected cell lines were monitored for viral expression by detecting reverse transcriptase (RT) activity in the supernatants (30) and for production of other viral proteins. When an adequate level of viral

Abbreviations: PBMC, peripheral blood mononuclear cells; RT, reverse transcriptase; HIV-1 and -2, human immunodeficiency virus type 1 and type 2, respectively; SIV, simian immunodeficiency virus; *vpr*$^-$, mutated *vpr*; *nef*$^-$, mutated *nef*; *vpr*$^-$–*nef*$^-$, mutated *vpr* plus mutated *nef*.

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production was obtained, viral stocks were prepared from supernatants of the infected cells.

Infection of Peripheral Blood Mononuclear Cells (PBMCs) and Monocytes. PBMCs were isolated by isopycnic centrifugation of Lymphoprep (Organon Teknika–Cappel) and depleted of monocytes by overnight adherence. Nonadherent cells were isolated, stimulated with phytohemoagglutinin for 3 days, and infected with an amount of virus equivalent to 2 $\times 10^5$ cpm of RT activity per ml per 1.5×10^6 cells. Hut 78 cells were infected identically. After 1-hr viral exposure cells were washed and seeded in 5 ml of RPMI 1640 medium containing 20% fetal calf serum, penicillin at 50 units/ml, streptomycin at 50 μg/ml, and 5 μM L-glutamine. At the indicated sampling times, the medium was replaced, and RT activity was assayed in the culture supernatants as described (30). Monocytes were isolated from PBMCs by adherence to plastic as described (31). Cells were infected while in suspension by adding an amount of virus equivalent to 2 or 4 $\times 10^5$ cpm of RT to 1×10^7 monocytes for 1 hr with periodic agitation. The cells were washed once and seeded in RPMI 1640 medium/20% fetal calf serum containing penicillin at 50 units/ml, streptomycin at 50 μg/ml, and 5 μM L-glutamine. At the indicated sampling times, culture supernatants were removed and cleared by low-speed centrifugation. The supernatants were collected, and the pellet consisting of both viable and nonviable cells was returned to the original cultures; the amount of RT in the supernatants was determined as described (30).

Protein Analysis. At the times chosen for protein analysis by radioimmunoprecipitation assay, the culture medium was removed, and the cells were washed once with RPMI 1640 medium lacking cysteine and methionine but containing 10% dialyzed fetal calf serum. Cells were resuspended in 2 ml of methionine and cysteine-free medium containing [^{35}S]methionine and [^{35}S]cysteine at 100 μCi/ml (1 Ci = 37 GBq) each and incubated overnight. At the end of incubation, cells were washed and then lysed with 0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$; the cell lysate was then immunoprecipitated (32) with human serum (1:100) from an individual infected with HIV-2.

RESULTS

Generation of HIV-2 Mutants in *nef* and *vpr* Genes. Two overlapping pieces of proviral DNA from the recombinant phage were subcloned separately in a Bluescript vector (Fig. 1) because plasmids containing the entire provirus were unstable in *Escherichia coli*. Plasmid EGP DNA carrying the *vpr* mutation was ligated either with plasmid KF-3 DNA or with plasmid KF-3 DNA carrying the mutated *nef* gene to generate an HIV-2 vpr^- or an HIV-2 vpr^-–nef^- mutant, respectively. The HIV-2 nef^- mutant was obtained by ligating plasmid EGP DNA and plasmid KF-3 DNA with the mutated *nef* gene. The ligated DNA was transfected into Hut 78 cell line, which is $CD4^+$ and can support HIV-2 replication.

To monitor viral production in Hut 78-transfected cells we followed RT activity and the presence of metabolically labeled viral antigen in the medium of the transfected cells. RT activity was detectable, on the average, at 15 days after transfection, and labeled gp120 and p24 were present in the supernatant of the cells transfected with all HIV-2 mutants as well as with HIV-$2_{sbl/isy}$ (data not shown), indicating that abolishing *nef* and *vpr* function did not impair production of extracellular virus in this human T-cell line. Furthermore, all HIV-2 mutants were as cytopathic as the wild type and induced very large syncytia in the transfected cells (data not shown).

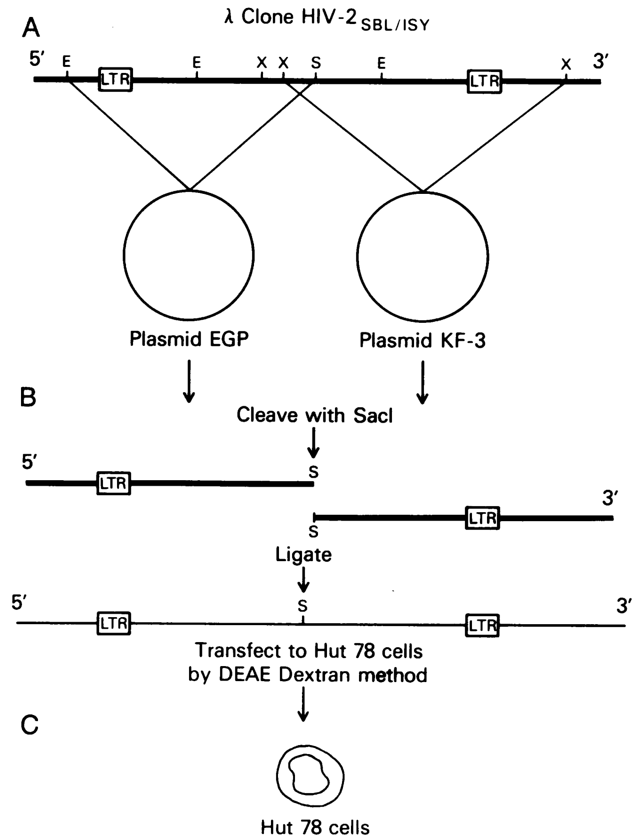


FIG. 1. Schematic representation of the strategy used to reconstitute biologically active proviral DNA from two plasmids containing overlapping regions of HIV-$2_{sbl/isy}$. E, *Eco*RI; S, *Sac* I; X, *Xba* I.

Infectivity of HIV-2 Viral Mutants. To measure the relative infectivity of the mutated viruses we prepared cell-free supernatants from the transfected Hut 78 cells, normalized them for equal content of viral RT activity (2×10^5 cpm/ml), and applied them to phytohemagglutinin-stimulated (for 3 days) human PBMCs enriched for T lymphocytes. The kinetics of viral infection were studied by following the amount of viral RT activity in the medium and the synthesis of viral p24 protein in the infected cells every 3 days after infection. The results of three experiments on three different blood donors are presented in Fig. 2. In Fig. 2*A*, RT activity was detected in both cultures infected with HIV-2 nef^- and HIV-2 nef^-–vpr^- mutants at day 9, but expression of p24 was seen with the HIV-2 nef^- virus as early as day 6 after infection (Fig. 2*A*). Similarly, in the HIV-2 vpr^--infected culture, p24 was evident at day 9, and RT activity was evident at day 12. Wild-type HIV-$2_{sbl/isy}$ scored positive for RT at day 15, but low levels of p24 were already detected at day 9. In Fig. 2*B*, higher expression of viral RT was detected in both HIV-2 vpr^- and nef^- than in the wild type at day 11, although the kinetics of viral expression were slightly different from Fig. 2*A*. Because use of equal amounts of viral RT activity in the infectivity assay does not necessarily reflect the amount of biologically active virus in the viral stock used, we tested the infectivity of serial dilutions (from 10^{-1} to 10^{-9}) of each viral stock in Hut 78 cell line. We followed the RT activity produced in the medium of duplicate cell culture for each virus for 1 month. One $TCID_{50}$ (tissue culture 50% infectious dose) corresponded to the minimal amount of each viral stock that yielded a productive infection in 50% of the culture. The PBMCs of a third donor were then infected at a multiplicity of infection (moi) of 0.01 [one viral infectious dose ($TCID_{50}$) every 100 cells]. The results of the third experiment are

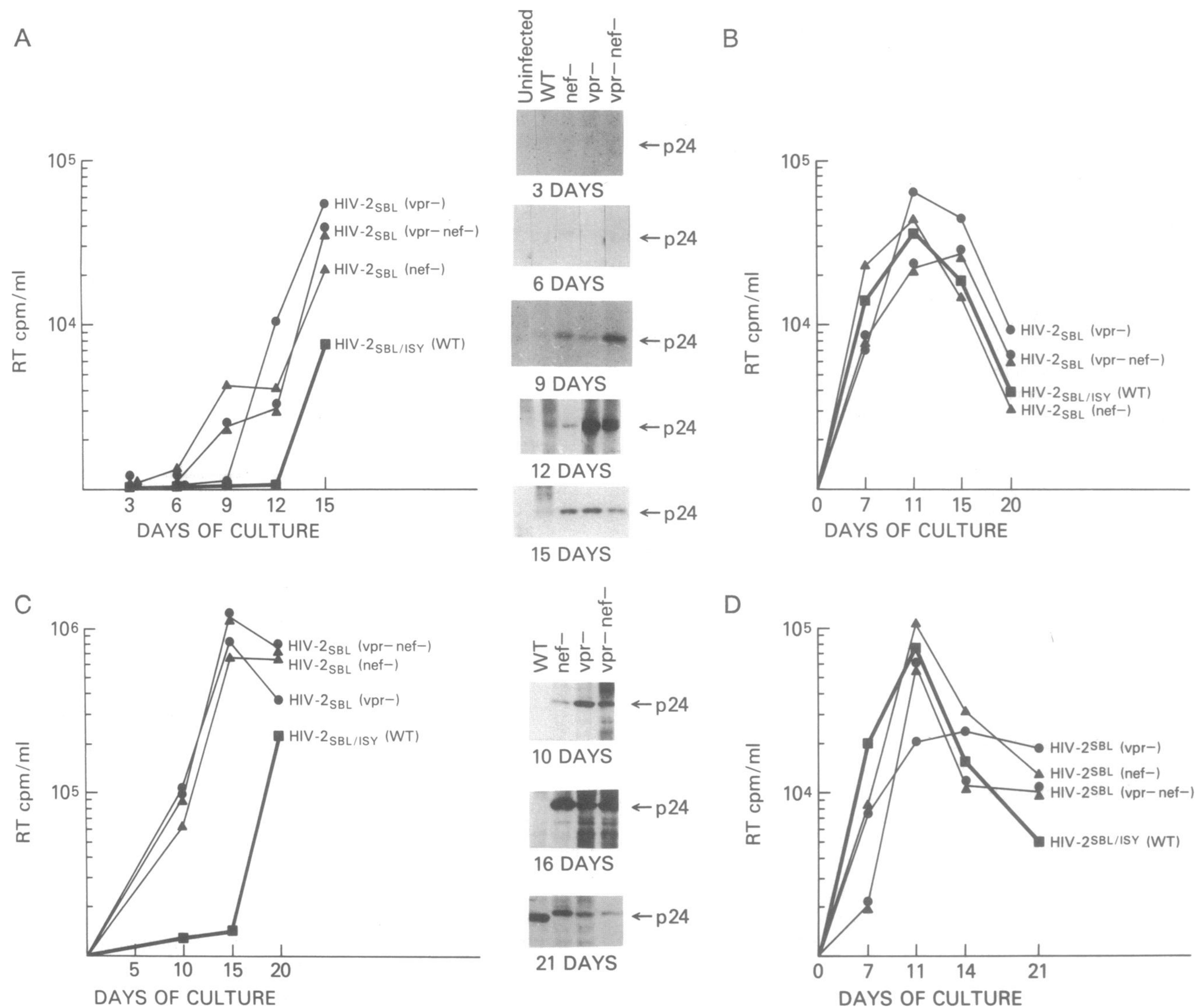


FIG. 2. Kinetics of viral infection of human PBMC (*A*, *B*, and *D*) and Hut 78 cells (*C*). *A* and *C* include radioimmunoprecipitation of viral proteins various days after infection. WT, wild type.

shown in Fig. 2*D*. Slightly higher expressions of the HIV-2 *nef*$^-$ virus were seen at day 10 when compared with the wild type. Taken together, the data indicate that the abrogation of *nef* function conferred a slight growth advantage to HIV-2 in primary human cells. Absence of the *vpr* gene *per se* appeared also to confer a slight increase in viral replication in two of three experiments. Such an effect of *vpr* on viral expression was even more evident when a similar experiment was conducted using Hut 78 as target cell. Fig. 2*C* shows that RT activity as well as p24 protein were detected at day 10 in the HIV-2 *vpr*$^-$ mutant, whereas wild-type HIV-$2_{sbl/isy}$ became positive for both parameters only at day 21. The kinetics of Hut 78 infection of the HIV-2 *nef*$^-$ and *vpr*$^-$–*nef*$^-$ mutants mirrored the kinetics seen when PBMCs (from donor 1) were used as target cells (Fig. 2 *A* and *C*).

Stability of the HIV-2 Mutant Genotypes. To ascertain whether the *nef* mutation introduced in the proviral DNAs was retained after many cycles of viral replication, we cleaved the genomic DNAs of the infected Hut 78 cells with *Eco*RI and *Cla* I. The *Cla* I recognition site is not present in HIV-$2_{sbl/isy}$ DNA but was inserted in the proviral DNA of the HIV-2 *nef*$^-$ (see *Materials and Methods*) and is therefore diagnostic for mutation in *nef*. Fig. 3 shows that the mutation in *nef* in both HIV-2 *nef*$^-$ and *vpr*$^-$–*nef*$^-$ was readily detected as a 1.4-kb band derived from the double digestion of genomic DNA with *Eco*RI and *Cla* I. The HIV-2 *vpr*$^-$ DNA, which is isogenic in *nef* with wild-type HIV-$2_{sbl/isy}$, lacks the 1.4-kb internal band.

To monitor whether reversion of the nucleotide change introduced in *vpr* occurred, we performed polymerase chain reaction analyses (29) on the DNA of infected cells with two HIV-$2_{sbl/isy}$-specific primers (5′-GTCCAAGGTACCGT-CAC-3′; 5′-CCCTAATGAGCTCTCTGGC-3′) and obtained the nucleotide sequence of the mutated region in seven clones of the amplified DNA fragment. No evidence of reversion was detected (data not shown).

Infection of Primary Macrophages. HIV-$2_{sbl/isy}$ infects not only human primary T cells but also human primary macrophages. To investigate whether the abrogation of *nef* and *vpr* function affected the capability of HIV-2 to infect both types of target cells, we infected human macrophages obtained from peripheral blood with the HIV-$2_{sbl/isy}$ and the HIV-2 mutants by using a described procedure (31). Parallel experiments with PBMCs from the same donor were also done as a positive control on the biological activity of the viral stocks used. RT activity in the supernatant of the infected macrophages was measured every few days; results are shown in Fig. 4. In Fig. 4*A* we used 2×10^5 cpm of RT activity for each virus. High levels of viral replication in macrophages were seen with the HIV-$2_{sbl/isy}$ and HIV-2 *nef*$^-$ mutant. Surpris-

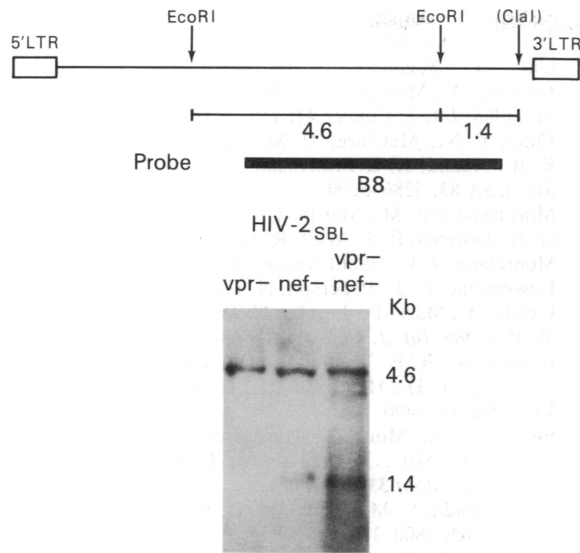


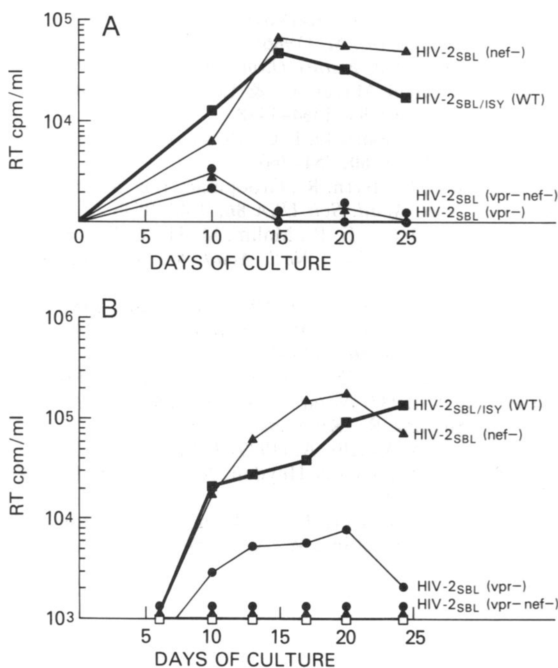
FIG. 3. Southern blot analysis of the DNA of chronically infected Hut 78 cells. Restriction enzyme and Southern blot (28) analyses of the DNA of infected Hut 78 cells were done after several months of culture. Because a *Cla* I site was introduced into the *nef* gene, we restricted the DNAs of HIV-2 *nef*$^-$, HIV-2 *vpr*$^-$, and HIV-2 *vpr*$^-$–*nef*$^-$ with *Cla* I and *Eco*RI to generate an internal viral band of 1.4 kb in the proviruses carrying the mutation presented schematically above. Filters carrying the genomic DNAs were hybridized according to described procedures (28) with B8 probe (6). LTR, long terminal repeat.

ingly, neither HIV-2 *vpr*$^-$ nor HIV-2 *vpr*$^-$–*nef*$^-$ mutants produced significant levels of RT in the supernatant. Results of a parallel culture of the PBMCs of the same individual are shown in Fig. 2*B*. As shown, the same viral stock of HIV-2 *vpr*$^-$ and HIV-2 *vpr*$^-$–*nef*$^-$ promptly replicated in PBMCs but failed to induce significant RT activity in the supernatant of the macrophage cultures. A second experiment was performed with more virus (4×10^5 cpm of RT activity), and the results represented in Fig. 4*B Left* again indicated failure of the HIV-2 *vpr*$^-$ and HIV-2 *vpr*$^-$–*nef*$^-$ viruses to productively replicate in human macrophages.

To determine whether the low level of RT activity in the supernatants of the macrophage culture infected with the HIV-2 *vpr*$^-$ and the HIV-2 *vpr*$^-$–*nef*$^-$ was from delayed virus release rather than from impaired viral expression, we metabolically labeled the macrophage culture 20 days after infection and scored for viral proteins by immunoprecipitation. Supernatants and total cellular lysates were immunoprecipitated by using a human serum positive for antibodies against HIV-2 proteins. Fig. 4*B* (*Right*) indicates that viral proteins could be readily detected in both cell lysate and supernatant of HIV-$2_{sbl/isy}$ and HIV-2 *nef*$^-$ mutant-infected macrophages but not in macrophages infected with HIV-2 *vpr*$^-$ or HIV-2 *vpr*$^-$–*nef*$^-$ mutants. Thus, the defect in the *vpr* gene appeared to impair viral expression rather than viral release from human macrophages. Viral entry and reverse transcription were apparently not inhibited by the mutation introduced in the *vpr* gene because we demonstrated a specific fragment of viral DNA of 0.591 kb by polymerase chain reaction analysis of the DNAs of the macrophages infected with the HIV-2 *vpr*$^-$, as well as in the wild type and the HIV-2 *nef*$^-$ (Fig. 4 *Right*). A low level of viral DNA was also detected in the macrophage infected with the HIV-2 *vpr*$^-$–*nef*$^-$ (Fig. 4). Why a lower level of viral DNA and no RT activity can be found in the culture infected with the HIV-2 lacking both the *vpr* and the *nef* genes is not clear because lack of the *nef* gene *per se* does not appear to interfere with HIV-2 expression in human macrophages. Understanding the mechanism of action of both *vpr* and *nef* will clarify whether the two gene products interact on a common pathway in the HIV-2 replicative cycle in human macrophages.

DISCUSSION

Our results show that HIV-2 defective in *nef* gene function can replicate efficiently in human macrophages and yield extracellular viruses. In contrast, when a defect is introduced in the *vpr* gene, no viral replication can be detected in human macrophages. The importance of the *vpr* gene for viral replication in human macrophages is also further confirmed by failure of the HIV-2 *vpr*$^-$–*nef*$^-$ mutant to productively infect primary macrophages.



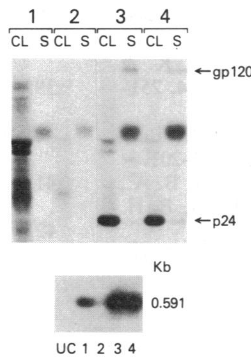


FIG. 4. Viral infection of human primary macrophages. (*A* and *B Left*) RT activity in the supernatants of macrophage cultures after infection with HIV-$2_{sbl/isy}$ or HIV-2 mutants of two different blood donor macrophages. ▲, HIV-2 *nef*$^-$; ■, HIV-$2_{sbl/isy}$; ●, HIV-2 *vpr*$^-$; ♝, HIV-2 *vpr*$^-$–*nef*$^-$; □, uninfected control. (*B Right upper*) Radioimmune precipitation of viral protein 20 days after infection from the experiment of *B* was done as described. CL, cell lysate; S, supernatant; 1, HIV-2 *vpr*$^-$; 2, HIV-2 *vpr*$^-$–*nef*$^-$; 3, HIV-2 *nef*$^-$; 4, HIV-$2_{sbl/isy}$. (*B Right*) Autoradiography of hybridization with the B8 probe (6) of polymerase chain reaction amplified 591 bp of HIV-2 sequence from infected macrophage DNA (24 days after infection) of the experiment of *B*. Primers used in the polymerase chain reaction are as described in text. UC, uninfected control DNA.

The fact that HIV-2 *nef*$^-$ virus replicated slightly better than did the parental virus in human T cells suggests that HIV-$2_{sbl/isy}$ carries a functional *nef* gene which, as has been suggested for HIV-1 (33, 34), exerts a negative effect on viral expression *in vitro*. More importantly, a similar effect is evident in primary human T cells. Considerable disagreement exists among different laboratories regarding the effect of the *nef* gene on viral growth (33–37) and viral transcription (35–38). Nevertheless, the HIV-$2_{sbl/isy}$ *nef*$^-$ mutant under our experimental conditions displays a slight growth advantage when compared with wild-type HIV-$2_{sbl/isy}$.

The *vpr* gene of HIV-2 is dispensable for viral replication in human T cells, as has been reported for HIV-1 (39, 40) and HIV-2 (41), but our data indicate that its expression is required for viral propagation in primary macrophages.

The HIV-1 *vpr* gene has been shown to be a trans-activator of the HIV-1 long terminal repeats as well as of heterologous promoters (42). The HIV-2 *vpr* gene also trans-activates *in vitro* (N.H., R.C.G., and G.F., unpublished observation), and this function might play an important role in viral replication in human macrophages. Because the molecularly cloned HIV-$2_{sbl/isy}$ virus infects rhesus macaques (25), the use in animals of HIV-2 mutants that replicate efficiently in both macrophages and T cells (*nef*$^-$) or in T cells only (*vpr*$^-$ and *vpr*$^-$–*nef*$^-$) will hopefully provide useful insights into viral tropism in the establishment of persistent infection in the host and allow evaluation of vaccine efficacy.

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