VIROLOGY 206, 935–944 (1995)

Vpr Is Required for Efficient Replication of Human Immunodeficiency Virus Type-1 in Mononuclear Phagocytes

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Received April 20, 1994; accepted November 11, 1994

HIV-1 *vpr* encodes a 96-amino acid, nuclear protein whose function is not well understood. Unlike the other lentivirus regulatory proteins, Vpr is present in virions at relatively high copy number. In cells, Vpr is localized to the nucleus. Possible functions for *vpr* consistent with these findings include the nuclear import of preintegration complexes, transactivation of cellular genes, or induction of cellular differentiation. We show here, using both replication competent, macrophage-tropic virus and a sensitive, single-cycle luciferase HIV-1 reporter vector, that *vpr* is important for efficient viral replication in primary monocyte/macrophages, but appears to play no role in activated or resting T cell infection. The block to infection in monocytes was localized by PCR analysis of newly synthesized viral DNA and with the luciferase reporter vector to a stage in the viral life cycle after entry and reverse transcription, yet prior to, or at the time of, proviral transcription. In addition, infection of mononuclear phagocytes with virions that had been loaded with Vpr molecules in the producer cells by trans-complementation still showed a *vpr*-phenotype. These data suggest a role for *vpr* molecules produced in newly infected cells, in addition to its presumed function in the virion.

INTRODUCTION

HIV and SIV encode regulatory molecules, some of which appear to be essential for viral replication (*tat*, *rev*) and others which are, at least in certain cell-types, dispensable (*vpr*, *vpx*, *nef*, *vif*, *vpu*). For HIV-1 replication in $CD4^+$ cell lines, *vpr* is dispensable. Ablation of the open reading frame results in only a slight decrease in the rate or extent of viral replication (Adachi *et al.*, 1991; Akari *et al.*, 1992; Cohen *et al.*, 1990a; Dedera *et al.*, 1989; Ogawa *et al.*, 1989). It is clear, however, that the protein is important *in vivo*. HIV-1-infected individuals, as well as SIV-infected monkeys, make antibodies against Vpr, indicating that it is in fact expressed (Wong-Staal *et al.*, 1987; Yu *et al.*, 1988). Furthermore, the amino acid sequence encoded by *vpr* is well conserved among various isolates of HIV, SIV, and other lentiviruses (Tristem *et al.*, 1992). The importance of *vpr* for viral persistence, replication, and pathogenesis is suggested by studies in which monkeys were infected with SIVmac239 containing an experimentally introduced mutation of the ATG initiation codon of *vpr* (Lang *et al.*, 1993). In these monkeys, reversion of the ATG mutation was associated with increased viral burden and rapid disease progression.

Unlike the other HIV-1 regulatory gene products (*tat*, *rev*, *vpu*, *nef*, and *vif*), Vpr and the related SIV/HIV-2 gene product, Vpx, are present at high copy number in viral particles (Cohen *et al.*, 1990b; Yu *et al.*, 1988; Yu *et al.*, 1990; Yuan *et al.*, 1990). Incorporation of Vpr into HIV-1 particles was found to be mediated by a specific interaction with the carboxy terminus of the Gag precursor which corresponds to the p6 protein (Paxton *et al.*, 1993). Mutational analyses indicate that Vpr incorporation requires the arginine-rich, carboxy-terminus of the protein. Vpr is localized within the virion core, most likely due to its association with p6 at the carboxy terminus of Gag. Vpx differs in this respect in that it appears to be exposed on the outside of the core particle (Yu *et al.*, 1993), and to associate with CA in the virion (Horton *et al.*, 1994).

The presence of Vpr in virions suggests that it may play a role in viral replication early, prior to synthesis of new viral proteins, or alternatively, late in the viral life cycle, during assembly and budding. The former possibility is more likely since *vpr* does not appear to affect assembly or budding of virus (N. Landau and S. Choe, unpublished data). In infected or transfected cells, Vpr and Vpx are localized to the nucleus (Lu *et al.*, 1993; M. Scott and N. Landau, unpublished observations). Vpx has been shown to bind RNA in an artificial system (Henderson *et al.*, 1988), but whether it or Vpr is bound to the viral genomic RNA in viral particles is unknown. Early studies suggested that Vpr transactivates the HIV-1 LTR as well as other viral and cellular promoters; however, this effect was modest (Cohen *et al.*, 1990b). In other studies, expression of HIV-1 *vpr* either from an integrated provirus or from a transfected *vpr* expression vector was found to induce differentiation of a human muscle cell

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0042-6822/95 $6.00

line, indirectly suggesting that *in vivo*, *vpr* might influence the differentiation state of infected cells (Levy *et al.*, 1993). Recently, Vpr has been shown to influence the nuclear transport of HIV-1 prior to integration in nondividing cells (Heinzinger *et al.*, 1994), including macrophages.

Studies on the effect of *vpr* and *vpx* on viral replication in cultured and primary cells have produced widely different results depending upon the cell type and viral strain. In T cell lines, *vpr* and *vpx* appear to be dispensable for replication (Cohen *et al.*, 1990a; Dedera *et al.*, 1989; Guyader *et al.*, 1989; Hu *et al.*, 1989; Kappes *et al.*, 1991; Marcon *et al.*, 1991). Some reports have shown that for HIV-2, mutation of *vpx* markedly reduces the ability of the virus to replicate in primary peripheral blood mononuclear cells (PBMC) (Guyader *et al.*, 1989; Kappes *et al.*, 1991; Yu *et al.*, 1991). However, other reports have shown that mutation of *vpx* had very little effect on virus replication in primary cells (Dedera *et al.*, 1989; Hu *et al.*, 1989; Marcon *et al.*, 1991).

Because the effect of HIV-1 *vpr* on viral replication in cell lines is small, we were interested to determine whether *vpr* would have a more pronounced effect on replication in primary cells, which are the natural targets for HIV *in vivo*. The results of our study suggest that *vpr* is important for efficient replication of HIV-1 in mononuclear phagocytes, but not for replication in resting or stimulated primary T cells. By comparing the relative amounts of newly synthesized viral DNA, and using a sensitive single-cycle replication assay, we show that in mononuclear phagocytes *vpr* appears to act after reverse transcription and at, or prior to, mRNA synthesis. Finally, we present evidence that a substantial portion of the *vpr* effect in mononuclear phagocytes is due to Vpr that is synthesized in the infected target cell. Thus, the role of *vpr* is not solely mediated by *vpr* molecules contained in the infecting virions. These results suggest a function for *vpr* expressed in infected monocyte/macrophages in addition to its presumed function in the virion.

MATERIALS AND METHODS

Plasmids

To facilitate plasmid growth and manipulation, the HIV-1 provirus HXB2 was cloned into pBS-KS (Stratagene) at the *Xba*I and *Hin*cII sites, resulting in the plasmid pHIV. HXB2 *vpr* contains a single base insertion at nt 5320 that results in a frameshift at amino acid 72. This defect was corrected by PCR mutagenesis. A downstream primer (TGTCGACACCCAATTCTGAAATGGAT) that hybridized to the frameshifted portion of *vpr* and removed the additional T and an upstream primer (CCTAGGATTTGGCTCCATGGCTTA) that hybridized near the 5′ end of *vpr* (nt 5207 to 5230) were used to amplify pHIV. The resulting DNA fragment was digested with *Nco*I and *Sal*I. This fragment was used to replace the corresponding restriction fragment of pHIV, resulting in the plasmid pHIV-R^+. Restoration of the *vpr* open reading frame was confirmed by nucleotide sequencing. Macrophage-tropic vpr^+ and vpr^- proviruses pHXB(BAL)-R^- and pHXB(BAL)-R^+ were constructed by replacing the *Sal*I to *Bam*HI fragment (nt 5332 to 8021) of pHIV and pHIV-R^+ with the corresponding fragment of the macrophage-tropic provirus, HIV-BaL (Hwang *et al.*, 1991), provided by D. Trono (Salk Institute).

pNL-Luc-E^-R^+ and pNL-Luc-E^-R^- are similar to the HIV-1 luciferase reporter vector described by Chen *et al.* (1994), except that they are based upon the infectious vpr^+ provirus NL4-3 and are env^-. To construct these vectors, a frameshift was introduced near the 5′-end of *env* by using T4 DNA polymerase to fill in the *Nde*I site (nt 5950) of pNL4-3. The firefly luciferase gene was then inserted into the *nef* gene by removing the *Bam*HI (nt 8021) to *Xho*I (nt 8443) fragment of pHXB-Luc (Chen *et al.*, 1994) and ligating it to the same sites in env^- pNL4-3. In pNL-Luc-E^-R^-, a frameshift was introduced into *vpr* by filling-in the *Afl*II site (nt 5180) at a position corresponding to amino acid 26.

Construction and characterization of Vpr expression vectors, pcDNA-tag-Vpr and pcDNA-Vpr, has been described previously (Paxton *et al.*, 1993).

Transfection

COS, Hela, and 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37° and 5% CO_2. Cells (1.2×10^6) seeded the previous day in 10-cm culture dishes were transfected with 20 μg of plasmid DNA by calcium phosphate precipitation as described previously (Chen and Okayama, 1987). The culture medium was changed 24 and 48 hr post-transfection, and virus-containing supernatants were collected at 72 hr. The amount of virus present in the transfected supernatants was determined by measuring $p24^{gag}$ using a commercial ELISA (Abbott Laboratories). Luciferase reporter viruses were prepared by cotransfecting 293 cells with pNL-Luc-E^-R^+ or pNL-Luc-E^-R^- (10 μg) and the amphotropic envelope glycoprotein expression vector pSV-A-MLV-env (10 μg). For complemented viruses, 293 cells were cotransfected with pNL-Luc-E^-R^+ or pNL-Luc-E^-R^- (2.5 μg), pSV-A-MLV-env (7.5 μg), and pcDNA-Vpr or pcDNA (15 μg).

Immunoblot analysis

Supernatants (10 ml) were harvested 72 hr post-transfection, filtered (0.45-μm filters), and centrifuged for 45 min at 100,000 *g* to pellet virions. Pelleted material was dissolved in 100 μl lysis buffer (1% Triton X-100, 0.15 *M* NaCl, 10 m*M* tris, pH 8.0) and the amount of reverse transcriptase present was measured as described previously (Goff *et al.*, 1981). Amounts of virus lysate containing 100 units reverse transcriptase were separated on 17.5% SDS–PAGE. The proteins were transferred to

an Immobilon filter (Millipore Corp.) which was probed with rabbit anti-Vpr serum diluted 1:5000 followed by alkaline phosphatase-conjugated goat anti-rabbit serum diluted 1:7000 (Tago) as described previously (Paxton *et al.*, 1993). Anti-Vpr serum was raised by multiple injections of a rabbit with recombinant Vpr prepared as a glutathione S-transferase fusion protein in *Escherichia coli*.

Primary cell culture

PBMC from normal HIV-1 seronegative donors were isolated by Ficoll-Hypaque density centrifugation. In some experiments, PBMC were activated by culturing for 24–48 hr in the presence of 50 μg/ml phytohemagglutinin (PHA). Stimulated cells were added to 24-well plates at a density of 3×10^6/well in 1.5 ml RPMI/10% FCS containing 10 U/ml IL-2 and streptomycin (100 μg/ml).

Peripheral blood monocytes were isolated from fresh PBMC by adherence to plastic at 37°. Following overnight culture, the adherent cells were removed from the flask by gentle scraping and residual T-lymphocytes were further depleted using Pan-T (anti-CD2) immunomagnetic beads according to the manufacturer's instructions (Dynal Inc.). This purified monocyte population was typically 91% $CD14^+$ as assayed by flow cytometry. The cells were cultured in 24-well plates at a density of 2×10^6 cells/well in medium without added growth factors.

Mature macrophages were derived by culturing the purified monocytes 7 to 14 days in RPMI/10% FCS without additional cytokines. At this time, >95% of the cells had become enlarged or spindle shaped with extended processes. The cells were removed from flasks by gentle scraping and cultured at a density of 5×10^5 cells/well in 24-well plates.

Infectivity assays

For infections with replication competent virus, cells in 24-well dishes were inoculated with 10 ng $p24^{gag}$ of virus obtained by transfection of COS, HeLa, or 293 cells. This amount of virus was equivalent to $5–7 \times 10^3$ TCID (determined by limiting dilution analysis of the virus stocks on activated PBMC) and corresponded to an m.o.i. of about 0.04. Following overnight incubation at 37°, the cells were washed twice and resuspended in 1.5 ml of either RPMI/10% FCS (mononuclear phagocytes, resting T-lymphocytes, Jurkat cells) or medium supplemented with 10 U/ml IL-2 (stimulated PBMC and stimulated T-lymphocytes). Samples of the culture supernatants were taken immediately after washing (Day 0) and on designated days thereafter and assayed for the presence of HIV-1 $p24^{gag}$ antigen by ELISA.

For single cycle infections with NL-Luc viruses, cells were infected as described above except that the medium contained 4.0 μg/ml polybrene. At designated time points, the cells were lysed in 200 μl lysis buffer (Promega) and stored at −70°. The amount of luciferase activity in 20 μl of each lysate was determined using commercially available reagents (Promega) in a Lumat LB 9501 luminometer.

PCR analysis of HIV-1 reverse transcripts

Virus supernatants, free of contaminating plasmid DNA, were prepared by infecting PHA-stimulated PBMC with virus produced from transfected COS cells. At the peak of virus production (Day 9), supernatants were harvested, filtered (0.45-μm pore size), and treated with DNase (25 U/ml) for 30 min at 37°. The virus was stored in aliquots at −70° and quantitated by $p24^{gag}$ ELISA. Monocytes and PHA-stimulated PBMC were inoculated with HXB (BAL)-R^+ or HXB (BAL)-R^- (5 ng of $p24^{gag}$) in a volume of 1.0 ml. The cells were harvested 10, 24, and 36 hr after infection and washed twice with ice-cold PBS, and DNA was isolated by standard methods. HIV-1 sequences were amplified using primers M-667 (GGCTAACTAGGGAACCCACTG) and M-661 (CCTGCGTCGAGAGAGCTCTGG) which hybridize within the long-terminal repeat (LTR)/gag region of the HIV-1 genome (Pang *et al.*, 1990). These primers span the primer-binding site, and therefore measure only complete or nearly complete HIV-1 DNA (Zack *et al.*, 1990). Primer M667 was end-labeled with [γ-^{32}P]ATP to a specific activity of 1×10^7 ng/μg. HIV-1 sequences were amplified by PCR for two cycles (94°, 2 min; 63°, 30 sec; 72°, 2 min) and then for an additional 30 cycles (94°, 1 min; 63°, 30 sec; 72°, 1 min). Parallel PCR reactions were run with primers PC03 and PC04 to the human β-globin gene (Saiki *et al.*, 1985). PCR products were then analyzed on 8% native polyacrylamide gels.

RESULTS

Replication of HIV-1 *vpr*$^+$ and *vpr*$^-$ macrophage-tropic viruses in primary cells

To study the role of *vpr* in HIV-1 replication, we constructed a pair of isogenic proviruses that differed only in their ability to encode a full-length *vpr* product. HXB2 *vpr* has an inserted T nucleotide, not present in other HIV-1 strains, which results in a frameshift at amino acid 72. This protein is apparently unstable; it cannot be found in cells that are producing large quantities of HXB2 particles, nor is it present at detectable levels in virions (Paxton *et al.*, 1993; Yuan *et al.*, 1990). This finding is not likely to be due to an inability of the frameshifted molecule to be recognized by anti-Vpr antibodies since both rabbit polyclonal anti-Vpr serum and a monoclonal antibody against an epitope tagged form of the frameshifted Vpr were unable to detect this molecule (Paxton *et al.*, 1993). To correct the defective HXB2 *vpr*, we deleted the additional T nucleotide. This restores *vpr* to the full-length, 96-amino acid open reading frame. In initial studies we

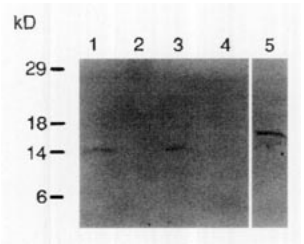

FIG. 1. Immunoblot analysis of HXB(BAL)R$^+$, HXB(BAL)R$^-$, and NL4-3-R$^-$ Vpr-complemented virions. COS cells were transfected with pNL4-3 (lane 1), pNL4-3-R$^-$ (lane 2), pHXB(BAL)R$^+$ (lane 3), or pHXB(BAL)R$^-$ (lane 4) or were cotransfected with pNL4-3-R$^-$ and pcDNA-tag-Vpr (lane 5). Virions in the cell culture supernatants were pelleted 72 hr post-transfection and equivalent amounts of virions (100 U reverse transcriptase) were analyzed on an immunoblot probed with rabbit anti-recombinant-Vpr serum and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. Vpr runs at 14 kDa while the epitope-tagged molecule is at 18 kDa. Molecular size markers are indicated at the left.

were unable to show any significant difference between the *vpr*$^+$ and *vpr*$^-$ viruses derived from these infectious proviruses. The viruses showed similar replication kinetics in CD4$^+$ T cell lines and a low but equivalent ability to replicate in activated PBMC (data not shown). We therefore further modified the proviruses to expand their tropism by exchanging the *env* gene with that of the macrophage-tropic virus, HIV-BAL (pHXB(BAL)-R$^+$ and pHXB(BAL)-R$^-$). The viruses produced by transfecting COS cells with these plasmids replicated in PHA-activated PBMC to titers approximately 10-fold higher than the parental viruses (data not shown). These viruses were used in all subsequent studies.

To verify that the viruses produced the appropriate *vpr* product, equivalent amounts of virions pelleted from the transfected COS cell culture supernatants were analyzed on immunoblots probed with anti-Vpr serum. This analysis showed that HIV(BAL)-R$^+$ virions contained Vpr molecules whose mobility on SDS–PAGE and copy number was similar to that of the NL4-3 strain of HIV-1, while HIV(BAL)-R$^-$ particles did not contain a detectable *vpr* product (Fig. 1; lanes 1–4).

To assess the influence of *vpr* on HIV-1 replication in primary cells, populations of PBMC and purified monocytes were prepared from uninfected normal donors. The cells were infected 24 hr after isolation with virus produced by either transfected COS or HeLa cells (10 ng p24^{gag}). The results showed that the level of replication of *vpr*$^+$ and *vpr*$^-$ viruses varied depending on which cell type was infected, but was not influenced by the producer cell-type (Fig. 2). Monocytes infected with *vpr*$^+$ virus produced 10- to 30-fold higher levels of p24^{gag} in culture supernatants at the peak of virus replication compared with those infected with *vpr*$^-$ virus. In contrast, the viruses replicated with similar efficiencies in PHA-stimulated PBMC and in unstimulated PBMC. These results are not likely to be due to differences in the amount of input virus since the inoculum was judged to be similar by both p24^{gag} assay and infectious titer (5×10^3 and 7×10^3 TCID input, HIV(BAL)-R$^-$ and HIV(BAL)-R$^+$, respectively). In addition, it is not the case that HIV(BAL)-R$^-$ failed to establish an infection in the monocyte culture, since a small but significant amount of viral replication was apparent (Fig. 2). Thus, in the absence of a functional *vpr*, HIV-1 replicated to low levels in monocytes. This is in contrast to our findings in activated PBMC where both *vpr*$^+$ and *vpr*$^-$ viruses replicated to high levels. Therefore,

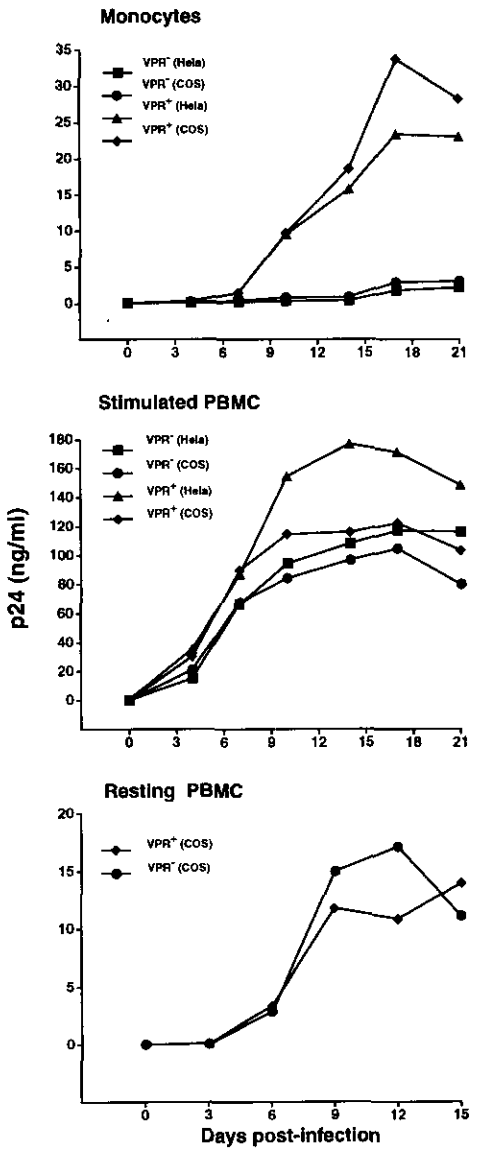

FIG. 2. Replication kinetics of *vpr*$^+$ and *vpr*$^-$ macrophage-tropic viruses in monocytes and PHA-stimulated or unstimulated PBMC. Virus stocks were prepared by transfecting COS or HeLa cells with pHXB(BAL)-R$^+$ or pHXB(BAL)-R$^-$ (20 μg). Cells were infected with virus (10 ng p24^{gag}, corresponding to an m.o.i. of about 0.04), washed, and incubated for 21 days. Supernatants were sampled on the days indicated and the amount of virus present was measured using a p24^{gag} ELISA. Similar results were observed with cells from three different donors.

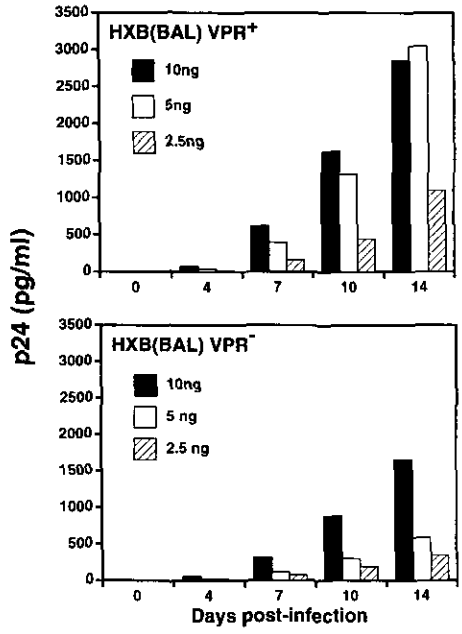

FIG. 3. Replication kinetics of vpr^+ and vpr^- macrophage-tropic viruses in differentiated macrophages. Macrophages were prepared by allowing freshly isolated monocytes to differentiate in culture for 10 days. The cells were then infected with different amounts of HXB(BAL)-R^+ or HXB(BAL)-R^- (2.5, 5.0, or 10 ng $p24^{gag}$) produced by transfected COS cells. The amount of virus present in culture supernatants was determined by $p24^{gag}$ ELISA.

vpr appears to play an important role in facilitating virus replication in peripheral blood monocytes.

In the absence of added cytokines, monocytes spontaneously differentiate into cells resembling mature macrophages after a period of 7 to 10 days in culture (Davies and Lloyd, 1989; Johnson *et al.*, 1977). The cells enlarge, and express increased levels of macrophage surface proteins such as Fc receptor type III, and increased amounts of enzymes such as acid phosphatase, lysozyme, and 5′-nucleotidase (Johnson *et al.*, 1977). To test whether *vpr* plays a role in infection of these more differentiated cells, we infected cultured macrophages. Monocytes were isolated from fresh PBMC, allowed to differentiate for 10 days, and then infected with three different amounts of vpr^+ and vpr^- virus (2.5, 5.0, and 10 ng $p24^{gag}$; Fig. 3). The results showed that in each case the vpr^+ virus replicated to higher levels (2- to 5-fold) than the vpr^- virus at the corresponding inoculum. This result could not be ascribed to small differences in input virus, since at each of the input doses, vpr^+ virus replicated better than vpr^- virus. The difference in ability of the two viruses to replicate in the cultured macrophages appeared to be smaller than that for the fresh cells in which 30-fold differences were observed (Fig. 2). This result suggests *vpr* plays a role in infection of macrophages, but is not required for infectivity of these cells.

PCR analysis of reverse transcription

To determine whether the block to infection of the vpr^- virus was at or prior to reverse transcription, we measured the amount of viral DNA synthesized in monocytes shortly after infection. To do this, we infected monocytes or PHA-stimulated PBMC with equivalent amounts of HXB(BAL)-R^+ or HXB(BAL)-R^-. At designated times after infection, total cellular DNA was extracted and analyzed by PCR using primers that detected complete or nearly complete reverse-transcribed HIV-1 DNA (M667 and M661; Zack *et al.*, 1990). After 10 hr, only low levels of HIV-1 reverse transcription products were detected in monocytes and PBMC infected with vpr^+ or vpr^- virus (Fig. 4). After 24 hr, the amount of HIV-1 DNA increased in the PBMC, and to a lesser extent in monocytes. This finding is consistent with the slower rate of reverse transcription in mononuclear phagocytes that has been previously reported (Collin and Gordon, 1994). At each time point, roughly equivalent amounts of viral DNA were detected in cells infected with vpr^+ and vpr^- virus. In the presence of 20 μM AZT, only low levels of HIV-1 DNA were detected, confirming that the analysis measured newly synthesized molecules and not contaminating input viral DNA. Thus, the block to infection of the vpr^- virus does not appear to be at the level of completion of reverse transcription, but at a later step in the virus life cycle.

The effect of *vpr* on HIV-1 infection in cell lines and in primary cells as detected with an HIV-1 luciferase reporter vector

To further examine the effect of *vpr* on viral replication, we used an HIV vector similar to that reported by Chen *et al.* (1994) in which a portion of *nef* has been replaced by the gene for firefly luciferase (Fig. 5). This vector was useful for our studies because the high sensitivity with which luciferase can be detected permits quantitation of infection in a single cycle of viral replication in primary cells, with or without mitogenic stimulation. The amount of luciferase activity in cells infected with this vector re-

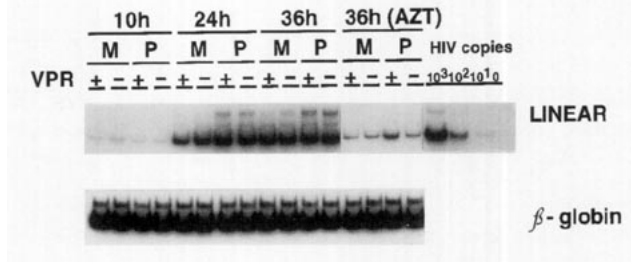

FIG. 4. PCR analysis of reverse transcripts produced by monocytes and activated PBMC shortly after infection with macrophage-tropic vpr^+ or vpr^- virus. Monocytes (M) and PHA-stimulated PBMC (P) were infected with plasmid DNA-free HXB(BAL)-R^+ and HXB(BAL)-R^- virus stocks (10 ng $p24^{gag}$), washed, and harvested at the indicated times postinfection. DNA (1 μg) was amplified with ^{32}P-end-labeled primers to detect completed reverse transcripts. Linearity of the PCR amplification was determined using serially diluted DNA from 8E5 cells that contain a single integrated provirus per cell (Gendelman *et al.*, 1987).

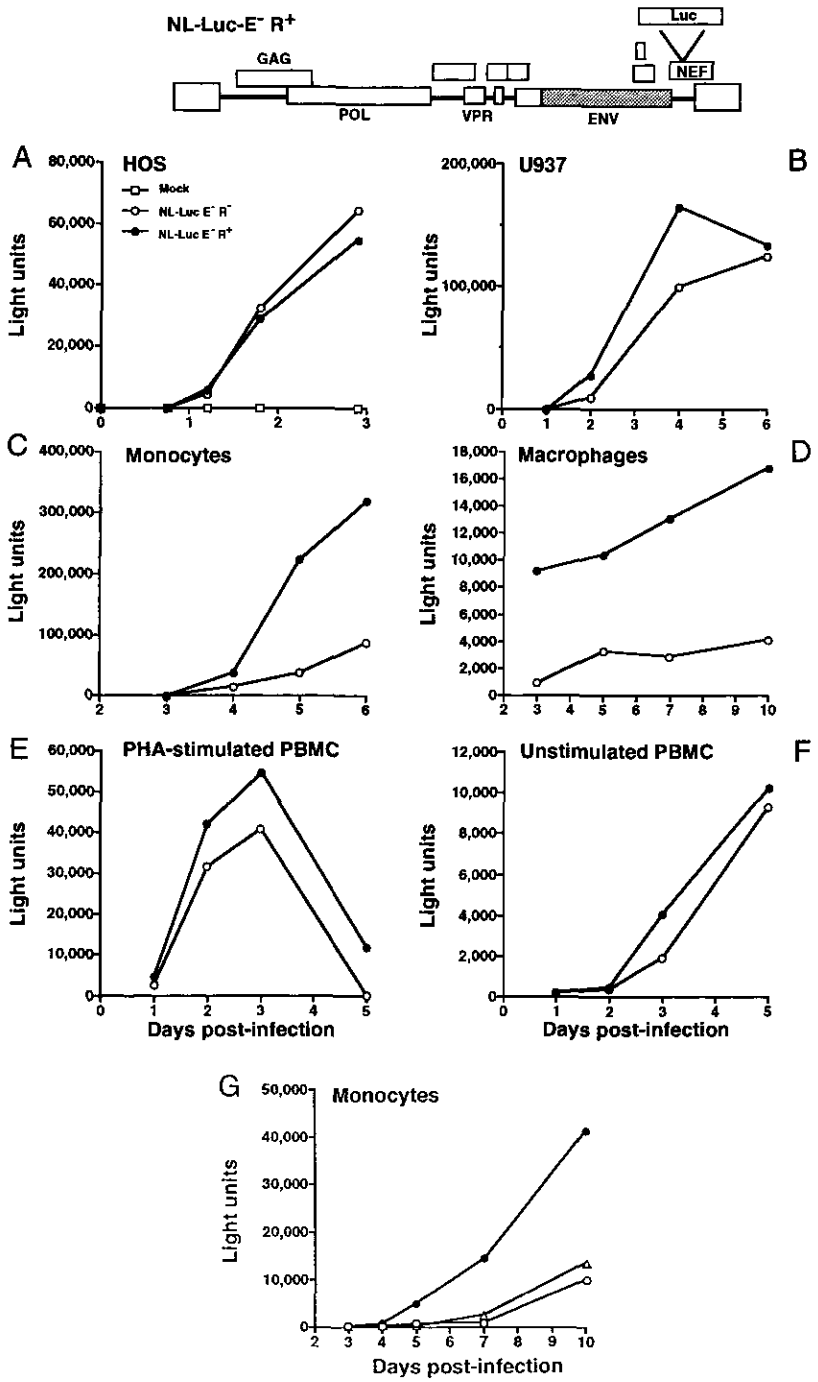

FIG. 5. Infection of cell lines and primary cells with vpr^+ and vpr^- luciferase reporter viruses. Structure of the luciferase reporter vector, pNL-Luc-E^-R^+, is shown at the top. pNL-Luc-E^-R^- differs by the insertion of four nucleotides in *vpr* at amino acid 26. Shading of *env* indicates the region frameshifted due to insertion of two nucleotides at codon 61. HOS cells (A), U937 (B), monocytes (C), macrophages (D), PHA-stimulated PBMC (E), and unstimulated PBMC (F) were infected with (10 ng $p24^{gag}$) amphotropic NL-Luc-E^-R^+ (●), NL-Luc-E^-R^- (○), or mock virus (□). Macrophages were cultured for 14 days prior to infection. For complementation *in trans* (G), viruses were produced by cotransfecting 293 cells with pNL-Luc-E^-R^+, pSV-A-MLV-env, and pc-DNA (●); pNL-Luc-E^-R^-, pSV-A-MLV-env, and pc-DNA (○); pNL-Luc-E^-R^-, pSV-A-MLV-env, and pcDNA-Vpr (△) at a ratio of 2.5:7.5:15. Cell cultures were harvested at the indicated days postinfection and luciferase activity was determined as described under Materials and Methods. Similar results were obtained with cells from at least four other donors with three independent preparations of viruses.

flects both the number of integrated proviruses and their transcriptional activity. We constructed vectors containing either a full-length (pNL-Luc-E^-R^+) or frameshifted *vpr* (pNL-Luc-E^-R^-). To ensure that the analysis would examine only the early events in the viral life cycle, we introduced a frameshift into *env* of both vectors, thus

blocking subsequent rounds of viral replication. To produce infectious virus, 293 cells were cotransfected with luciferase reporter vector DNA and, to complement the defect in *env*, an expression vector for murine leukemia virus amphotropic envelope glycoprotein, pSV-A-MLV-env (Chen *et al.*, 1994; Landau *et al.*, 1991). The resulting HIV(MLV) pseudotypes allowed infection of cells irrespective of the amount of cell surface CD4 and, in addition, consistently resulted in higher luciferase activity than virus produced by cotransfection with an HIV envelope glycoprotein expression vector (data not shown).

To test the relative infectivity of the amphotropic vpr^+ and vpr^- reporter viruses, we infected cell lines and primary cell populations with equivalent amounts of virus and measured the amount of luciferase produced at designated times postinfection. Infection of a human osteosarcoma cell line, HOS, which is unrelated to the natural targets of HIV infection (Fig. 5A), showed no significant difference in infectivity between the vpr^+ and vpr^- viruses. This suggested that *vpr* plays no role in infection of these cells (at least up to the point of expression of the integrated provirus) and also confirms that the two virus preparations were equally infectious. Infection of the human leukemic promonocytic cell line U937 with the reporter viruses (Fig. 5B), similarly, showed only small differences in luciferase activity (less than twofold in this experiment and in subsequent repetitions). Both viruses infected stimulated and unstimulated cells to a similar extent, although, as expected, the unstimulated cells produced only small amounts of luciferase (Figs. 5E and 5F).

In contrast, in monocytes, levels of luciferase activity differed significantly (five- to eightfold higher for vpr^+ virus) by Days 5 and 6 (Fig. 5C). Such differences in luciferase activity occurred reproducibly with six different donor cell populations, although for two of these the differences between vpr^+ and vpr^- were smaller than those shown here (three to fourfold higher for vpr^+ virus). The difference in luciferase activity between vpr^+ and vpr^- virus tended to decrease from Days 6 to 10, with the vpr^- virus-infected cells starting to produce increased amounts of luciferase (Figs. 5C and 5G). This could indicate that as the mononuclear phagocytes differentiated over the course of the experiment, *vpr* became less important for expression of the reporter virus luciferase gene.

Fully differentiated macrophages infected with vpr^+ reporter virus showed significantly higher levels of luciferase compared to those infected with vpr^- virus (Fig. 5D). This approximately 4-fold difference was similar to the difference in infectivity found with the replication-competent HIV(BAL) viruses in macrophages (Fig. 3). The absolute amount of luciferase activity is about 10-fold lower than that of the immature cells. This is presumably related both to the smaller number of cells present following prolonged culture in the absence of cytokines (about 25% of the original number) and to their reduced metabolic activity following differentiation (Zuckerman *et al.*, 1979).

Overall, expression of the reporter virus luciferase gene reflects only the steps of viral replication up to the point of proviral transcription. Therefore, the block to HIV replication in mononuclear phagocytes that occurs in vpr^- virus is at, or prior to, transcription of the integrated provirus.

The presence of Vpr in viral particles is not sufficient for maximum reporter virus expression in monocytes

Because Vpr is packaged in large amounts into viral particles, we were interested to determine whether its presence in the virion would complement in trans the vpr^- phenotype in mononuclear phagocytes. To test this, we prepared luciferase reporter virus in 293 cells that had been cotransfected with pNL-Luc-E^-R^-, pSV-A-MLV-env, and the Vpr expression vector, pcDNA-Vpr. Immunoblots on virions generated by this method show that they contain Vpr at levels equivalent to, or greater than, those found in wild-type, NL4-3 virions (Fig. 1, lanes 1 and 5). When cells are infected by the complemented virus, Vpr is initially present; however, once the provirus integrates into the target cell chromosome, new Vpr cannot be synthesized. Thus, this virus allowed us to determine whether the effect of *vpr* on luciferase expression in mononuclear phagocytes was due to Vpr molecules contained in the virions or those expressed from the provirus in the newly infected cells.

Representative results of one such experiment (Fig. 5G) showed that cells infected with the complemented virus produced amounts of luciferase activity similar to those of the vpr^- virus. This lack of complementation suggests that the effect of *vpr* in mononuclear phagocytes is due to Vpr molecules expressed by the provirus, and not those contained in the virions. This does not exclude a role for *vpr* in nuclear transport (Heinzinger *et al.*, 1994), but may indicate a second function for this gene.

DISCUSSION

The results presented here show that HIV-1*vpr* is important for virus replication in monocytes and in differentiated macrophages, but does not appear to play a role in infection of activated or unstimulated T-cells or cell lines. These conclusions were derived from our finding that *vpr*-deficient, macrophage-tropic HIV-1 replicated efficiently in $CD4^+$ cell lines, in primary T-lymphocytes, and PHA-activated PBMC, but was impaired in freshly isolated peripheral blood monocytes and cultured macrophages. PCR analysis of the reverse transcription products at times shortly after infection showed no significant differences in the levels of full-length HIV-1 DNA in infected monocytes. In a single-cycle replication assay using an HIV vector containing a luciferase reporter gene,

cells infected with *vpr*$^+$ virus expressed significantly higher levels of the reporter gene than those of the isogenic *vpr*$^-$ virus. This could result from infection of more cells, increased expression of the provirus in each cell, or the combination of both effects. Furthermore, providing Vpr *in trans* in the transfected producer cells failed to complement the *vpr*$^-$ phenotype in mononuclear phagocytes. These results suggest that *vpr* has a function that is mediated by Vpr molecules newly synthesized from the integrated provirus. In addition, *vpr* function did not appear to be mediated through the HIV envelope glycoprotein, gp160, since the luciferase reporter virus pseudotyped with the amphotropic MLV envelope glycoprotein was still influenced by the *vpr* genotype of the reporter vector.

An earlier report suggested that *vpr* of HIV-2 facilitated viral replication in macrophages (Hattori *et al.,* 1990); however, the mutation used in that study also affected the *vpx* open reading frame, whose termination codon overlaps the *vpr* initiation codon. Therefore, this phenotype may have been caused by the alteration of *vpx* or of both gene products. A second study in which *vpr* in conjunction with *vpu* was shown to affect the ability of HIV-1 to replicate in mononuclear phagocytes (Westervelt *et al.,* 1992) is consistent with our findings.

In our studies, as well as those previously reported by others (Kazazi *et al.,* 1989; Valentin *et al.,* 1991), fresh monocytes supported higher levels of viral replication than differentiated macrophages. In other reports, however, different results were obtained. Rich *et al.* (1992) found that the macrophage-tropic strain HIV-$1_{JR\text{-}FL}$ infected fresh mononuclear phagocytes with very low efficiency, but that the infectability of the cells increased considerably following differentiation in culture for 7 to 10 days. The reasons for these differences are not clear but could be due to differences in culture conditions, methods for purifying monocytes, or viral strain differences. These differences do not affect the conclusions of our experiments, since we compared the relative infectivity of *vpr*$^+$ and *vpr*$^-$ virus within a given cell population.

Presumably, the Vpr molecules that are packaged in the virion play a role early in the viral life cycle (entry, uncoating, reverse transcription, nuclear transport, or proviral integration). A role in nuclear transport is particularly attractive given that Vpr is strongly localized to the nucleus (Lu *et al.,* 1993). We were therefore surprised that the *vpr*$^-$ phenotype was not complemented by supplying Vpr in trans in the producer cells. If nuclear transport were the only function of *vpr*, this virus would have resulted in luciferase activity equivalent to that of the *vpr*$^+$ virus. This lack of complementation was probably not simply caused by an insufficient amount of Vpr in the virions, since the cotransfection protocol resulted in virions containing Vpr in quantities that were equivalent to, or higher than, wild-type levels. In addition, by PCR analysis with appropriate primers we observed only a small (twofold) decrease in the amount of circular viral DNA molecules formed in monocytes infected with the *vpr*$^-$ virus (unpublished observations). The effects of *vpr* in this assay are therefore likely to be due to Vpr molecules encoded by the newly integrated provirus.

It is possible that *vpr* has a dual function, one involving nuclear transport (Heinzinger *et al.,* 1994), and a second that acts after integration of the provirus. The relative contribution of the two effects might vary in different cell types and could be influenced by the differentiation state of the cells. The possibility that a single HIV gene product could play two different roles is not unprecedented. For example, *vpu* has been shown to have two separable roles in viral replication (Schubert and Strebel, 1994).

Subsequent to integration, *vpr* might act to increase viral production by affecting the levels of various cellular transcription factors. A role for *vpr* in transcription is consistent with the findings of Levy *et al.,* who suggested that *vpr* induces the differentiation of immature muscle cells (Levy *et al.,* 1993). In an analogous fashion, *vpr* may transcriptionally activate cellular genes in mononuclear phagocytes, thereby activating transcription of the provirus. However, the function of *vpr* is not solely to induce macrophage differentiation, since, as we showed in this report, *vpr* functions in fully differentiated cells. Increased HIV-1 transcription upon activation of mononuclear phagocytes has been previously reported (Griffin *et al.,* 1989; Moses *et al.,* 1994). In unstimulated mononuclear phagocytes, the basal level of transcription of the HIV-1 LTR is limited, apparently due to low levels of transcription factors that interact with the HIV LTR (Moses *et al.,* 1994). Upon stimulation, transcriptional activity of the LTR is elevated due to an increase in the levels of several transcription factors and, perhaps, due to a decrease in negative regulators of transcription. A role for *vpr* in transcription was also suggested by Cohen *et al.* (1990b) who showed that Vpr has a modest ability to transactivate several promoters.

The ability of HIV to infect monocyte/macrophages has been shown *in vitro* and *in vivo* (Ho and Hirsch, 1986), where HIV-infected cells of the monocyte-macrophage lineage in the brain, skin, lungs, and lymph nodes are believed to play an important role in HIV pathogenesis (Gartner *et al.,* 1986; Gendelman *et al.,* 1989). Because infection of these long-lived cells is not cytopathic, they may serve as an important source of virus production *in vivo.* Infected mononuclear phagocytes harbor HIV-1 in the brain and are implicated in the development of neurological dysfunction (Ho *et al.,* 1987; Koenig *et al.,* 1986). In addition, macrophage tropism appears to be important for transmission of HIV-1, since it is characteristic of the viruses present in newly infected individuals (Zhu *et al.,* 1993). It is therefore likely that *vpr*, by increasing the efficiency with which macrophages become productively

infected, plays an important role in the pathogenicity of HIV-1.

ACKNOWLEDGMENTS

We thank Yao-Xing Huang and Emily Tsai for technical assistance, Wendy Chen for graphics and David Baltimore, Paola DiMarzio, David Ho, and Dan Littman for critical reading of the manuscript. This work was supported by the Aaron Diamond Foundation and Grant AI27742 to N.R.L. from the Center for AIDS Research at N.Y.U. Medical Center. B.K.C. was supported by a Medical Scientist Training Program grant and NIAID Grant AI22346.

Note added in proof. Since submission of this manuscript, Balliet *et al.* (1994) similarly reported that *vpr* increases HIV replication in monocytes.

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