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Fate of the Human Immunodeficiency Virus Type 1 Provirus in Infected Cells: a role for *vpr*

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We investigated the fate of human immunodeficiency virus type 1 (HIV-1) viral DNA in infected peripheral blood lymphocytes and immortalized T-cell lines by using a replication-defective HIV-1. We observed that integrated HIV-1 DNA and viral gene expression decrease over time. A frameshift mutation in *vpr* resulted in maintenance of the HIV-1 provirus and stable persistence of viral expression. Transfection of *vpr* together with the neomycin resistance gene in the absence of other viral genes decreased the formation of geneticin-resistant colonies, indicating either a cytotoxic or a cytostatic effect upon cells. Therefore, maintenance of HIV-1 infection within an infected proliferating population is due to two competing processes, the rate of viral spread and the degree of cell growth inhibition and/or death induced by Vpr.

Infection with human immunodeficiency virus type 1 (HIV-1) is characterized by a progressive decline of $CD4^+$ lymphocytes, ultimately leading to an inability of the body to mount an effective immune response (11, 36). The means by which HIV-1 leads to $CD4^+$ cell depletion is unknown. One of the key approaches to understanding HIV-1 pathogenesis is elucidation of interactions between specific HIV-1 gene products and the host cell, which contribute to replication and persistence of HIV-1 in the host and result in deleterious effects on $CD4^+$ lymphocytes. The HIV-1 genome has a number of auxiliary genes that are not present in other less complex retroviruses, and these play a role in regulating viral replication and persistence in the host (12, 13). The function of some of these genes has been described; however, others remain poorly characterized. To investigate early events in a single-step infection process, we constructed a defective HIV-1 [HIV-$1_{JR\text{-}CSF}$env(−)] by deleting a restriction fragment of the *env* gene between two *Afl*III restriction enzyme sites located at nucleotides 6514 and 7486 of the HIV-$1_{JR\text{-}CSF}$ genome (20). In this manner, defective virions are capable only of a single round of infection; therefore, events following infection can be studied in the absence of reinfection. In addition, viruses that are deficient in *env* do not induce fusion between infected and uninfected cells. Thus, the fate of infected cells can be studied in the absence of early cell death because of syncytium formation.

Production of infectious virions was accomplished by cotransfection by the electroporation method (3) of HIV-$1_{JR\text{-}CSF}$ env(−) with the expression plasmid pJD1 (9), which encodes the amphotropic murine leukemia virus *env* gene to generate the pseudotype HIV-$1_{JR\text{-}CSF}$env(−)/ampho-env. We examined the fate of HIV-1 viral DNA and viral gene expression in peripheral blood mononuclear cells (PBMC) infected with HIV-$1_{JR\text{-}CSF}$env(−)/ampho-env (Fig. 1). Unexpectedly, the viral DNA resulting from infection with a defective HIV-1 was not maintained in the infected cell population (Fig. 1A). Concomitant with a decrease in viral DNA, a decrease in p24 production from infected cells was also observed (Fig. 1B). The viral DNA level decreased from 950 copies per μg of cell DNA (cell equivalent, 1.2×10^5) at day 3 to 20 copies at day 7 postinfection. Similar results were observed with pseudotypes with the HIV-$1_{JR\text{-}CSF}$ and HIV-1_{LAI} envelope (data not shown), indicating that the failure of HIV-1 to persist is not related to the host range of the virus. These results were unexpected because infection with retroviruses generally leads to formation of integrated proviruses which are stably maintained through cell generations.

We investigated the expression of HIV-1 in this system by engineering HIV-1-defective viruses to express a reporter gene, luciferase (*luc*) (8), in place of *nef* in the HIV-$1_{JR\text{-}CSF}$ (20) and HIV-$1_{NL4\text{-}3}$ (1) molecular clones. Infection of PBMC and the T-cell lines SupT1 and MT-2 with pseudotype viruses HIV-$1_{JR\text{-}CSF\text{-}luc}$env(−)/ampho-env (4) and HIV-$1_{NL4\text{-}3\text{-}luc}$env(−)/ampho-env, bearing *luc*, showed high levels of luciferase activity which then decreased over time (Fig. 1C). DNA levels of HIV-$1_{NL4\text{-}3\text{-}luc}$env(−) also decreased over time (data not shown), as described above for HIV-$1_{JR\text{-}CSF}$env(−)/ampho-env. Thus, expression of HIV-1, as measured by luciferase activity, was also not maintained over time.

Two explanations could be given for the loss of the HIV-1 DNA and viral gene expression in infected cells. Although contrary to most reports of the role of unintegrated viral DNA, it is possible that the unintegrated DNA intermediate is expressed but does not efficiently integrate into the host cell chromosome and is gradually diluted because it is unable to replicate in cells that are dividing. The other possibility is that integration occurs efficiently but that the observed loss in viral DNA occurs subsequent to integration, because of growth inhibition or the death of infected cells.

We tested the former possibility directly by quantitating the relative amounts of viral integrated and unintegrated DNA in a single-step infection (Table 1). Phytohemagglutinin (PHA)-stimulated peripheral blood cells (5×10^6) were infected with 5 ml of HIV-$1_{NL4\text{-}3\text{-}luc}$env(−)/ampho-env, containing 160 ng of

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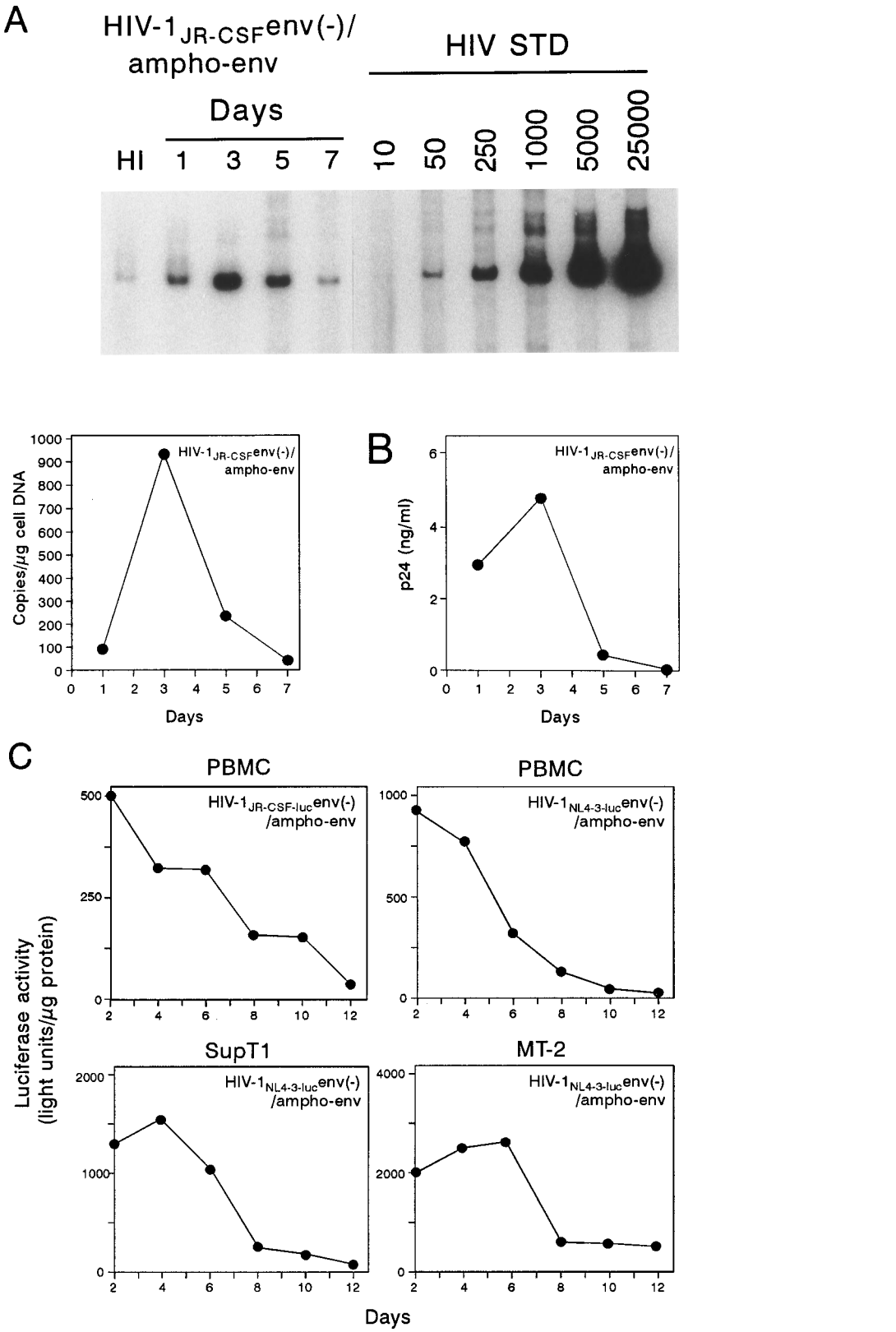


FIG. 1. Kinetic analysis of viral DNA, p24 production, and luciferase activity following infection with a defective HIV-1. Infectious virions were obtained by cotransfection of HIV-$1_{JR\text{-}CSF}$env(−) and HIV-1ampho-env. Transfection supernatants (5 ml) containing 80 ng of p24 per ml were digested with DNase I (45) and mixed with 10 μg of Polybrene per ml, and the mixtures were incubated with 5×10^6 PHA-stimulated PBMC for 1 h at 37°C. Cells were washed and resuspended in culture medium. (A) Infected cells harvested at various time points, lysed, and analyzed for viral DNA by quantitative PCR with primers and under conditions which were described previously (45). The HIV DNA standards (STD) used were derived from dilutions of the plasmid pYKJRCSF, containing the indicated number of copies, which had been linearized with *Eco*RI. All samples were previously adjusted to cell number by quantitative PCR for β-globin, and 1 μg of cellular DNA (cell

TABLE 1. Quantitation of integrated and unintegrated viral DNA in cells infected with a defective HIV-1

Expt[a]	Days postinfection	Amt of viral DNA species	
		Integrated[b]	Unintegrated[c]
Uninfected PBMC	NA[d]	<10	<10
HIV-$1_{NL4-3-luc}$env(−)/ampho-env	1	4.3×10^3	8.8×10^2
HIV-$1_{NL4-3-luc}$env(−)/ampho-env	3	6.0×10^3	1.3×10^2
HIV-1_{NL4-3}D116G/ampho-env	1	<10	67
ACH2	NA	9.6×10^4	2.8×10^2

[a] HIV-1_{NL4-3}D116G env(−) is an integrase-defective vector derived from HIV-$1_{NL4-3-luc}$env(−) by mutagenesis of codon 116 (aspartic acid) of the integrase gene to glycine. ACH2 is a cell line chronically infected with HIV-1 (5).

[b] Integrated viral DNA was normalized to 1 μg of β-globin DNA (cell equivalent = 1.2×10^5).

[c] Recovery of low-molecular-weight DNA was monitored by measuring recovery of 500 copies of a linearized 10-kb plasmid containing human T-cell leukemia virus type 2 sequences (14), which was added to samples prior to agarose gel electrophoresis. Recovery was between 10 and 30%. Unintegrated DNA was normalized to 1 μg of cellular DNA.

[d] NA, not applicable.

p24 per ml or were mock infected. One or three days postinfection, the cells were lysed, and total cellular DNA was purified as described previously (31). Purified DNA (5 μg) was subjected to agarose gel electrophoresis. Agarose sections corresponding to low (6 to 13 kb)- and high (>25 kb)-molecular-weight DNA were isolated, and DNA was purified and analyzed for HIV and cellular DNA content by quantitative PCR. HIV PCR was performed as described in the legend to Fig. 1, and PCR specific for β-globin was performed as described previously (45). Quantitations were performed by radioanalytical imaging of gels (Ambis, San Diego, Calif.), and values were obtained by interpolation from standard curves. The levels of integrated viral DNA after infection were 4.3×10^3 copies at day 1 and 6×10^3 copies at day 3 per μg of total cell DNA. The unintegrated viral DNA copy numbers were 8.8×10^2 at day 1 and 1.3×10^2 copies at day 3 per μg of cell DNA. Thus, by day 3 postinfection, unintegrated DNA represents only a minimal fraction of the viral DNA, and the majority of viral DNA is integrated. A parallel infection with an integrase-defective mutant, HIV-1_{NL4-3}D116G/ampho-env (10, 28), produced no detectable integrated DNA and 67 copies of unintegrated viral DNA per μg of cell DNA at day 1.

We examined the second possibility, i.e., that the loss of viral DNA may occur as a postintegration event, by directly quantitating total and integrated viral DNA relative to cell DNA over time. The levels of luciferase activity (Fig. 2A), total viral DNA levels, and high-molecular-weight viral DNA levels (Fig. 2B and C) decreased after day 3. Furthermore, the decrease in high-molecular-weight viral DNA paralleled that of total viral DNA. These results demonstrate that the integrated HIV-1 DNA is not maintained in a population of infected cells.

Retroviruses are not known to be capable of proviral excision. Therefore, a loss in integrated DNA from an infected cell population is likely to represent death or growth inhibition of cells carrying HIV-1 proviruses. Previous studies showed that the HIV-1 *vpr* gene product promoted growth disturbances (25, 35) and differentiation of human rhabdomyosarcoma cells (25). We addressed whether Vpr is involved in preventing viral persistence by introducing a frameshift mutation at codon 64 of the *vpr* open reading frame of HIV-$1_{NL4-3-luc}$env(−), which produced the construct designated HIV-$1_{NL4-3-luc}$env(−)vprX. The previous mutation was performed by digestion of HIV-$1_{NL4-3-luc}$env(−) with *Eco*RI, end filling, and religation. Infections of PBMC and SupT1 cells with HIV-$1_{NL4-3-luc}$env(−)/ampho-env or the *vpr* mutant, HIV-$1_{NL4-3-luc}$env(−)vprX/ampho-env, were performed in parallel, and luciferase activities were measured at various time points (Fig. 3). The levels of luciferase produced by the *vpr*(+) HIV-$1_{NL4-3-luc}$env(−)/ampho-env in PBMC and SupT1 cells decreased over time, as observed earlier. In contrast, the luciferase activities produced by the *vpr*(−) HIV-$1_{NL4-3-luc}$env(−)vprX/ampho-env in PBMC and SupT1 cells remained relatively stable throughout the course of the experiment (Fig. 3). It is noteworthy that HIV-$1_{NL4-3-luc}$env(−)/ampho-env reproducibly generated 5- to 10-fold-higher levels of p24 in the transfection supernatant relative to its *vpr*(−) counterpart when equal amounts of DNA were transfected (7). To exclude the possibility that the initial multiplicity of infection may be responsible for the observed differences in viral persistence, we performed PBMC infections with fivefold serial dilutions of an HIV-$1_{NL4-3-luc}$env(−)/ampho-env virus [*vpr*(+)] stock. For each virus dilution, we observed decreases in luciferase activity over time, even when the initial luciferase activity was lower than or equal to that of the *vpr*(−) virus (Fig. 3). These results indicate that Vpr is involved in the failure of HIV-1 DNA and luciferase expression to persist in infected cells.

A likely explanation for the lack of persistence of the HIV-1 proviral DNA could be the induction of a growth disadvantage or death of infected cells. The above results demonstrate that this phenotype is dependent on the presence of a complete *vpr* open reading frame. Therefore, it is possible that Vpr in the infected cells may induce growth inhibition or cytotoxicity. We tested the potential toxicity of Vpr to cell proliferation by measuring the ability of a *vpr*-expressing plasmid to generate stable transfectants in HeLa cells. The plasmid pvpr/neo^R contains the *vpr* open reading frame downstream of the cytomegalovirus immediate early promoter and the neomycin phosphotransferase gene downstream of the simian virus 40 early promoter (Fig. 4A). A control vector, pvpr$^-$/neo^R, was derived from pvpr/neo^R by deleting the amino-terminal *vpr* coding sequences (Fig. 4A). The frequency of geneticin-resistant colonies obtained with the *vpr*-expressing plasmid pvpr/neo^R was about 30-fold lower than that for the control *vpr*(−) plasmid (Fig. 4B, experiment A). We also tested the ability of *vpr* to inhibit colony formation by cotransfection (Fig. 4B, experiment B). We cotransfected pTK-*neo*, a plasmid in which the neomycin resistance gene is downstream of the herpes simplex thymidine kinase promoter, and a molar excess of pCMV-vpr or control plasmid pCMV-thy-1 (34). The number of resistant colonies was about 280-fold lower in the presence of pCMV-vpr than in the presence of pCMV-thy-1. Taken together with the data from experiments with the HIV-1 *vpr* mutant, these results are consistent with either a cytotoxic or a cytostatic

equivalent = 1.2×10^5 cells) was analyzed. (B) Production of p24 in culture supernatants from infected cells measured by enzyme-linked immunosorbent assaying (Coulter Immunology, Hialeah, Fla.). (C) Infectious virions were obtained by cotransfection of a proviral construct, HIV-$1_{JR-CSF-luc}$env(−) or HIV-$1_{NL4-3-luc}$env(−), as indicated, and HIV-1ampho-env. Transfection supernatants (5 ml) were mixed with 10 μg of Polybrene, and the mixtures were incubated with 5×10^6 PHA-stimulated PBMC, SupT1 cells, or MT-2 cells, as indicated. Transfection supernatants from HIV-$1_{JR-CSF-luc}$env(−) and HIV-$1_{NL4-3-luc}$env(−) contained 58 and 78 ng of p24 per ml, respectively. Infected cells (10^6) were harvested at various time points, lysed, and analyzed for luciferase activities and protein contents. Luciferase activity was normalized to 1 μg of cellular protein.

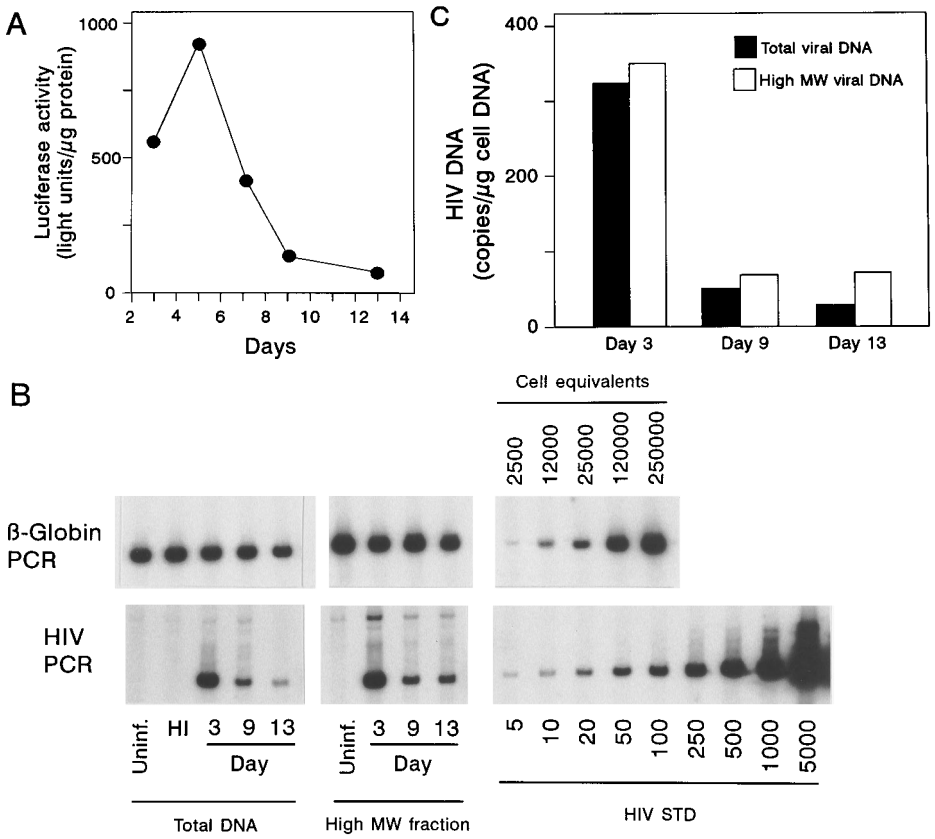


FIG. 2. Analysis of total and integrated viral DNA in infected cells over time. (A) PHA-stimulated PBMC (10^7) were uninfected or infected with 10 ml of HIV-$1_{NL4\text{-}3\text{-}luc}$env(−)/ampho-env, containing 95 ng of p24 per ml and analyzed for luciferase activity at the indicated time points. (B) Total cellular DNA or high-molecular-weight (MW) DNA from selected time points was isolated and quantitated for HIV DNA content and β-globin, as described for Table 1. Total cellular DNA (10 μg) was separated by agarose gel electrophoresis, and 50% of the resulting samples was analyzed by PCR. STD, standard. (C) Results of radioanalytical imaging of PCR gel shown in panel B. HIV copy numbers were normalized to 1 μg of cellular DNA. MW, molecular weight.

effect of the *vpr* gene in transfected and HIV-1-infected cells. This effect of Vpr provides an explanation for the failure of a defective HIV-1 carrying a functional *vpr* gene in persisting in single-step infections.

Our results indicate that establishment of a persistent infection by HIV-1 differs from that of less complex retroviruses. Establishment of infection typically results after integration of the viral DNA. The integrated form of viral DNA, which is known as the provirus, is then stably maintained as the infected cell continues to proliferate, resulting in a chronic infection. Indeed, the provirus was originally defined genetically as that retroviral genetic form which is stably heritable from one cell generation to the next (39). In contrast to this prototypical retroviral infection, the HIV-1 provirus is no longer stably inherited within a population, since integration and expression of *vpr* lead to impairment of cell multiplication or viability. Thus, effective maintenance of a chronic HIV-1 infection would require continuing viral spread to compensate for the loss of integrated viral DNA. The maintenance of HIV-1 infection, therefore, appears to be regulated by two competing processes: the rate at which virions can spread and establish new infections, and the rate at which integrated viral DNA is lost. In support of this idea, recent observations indicate that there is a continuing high level of HIV replication in lymphoid organs, even during the early asymptomatic phase of disease (11, 32).

Our results indicate that the failure of a defective HIV-1 to persist in vitro is a consequence of an inhibitory effect of Vpr on the growth or viability of infected cells. This inhibitory effect of Vpr may be exerted through either a cytotoxic or a cytostatic mechanism. Because the experiments presented here were conducted with proliferating cells, either of the above mechanisms would result in a decrease in the frequency of cells carrying integrated proviruses and inhibiting generation of stable transfectants expressing *vpr*. Thus, our results do not distinguish whether Vpr has a cytotoxic or a cytostatic effect. A recent report by Rogel et al. (35) showed that cells expressing *vpr* have an abnormal cell cycle profile, with a greater proportion of cells in G_2 than normal. However, they could not distinguish between arrest of cells in G_2 versus death of cells in G_1. Recent data (19) indicate that Vpr acts by arresting cells in the G_2/M phase of the cell cycle. The ultimate fate of the arrested cells is not yet known.

The growth-inhibitory effect of Vpr provides an explanation for the observation that molecular clones of HIV-1 and simian immunodeficiency virus (SIV) obtained from in vitro-cultured viruses often encode truncated versions of Vpr (23, 27, 35, 37, 44). It is noteworthy that a study utilizing an HIV-1_{HXB2} pseudotype vector (5) with luciferase as a reporter system, which was similar to our own, demonstrated persistence over time. HIV-1_{HXB2} is known to encode a truncated Vpr (29). Viruses carrying full-length *vpr* genes would have a reduced ability to persist in vitro, and, thus, truncation of *vpr* facilitates maintenance of the provirus and establishment of chronic in-

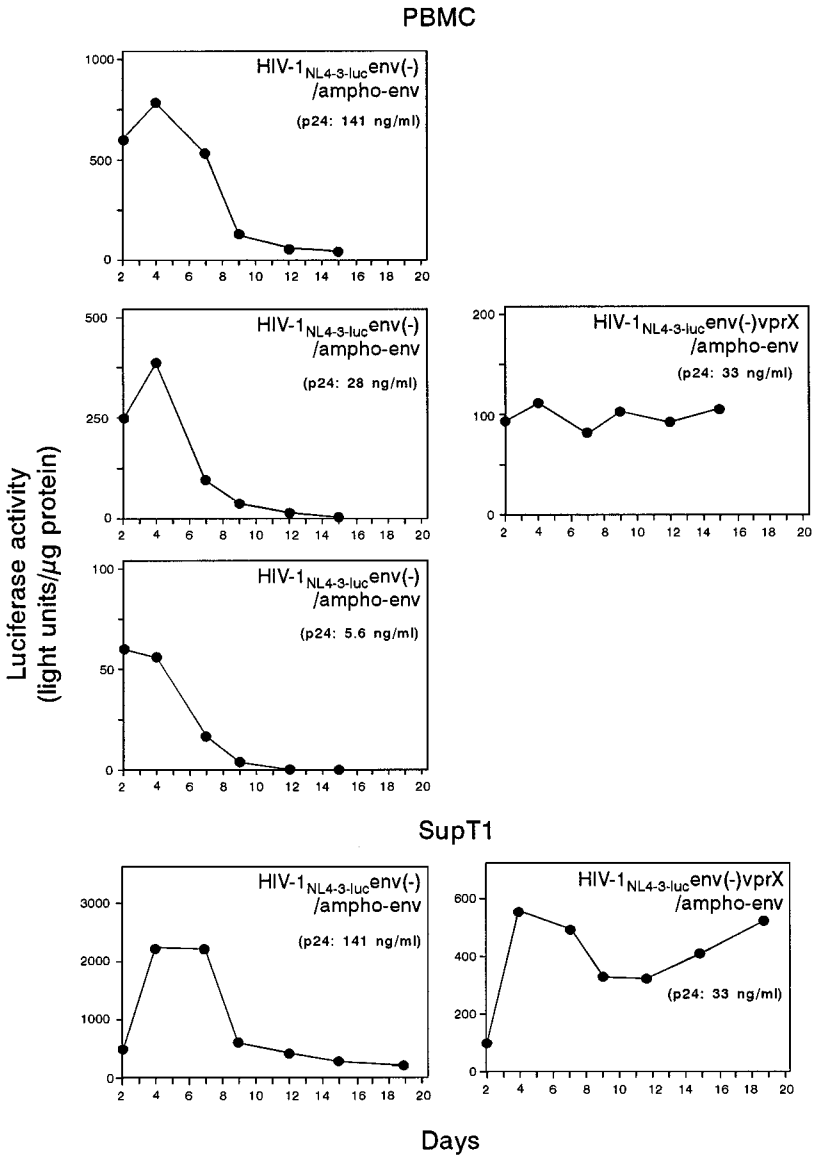


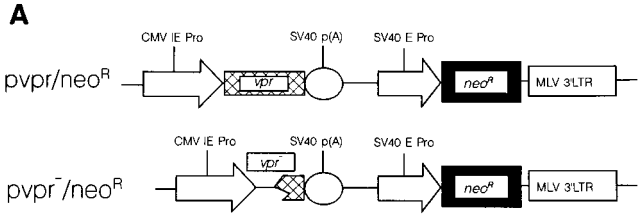
FIG. 3. Infections with viruses bearing full-length or truncated *vpr*. Production of infectious virions was achieved by cotransfection of the appropriate proviral constructs with ampho-env. The transfection supernatant for HIV-$1_{NL4\text{-}3\text{-}luc}$/ampho-env was fivefold diluted. Five milliliters of undiluted or diluted supernatants was added to 5×10^6 PBMC or SupT1 cells. At the indicated time points, 10^6 cells were harvested and analyzed for luciferase. The p24 values in undiluted transfection supernatants were 141 ng/ml in HIV-$1_{NL4\text{-}3\text{-}luc}$env(−)/ampho-env and 33 μg/ml in HIV-$1_{NL4\text{-}3\text{-}luc}$env(−)vprX/ampho-env. PBMC were also infected with 5 ml of 5-fold and 25-fold dilutions of HIV-$1_{NL4\text{-}3\text{-}luc}$env(−)/ampho-env.

fections. Rogel et al. (35) compared the abilities of replication-competent *vpr*(+) and *vpr*(−) viruses to establish chronic infections and showed a lack of viral maintenance in cells infected with *vpr*(+) virus, whereas *vpr*(−) viruses were able to establish chronic infections.

In contrast to these in vitro observations, *vpr* is conserved following replication in vivo (29, 40). Furthermore, introduction of point mutations that inactivate the *vpr* gene of SIV_{mac} resulted in a high rate of reversion in infected macaques (21). The discrepancy between the in vitro and in vivo results might be explained by negative effects of chronic HIV-1 infection on viral persistence in the host that are not yet known. For example, chronically infected cells may be better targets for the immune response. Nucleotide sequence analysis of HIV-1 quasi-species and drug-resistant variants over time indicates rapid turnover of HIV-1 genomes (15, 18, 22, 29, 38, 41, 42), in agreement with inefficient establishment of chronic infections. Alternatively, the selective pressures to maintain *vpr* in vivo may relate to other functions of this gene.

The *vpr* gene has been implicated in other aspects of the HIV-1 life cycle. The *vpr* gene product has been reported to act as a weak transcriptional *trans* activator on the HIV-1 long terminal repeat and to increase the rate of viral replication in vitro (7, 26, 30). In addition, Vpr was shown to be present in virions in equimolar amounts with the *gag* polypeptides (6, 24, 33, 43, 44). The presence of Vpr and the matrix protein in the virions appears to determine nuclear transport of the preintegration complex in nondividing cells (2, 16, 17). This function may play an important role for HIV-1 in establishing infection in nondividing cells such as macrophages. The function that we

A



B

	Plasmids transfected	Resistant colonies
Expt. A	pvpr$^-$/neo^R	6.4 x 10^3
	pvpr/neo^R	2.1 x 10^2
Expt. B	pCMV-thy-1 + pTK-neo	4.5 x 10^3
	pCMV-vpr + pTK-neo	16

FIG. 4. Stable transfection of *vpr* with the selectable marker for neomycin resistance. (A) Schematic representation of dual expression vectors bearing *vpr* and/or the neomycin resistance gene. The construct pvpr$^-$/neo^R was obtained by deleting codons 1 to 63 of the *vpr* open reading frame from the parental construct pvpr/neo^R. (B) Expression vectors pvpr/neo^R (20 μg), pvpr$^-$/neo^R (20 μg), pCMV-thy-1 (constructed by replacing murine *thy-1* cDNA in pCMV-thy-1 with *vpr*, by using the *Xho*I and *Mlu*I restriction enzyme sites [34]) (20 μg), pCMV-vpr (20 μg), and pTK-*neo* (2 μg) were transfected by electroporation in HeLa cells, and the cells were plated in 10-cm dishes. Posttransfection (36 h), 0.75 mg of geneticin per ml was added to the medium. Surviving colonies were counted visually 10 to 15 days posttransfection. Expt, experiment. CMV IE Pro, cytomegalovirus immediate-early promoter; SV40 E Pro, simian virus 40 early promoter; MLV 3′LTR, murine leukemia virus 3′ long terminal repeat; neomycin resistance gene.

describe here for the *vpr* gene products appears to be distinct from the other described functions of Vpr. The deleterious effects of Vpr upon cells appear to be independent of virion association, since expression of Vpr alone can induce inhibitory effects. Vpr has also been shown to induce differentiation of human muscle cell lines (25), an effect that is possibly related to the cell growth or viability effects of Vpr seen here.

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