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Distinct Modes of Human Immunodeficiency Virus Type 1 Proviral Latency Revealed by Superinfection of Nonproductively Infected Cell Lines with Recombinant Luciferase-Encoding Viruses

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To study the basis of cellular latency of human immunodeficiency virus (HIV), we have used a recombinant luciferase-encoding HIV (HXB-Luc) to superinfect nonproductively HIV-1-infected human leukemic cell lines. HXB-Luc contains the *Photinus pyralis* luciferase gene in place of the *nef* gene and provides a highly sensitive, simple assay for HIV infection and expression. To circumvent any superinfection block in latently infected cells, we also generated viruses pseudotyped with murine leukemia virus amphotropic envelope (HXB-Luc:ampho). The parental uninfected lines, U937 and A3.01, from which the latently infected cell lines U1 and ACH-2, respectively, were derived could be readily infected with pseudotyped or nonpseudotyped reporter viruses. However, superinfection of U1 cells with either HXB-Luc or HXB-Luc:ampho resulted in only low levels of luciferase activity. Like the endogenous provirus, HXB-Luc provirus could be efficiently activated by phorbol ester treatment of HXB-Luc:ampho-superinfected U1 cells. In contrast, superinfection of ACH-2 cells resulted in active expression of the secondarily introduced virus even in unstimulated cells and luciferase production higher than in the parental cell line A3.01. Thus, the proviral latency in U1 cells appears to result from a defect in the cellular environment (a *trans* effect), whereas the latency in ACH-2 is specific to the integrated provirus and is probably a *cis* effect due to the site of integration. These results demonstrate distinct modes of proviral latency in these two cell line models and may have implications in our understanding of the regulation and significance of cellular latency in HIV infection.

Acute infection of some human leukemic cell lines with human immunodeficiency virus (HIV) generates a cytopathic infection which leaves behind a heterogeneous population of chronically infected survivors (5, 14, 17). A number of cell lines have been derived from such chronically HIV-infected cells (7); a few of these have been found to have low basal expression of virus yet retain the ability to produce large quantities of infectious virus when stimulated (14, 16). These cell lines have been studied extensively as in vitro models for cellular latency. U1 is one such cell line that was cloned from the chronically infected promonocytic leukemic cell line U937, and ACH-2 was similarly derived from the T-lymphocytic leukemic cell line A3.01. Studies of U1 and ACH-2 have provided substantial information about the stimuli and regulatory pathways that may be involved in the activation of HIV replication in vivo.

In both the U1 and ACH-2 cell lines, infectious virus production can be induced with phorbol ester or specific cytokines such as tumor necrosis factor alpha (15–17, 34). This activation results in a large induction of HIV mRNA, a process believed to involve, at least in part, the action of transcription factor NF-κB (10, 19). Both cell lines have been found to express only low levels of predominantly multiply spliced HIV mRNA in the unstimulated state. Activated HIV gene expression results in an increase in the amounts of multiply spliced mRNA and the viral regulatory proteins which they encode (28, 33), including Rev, whose function is critical for subsequent expression of unspliced and singly spliced mRNAs which encode HIV structural proteins (26, 27, 32). In this regard, the sequence of events which follows activation of latent HIV in U1 and ACH-2 resembles the events that follow integration during acute infection (22).

Despite the increased understanding of the mechanisms involved in proviral activation in these latently infected lines, the underlying cause of their low basal HIV-specific transcription is not well understood. Because infectious virus can be obtained from both cell lines, it is unlikely that defective proviruses are the cause of low basal expression. Therefore, it is possible that their intracellular environments differ from the parental cell lines U937 and A3.01, such that viral replication cannot occur efficiently in the unstimulated state. Alternatively, low basal expression of the latent provirus could be due to a suboptimal site(s) of integration. Recently, the latter mechanism has been suggested for the ACH-2 cell line (41).

To directly address these questions we have constructed a reporter HIV-1 with the *Photinus pyralis* luciferase gene (9) cloned in place of the *nef* gene and examined the ability of U1, ACH-2, and their parental cell lines to support viral expression by measuring luciferase activity produced by the reporter virus on infection. Luciferase gene expression thus provides a highly sensitive, quantitative marker for HIV expression. The *nef* gene was chosen as the site for reporter gene insertion by virtue of its property of being expressed in a Rev-independent manner (23) and because it is known to be nonessential for in vitro infection (13, 40). To overcome a possible block to HIV superinfection in these chronically infected cells, we used reporter virus pseudotyped with murine leukemia virus (MuLV) amphotropic envelope (24, 25) in addition to nonpseudotyped reporter virus.

Superinfection of the U1 cells with the luciferase reporter virus has revealed a cellular environment that does not support

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expression of an additional HIV provirus. In contrast, we find that ACH-2 displays no such block to expression of the luciferase reporter. Our studies have thus revealed two distinct mechanisms of proviral latency in these nonproductively infected cell lines.

MATERIALS AND METHODS

Cell lines and plasmids. The luciferase reporter virus was derived from molecular clone R73 (provided by Mark Feinberg, The Gladstone Institute of Virology and Immunology, San Francisco, Calif.), a derivative of the HXB-2D isolate (38). A proviral construct with a deletion in *nef*, pHXBΔnef, was first constructed by introducing a *Not*I site into the 5′ region of the *nef* gene of R73 by using PCR as follows. A 5′ primer which overlaps with a unique *Bam*HI site and a 3′ primer which places *Not*I and *Xho*I sites at the 5′ end of the *nef* coding region were used. The resulting clone contains a deletion of the region encoding the first 34 amino acids of the *nef* gene. The *P. pyralis* luciferase gene was subsequently cloned between the *Not*I and *Xho*I sites of pHXBΔnef. The resulting proviral plasmid, pHXB-Luc, carries an inserted luciferase gene which utilizes the natural Nef translation start site. An envelope mutant variant, HXB-LucE$^-$, was created by the ablation of an *Nde*I site in the 5′ region of the *env* gene. Identical constructs were produced in the context of proviral clone NL43 (1) by cloning the *Bam*HI-*Xho*I fragment from HXB-Luc into corresponding sites in NL43. NL43 is a fusion of the proviral DNA from the NY5 isolate and the LAV-1 isolate (1).

Cell lines U1, ACH-2, U937, and A3.01 were obtained from Tom Folks through the AIDS Reference and Reagent Program. HOST4 cells and amphotropic envelope expression vector pSVL-MEA (24) were generous gifts from Ned Landau, Aaron Diamond AIDS Research Center, New York, N.Y. Nonadherent cell lines were grown in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum, penicillin, and streptomycin. Adherent cell lines 293, COS, HOS, and HOST4 were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Transfection, infection, and luciferase assays. Virus was produced by transfection of proviral DNA into the human embryonic kidney cell line, 293, or African green monkey kidney cell line, COS, by using a modified calcium phosphate method (31). Pseudotyped virus was similarly produced by cotransfection of reporter virus DNA with an expression vector for MuLV amphotropic envelope, pSVL-MEA. The medium was changed 18 to 24 h after transfection, and supernatants were harvested 2 days after transfection. Virus-containing supernatants were cleared of cellular debris by centrifugation at 500 × *g* for 5 min. Supernatant p24 was determined by antigen capture assay (Abbott Laboratories) to quantitate the virion content. Target cells of interest were infected for 1 to 2 h at 37°C with indicated amounts of p24 at a concentration of 2×10^6 to 4×10^6 cells per ml in the presence of 8 μg of Polybrene per ml. The cells were then washed with phosphate-buffered saline and resuspended to a concentration of 2.5×10^5 cells per ml in fresh medium immediately following the incubation. Cells (1×10^6 to 2×10^6) harvested at the indicated times postinfection were lysed with 100 μl of 1× luciferase lysis buffer (Promega). A 10-μl sample of each lysate was assayed for photon emission with a luminometer (Optocompl) by using the Promega Luciferase Assay System. Protein content in the cell lysates was determined by using the Bio-Rad protein assay reagent as specified by the manufacturer.

Quantitative PCR. Infected cells were harvested at 72 h postinfection, and genomic DNAs were prepared as previously described (2). DNAs were standardized by measuring UV A_{260}, and the integrity of samples was confirmed by visualization with ethidium bromide stain on agarose gel. Reactions were performed in a 50-μl volume containing 500 ng of cellular DNA, 100 ng each of luciferase PCR primers (5′-CAG AAT CGT CGT ATG CAG TGA-3′ and 5′-CTA CGG TAG GCT GCG AAA TGT-3′), 50 μM each deoxynucleoside triphosphate, 16.6 nM [α-^{32}P]dCTP (2.5 μCi), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 7.9), and 1.25 U of *Taq* polymerase (Boehringer Mannheim). PCR cycles consisting of 94°C for 60 s and 66°C for 90 s for 27 rounds were used.

RESULTS

To investigate the cause of low basal viral expression in the latently infected human leukemic cell lines, U1 and ACH-2, we engineered an infectious HIV proviral construct with the firefly luciferase gene cloned into the region which encodes the viral regulatory gene, *nef* (see Materials and Methods). The resulting virus, HXB-Luc, expresses luciferase upon infection and allows for highly sensitive, quantitative measurement of HIV gene expression. When pseudotyped with MuLV amphotropic envelope, the luciferase-encoding virus should overcome any superinfection block present in the latently infected cell lines, allowing for characterization of HIV expression in U1 and ACH-2 relative to the expression observed in their respective parental cell lines.

Luciferase-encoding HIV-1 pseudotyped with amphotropic envelope can infect cells independently of CD4 expression. To create an infectious luciferase-encoding pseudotyped virus (HXB-Luc:ampho), COS cells were cotransfected with the expression vector for the MuLV amphotropic envelope, pSVL-MEA, and luciferase-encoding proviral DNA, pHXB-Luc. Nonpseudotyped virus was similarly produced by transfection of the pHXB-Luc provirus alone. Fivefold dilutions of the supernatants from the transfected cells were used to infect human osteosarcoma (HOS) cells or the stably CD4-expressing HOS derivative, HOST4. Cells were harvested and tested for luciferase activity 48 h postinfection. As expected, the nonpseudotyped virus was unable to efficiently infect the CD4-negative HOS cells (Fig. 1). However, HXB-Luc-infected HOS cells did express minimal luciferase activity above background when infected with the highest concentration of virus tested (corresponding to approximately 10 ng of p24), indicating that very-low-level infection may occur in HOS cells that do not express CD4. In contrast, the HXB-Luc virus gave rise to abundant luciferase activity when used to infect the CD4-positive cell line, HOST4. When provided with the MuLV amphotropic envelope, HIV was able to efficiently infect both CD4$^-$ HOS cells and CD4$^+$ HOST4. Thus, as described previously (24, 25), the amphotropic envelope confers CD4 independence for HIV infection of human cells. In all infections, the observed luciferase activity was roughly proportional to the level of input p24, with fivefold dilutions resulting in corresponding decreases in luciferase activity.

Proviral latency in the U1 monocytic line is due to a cellular phenotype which does not support basal expression of HIV. To test whether the latently infected U1 cells could support the expression of an added HIV provirus, we infected the U1 cell line and its parental line, U937, with nonpseudotyped virus, HXB-Luc, and pseudotyped virus, HXB-Luc:ampho. Approximately one million cells were harvested at various times postinfection and assayed for luciferase activity. When superinfected with nonpseudotyped HXB-Luc virus, latently infected U1 cells expressed only low levels of luciferase activity

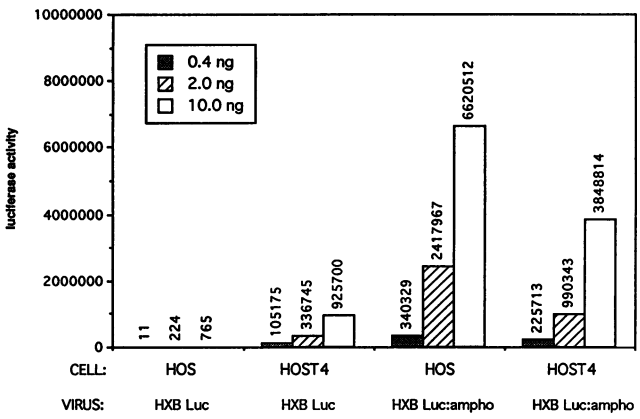

FIG. 1. Luciferase activity measured 48 h after infection of HOS and HOST4 cells with pseudotyped HXB-Luc:amphovirus or non-pseudotyped HXB-Luc virus as indicated. Cells were infected with virus supernatants containing the indicated quantity of viral p24 antigen in nanograms.

relative to U937 throughout the time course (Fig. 2A). This pattern of low-level expression could not be overcome by the use of virus pseudotyped with amphotropic envelope. In contrast, the parental cell line, U937, was efficiently infected with either HXB-Luc or HXB-Luc:ampho as indicated by significant luciferase activity from both viruses as early as 19 to 24 h postinfection. Thus, despite the abrogation of a possible superinfection block by using an amphotropic envelope-pseudotyped virus, the secondarily introduced virus expressed only very low levels of luciferase in the U1 cells relative to the U937 cells. In the U1 cells it must be either the presence of an inhibitory factor(s) or the absence of an appropriate positive factor(s) which limits virus expression in these cells. Equivalent results were obtained in repeated experiments with different virus stocks and with U1 and U937 cells obtained from different passage numbers (data not shown).

Proviral latency in ACH-2 is not due to a latent cellular phenotype. Like the U1 cells, the ACH-2 cells expressed only low levels of luciferase when infected with nonpseudotyped HXB-Luc (Fig. 2B). In contrast, when ACH-2 cells were infected with an HXB-Luc:ampho virus, levels of luciferase expression in the cells were high relative to those in A3.01 cells. Luciferase levels in the parental cell line A3.01 indicated that these cells could be infected by either HXB-Luc or HXB-Luc:ampho. These data indicate that the ACH-2 cell line has a strong superinfection block to HIV which can be overcome by the infection with amphotropic envelope, consistent with the previously reported low CD4 expression (14). However, the cellular environment of ACH-2 is highly permissive for HIV growth, implying that the defect responsible for the quiescence of the endogenous provirus must be the consequence of a *cis*-acting effect. The high expression in ACH-2 relative to that in A3.01 suggests that ACH-2 cells may be primed for high HIV expression, perhaps owing to preexisting HIV regulatory proteins or, alternatively, to clonal variation between the ACH-2 cells and parental cell line, A3.01. Again, equivalent results were obtained in repeated experiments with different virus stocks and ACH-2 and A3.01 cells of different passage number (data not shown).

Higher viral expression in U937 cells is not due to more efficient viral spread. Because the pseudotyped HXB-Luc:ampho virus contains an intact *env* gene, it was possible that the differences in expression of the luciferase-encoding virus in U1 and U937 cells were due to a greater efficiency of viral spread in the U937 cells. To control for this possibility, mutant luciferase-encoding reporter viruses with frameshift mutations in the *env* gene were tested in the latent cell lines and their respective parental cell lines. Luciferase-encoding envelope-deficient (*env* mutant) viruses were produced in the context of molecular clones HXB-2 and NL43 (see Materials and Methods). The availability of two different molecular clones containing the luciferase gene allowed us to also examine whether the patterns of luciferase expression were specific to one particular molecular clone of HIV. Infection with *env* mutant reporter viruses pseudotyped with amphotropic envelope (HXB-LucE$^-$:ampho or NL43-LucE$^-$:ampho viruses) yielded results similar to those obtained with HIV envelope-expressing virus (HXB-Luc:ampho). U1 cells exhibited a low expression phenotype compared with the high expression seen in U937 cells, and ACH-2 cells again displayed a high expression phenotype with luciferase synthesis consistently higher than that seen in A3.01 cells (Fig. 3). Thus, the differential ability of the reporter virus to spread in cell culture did not contribute to the observed differences in the luciferase expression between the latent lines and their parental lines.

We have also found that the envelope-expressing luciferase-encoding viruses do not spread efficiently in cell culture (data not shown), which most probably explains why the *env* mutant viruses do not show a lower luciferase expression in any of the cell lines. Identical results were obtained with *env* mutant luciferase-encoding viruses generated from the HXB-2-derived clone, HXB-LucE$^-$, and the NL43-derived clone, NL43-LucE$^-$, indicating that differences in expression are not specific to one molecular clone of HIV (data not shown).

Activation of latent virus by PMA-PHA in HIV Luc-superinfected U1 cells. To ascertain whether superinfected HIV-Luc in U1 cells could be activated in a manner similar to the endogenous U1 provirus, we stimulated U1 cells with phorbol myristate acetate (PMA)-phytohemagglutinin (PHA) (50 ng/ml and 2 μg/ml, respectively) 8 days following superinfection with HIV Luc:ampho. After stimulation for 24 h, luciferase expression from stimulated cells was compared with luciferase expression from identically superinfected, unstimulated cells. A dramatic 36-fold induction of luciferase activity was generated by PMA-PHA treatment of superinfected U1 (Fig. 4), indicating that the reporter virus can indeed be activated from its latent state in a manner similar to that for the endogenous virus. The induction of luciferase activity in the U1 cells also demonstrates that the low luciferase expression in the cells was not due to a failure to infect them efficiently. When similarly infected U937 cells were stimulated, only a modest twofold induction was evident (see Discussion).

Low expression in U1 cells is not due to poor efficiency of entry or reverse transcription. To monitor the efficiency of amphotropic envelope-mediated infection, we harvested cellular DNA from the latently infected cells and their respective parental lines 72 h after infection with NL43-LucE$^-$:ampho virus. All virus stocks used for experiments involving PCR were treated with 15 μg of bovine pancreatic DNase per ml for 45 min at 37°C in the presence of 10 mM $MgCl_2$ prior to infection to degrade any plasmid DNA contaminating the stock. Infection with NL43-LucE$^-$:ampho gave rise to the same pattern of low expression in U1 cells relative to U937 cells and to high expression in ACH-2 cells relative to A3.01 cells (data not shown). Cells infected with heat-inactivated virus (15 min at 65°C) showed no luciferase activity (data not shown). Quantitative PCR was performed on DNA samples with primers specific for luciferase (Fig. 5). U1 and U937 DNAs contained

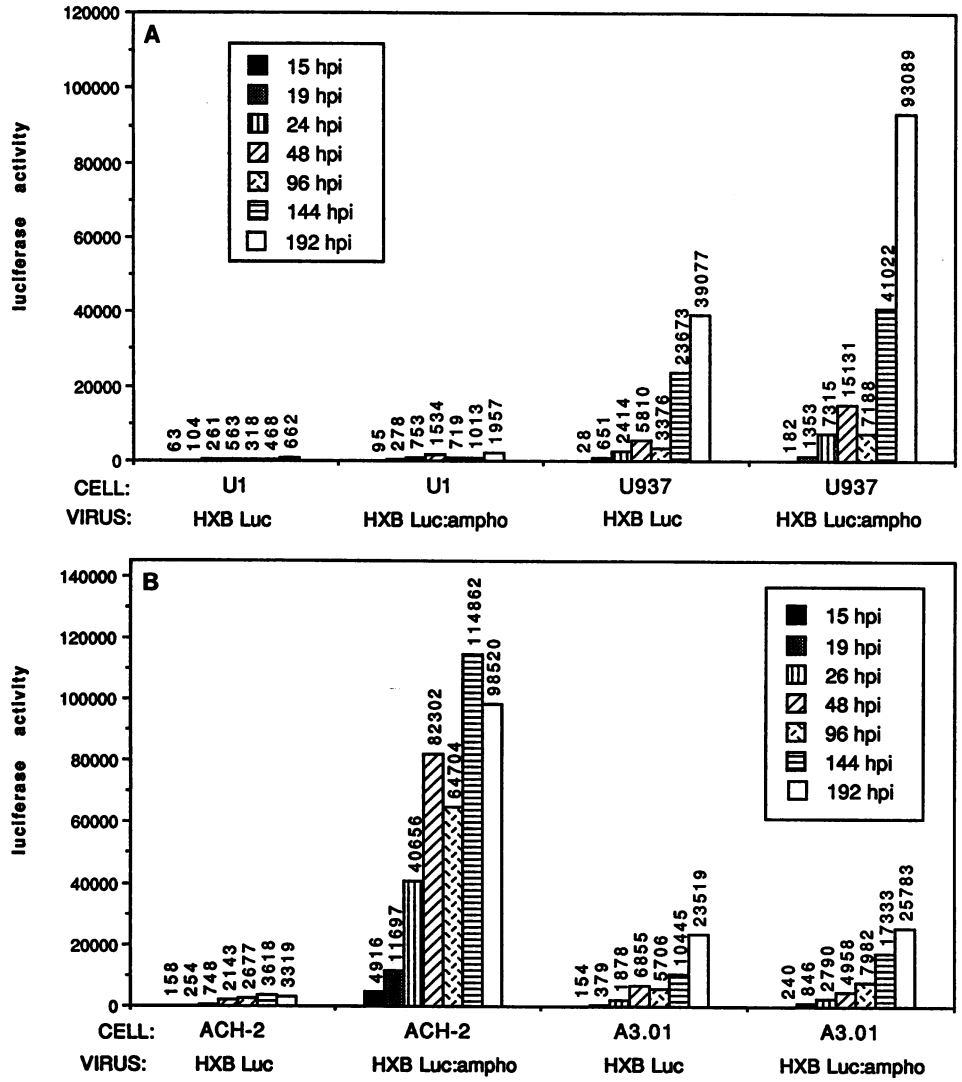

FIG. 2. (A) Luciferase activity following infection of 4×10^6 U1 or U937 cells with HXB-Luc or HXB-Luc:ampho virus containing 60 ng of p24 antigen, at successive time points (in hours) following infection (hpi). (B) Luciferase activity following infection of 4×10^6 ACH-2 or A3.01 cells with HXB-Luc or HXB-Luc:ampho virus containing 60 ng of p24, at successive time points following infection. Luciferase numbers shown in panels A and B correspond to activity measured from cell extracts containing 10 μg of protein.

comparable levels of luciferase DNA (Fig. 5, lanes 1 and 2). ACH2 and A3.01 DNAs also had similar levels of luciferase DNA by the PCR assay (lanes 3 and 4). Control infections of the cell lines with heat-inactivated HXB-Luc:ampho (lanes 5 through 8) showed that the PCR signal was not due to contamination of plasmid DNAs in the virus preparation and was dependent on infection with viable virus. Control PCRs with no DNA added or with mock-infected cellular DNA were negative for PCR signal (lanes 9 and 10). Tenfold serial dilutions of pHXB-Luc plasmid into equivalent amounts of mock-infected cellular DNA show that the signals are in the linear range of the PCR assay. These results demonstrate that U1 or ACH-2 cells were infected with efficiencies equal to those of their respective parental cell lines and that the observed differences in luciferase expression were not due to variable efficiency of infection of the cell lines.

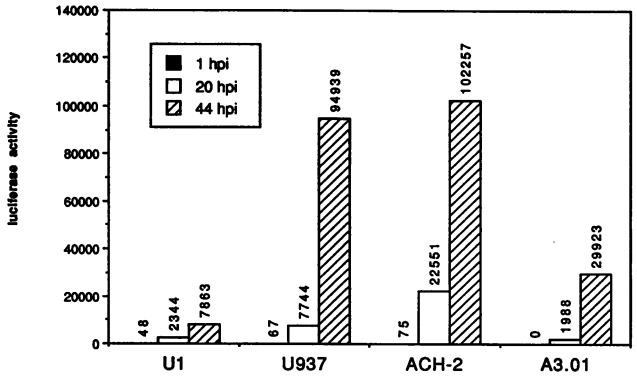

FIG. 3. Luciferase activity measured at indicated time points (hours) after infection (hpi) of 4×10^6 U1, U937, ACH-2, or A3.01 cells with envelope-deficient, pseudotyped virus, NL43-LucE$^-$:ampho (60 ng of p24 antigen). Luciferase numbers correspond to activity measured from cell extract containing 10 μg of protein.

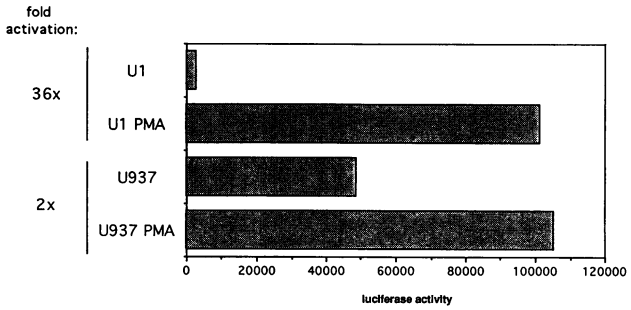

FIG. 4. Luciferase activities from U1 and U937 cells 8 days after infection with NL43-LucE$^-$:ampho virus are shown with PMA-PHA stimulation (50 ng/ml and 2 μg/ml, respectively, for 24 h) and without stimulation. Fold increase upon stimulation is indicated to the left. Luciferase numbers correspond to activity measured from cell extract containing 10 μg of protein.

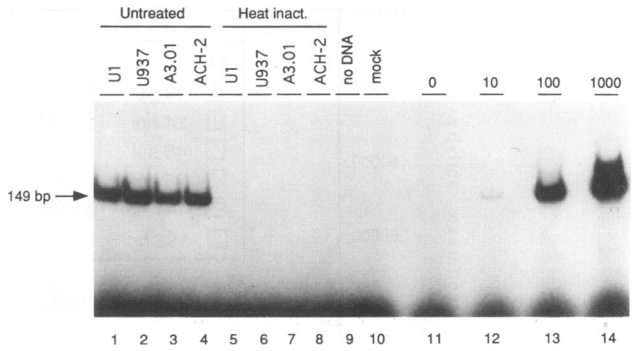

FIG. 5. Quantitative DNA PCR with luciferase-specific primers on 500 ng of cellular DNAs prepared from latently infected cell lines and their parental cell lines 72 h after infection with NL43-LucE$^-$:ampho. U1, U937, ACH-2, or A3.01 cells (10^6 cells) in 250 μl were infected with DNase-treated virus containing 200 ng of p24. Lanes: 1 to 4, PCR products produced from total DNA prepared from infected U1, U937, ACH-2, and A3.01 cells, respectively; 5 to 8, PCR products from the same cell lines infected with heat-treated NL43-LucE$^-$:ampho; 9, PCR without DNA; 10, mock-infected A3.01 DNA; 11 to 14, standards containing 500 ng of mock-infected cellular DNAs with 0, 10, 100, and 1,000 fg of pHXB-Luc plasmid DNA.

DISCUSSION

The U1 and ACH-2 cell lines have provided useful models for investigating the mechanisms of proviral latency and activation. Our study has revealed that proviral latency in these two cell lines is controlled by different mechanisms. We found that the latently infected U1 cells failed to support efficient expression of a superinfected, luciferase-encoding HIV reporter, suggesting that it is the availability of necessary *trans*-acting factors in U1 cells which maintains proviral latency in these cells. U1 cells may therefore lack some critical factor for viral expression or, alternatively, express a repressor of viral expression. In direct contrast, the ACH-2 cell line displayed no such intrinsic defect in supporting HIV replication. Rather, the ACH-2 cellular environment appeared to enhance the expression of the second virus, perhaps as a consequence of preexisting basal production of viral regulatory proteins or because of clonal variation. Proviral latency in ACH-2 cells may thus be categorized as a *cis*-acting effect, potentially a result of an unfavorable site of integration or hypermethylation (4) of the proviral DNA. This mode of latency in ACH-2 cells is also supported by a recent study by Winslow et al., who found high expression of a secondarily introduced provirus, but not of the endogenous one, in neomycin-selected ACH-2 cells following superinfection with a virus carrying a neomycin phosphotransferase gene (41).

Early data showed that even in patients with AIDS, only a very small fraction of peripheral blood mononuclear cells produce significant levels of HIV mRNA (20), a fraction believed to be significantly smaller than the number of DNA-positive cells (38). These findings were supported by subsequent studies which showed that a pool of latently infected cells does exist in infected patients (6, 37, 39). In recent work with sensitive in situ PCR techniques capable of quantifying single-copy HIV-infected cells, a larger number of latently infected cells have been detected in both peripheral blood and the lymph nodes than was previously estimated (11, 12, 30). While studying HIV-infected chimpanzees as a potential model for clinical latency of HIV disease, we recently demonstrated that unstimulated peripheral blood mononuclear cells from these animals do not show any detectable signs of viral replication yet can be rapidly induced to express viral mRNAs after exposure to PMA-PHA in vitro (36). Given such similarities to cells which exist in vivo, it is possible that the mechanisms of latency used by U1 and ACH-2 play a role in HIV infection of cells in vivo. On the other hand, irrespective of whether U1 and ACH-2 cells have direct in vivo counterparts, these cell lines pose interesting questions and should be useful models for the study of proviral regulation. Hence further investigation of the mechanisms of viral regulation in these models of cellular latency seems warranted.

Because of its potential to yield new insights into the cellular factors that regulate basal HIV expression, it is especially intriguing to speculate about the nature of the *trans*-acting defect found in the U1 cells. The Sp1- and NF-κB-binding sites have long been understood to be elements critical for HIV long terminal repeat activity (18, 21, 29). Sp1 site-binding proteins are believed to be involved in the basal transcription of the HIV long terminal repeat; therefore one might speculate that differences in Sp1 site-binding proteins may be involved in the maintenance of proviral quiescence in the U1 cells. However, electrophoretic mobility shift assays with Sp1 site-containing probes and nuclear extracts from the U1 and U937 cells have not revealed any qualitative or quantitative differences in the protein complexes interacting with these sites (8).

Activation of transcription in U1 cells has been correlated with an increase in NF-κB DNA-binding activity (19). Chronic HIV infection of certain human myeloid/monocytic cell lines, including U937, has been found to induce increased nuclear NF-κB activity even in the absence of any exogenous regulatory stimuli (3, 35). Interestingly, although U1 cells are derived from chronically infected U937 cells, their basal level of NF-κB activity does not appear to be upregulated, because they show only weak nuclear NF-κB-binding activity similar to that of uninfected, unstimulated U937 cells (8, 33). Furthermore, PMA treatment of the HXB-Luc-superinfected U1 cells induced a 36-fold stimulation in the luciferase activity, whereas only a 2-fold stimulation was observed in the similarly infected U937 cells, suggesting that, in the latter, HIV infection may have already resulted in a near-maximal NF-κB induction. Thus, a lack of such HIV-induced NF-κB activity could be one mechanism which restricts the viral replication in U1 cells.

Although the *trans*-acting mechanism of latency in U1 cells may draw more experimental attention because of the interest in identifying the critical *trans*-acting factors necessary for HIV expression, it seems equally likely that the *cis*-acting effect

found in ACH-2 cells could be used in cells in vivo. ACH-2 provides an interesting case in which cellular activation can overcome a *cis*-acting inhibitory effect and suggests that differences in the site of proviral integration can have a critical effect on the level of viral expression in the absence of activating stimuli. Such *cis*-acting mechanisms could be important in vivo where a suboptimal site of integration may limit viral expression to a level below a critical threshold level yet still permit viral upregulation by cellular activation.

In future studies, it will be interesting to test other latently infected cell lines to see whether the patterns revealed in U1 and ACH-2 may be generalized according to cell type. Additionally, characterization of other latently infected cell lines with *trans*-acting, U1-like defects may eventually lead to the identification of new cellular factors that are critical for regulation of HIV gene expression.

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