Human Immunodeficiency Virus Type 1 Replication in the Absence of Integrase-Mediated DNA Recombination: Definition of Permissive and Nonpermissive T-Cell Lines

JOURNAL OF VIROLOGY, Sept. 2001, p. 7944–7955 Vol. 75, No. 17
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.17.7944–7955.2001

Human Immunodeficiency Virus Type 1 Replication in the Absence of Integrase-Mediated DNA Recombination: Definition of Permissive and Nonpermissive T-Cell Lines

NORIKO NAKAJIMA,† RICHARD LU, AND ALAN ENGELMAN*

Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Received 27 March 2001/Accepted 1 June 2001

Functional retroviral integrase protein is thought to be essential for productive viral replication. Yet, previous studies differed on the extent to which integrase mutant viruses expressed human immunodeficiency virus type 1 (HIV-1) genes from unintegrated DNA. Although one reason for this difference was that class II integrase mutations pleiotropically affected the viral life cycle, another reason apparently depended on the identity of the infected cell. Here, we analyzed integrase mutant viral infectivities in a variety of cell types. Single-round infectivity of class I integration-specific mutant HIV-1 ranged from <0.03 to 0.3% of that of the wild type (WT) across four different T-cell lines. Based on this approximately 10-fold influence of cell type on mutant gene expression, we examined class I and class II mutant replication kinetics in seven different cell lines and two primary cell types. Unexpectedly, some cell lines supported productive class I mutant viral replication under conditions that restricted class II mutant growth. Cells were defined as permissive, semipermissive, or nonpermissive based on their ability to support the continual passage of class I integration-defective HIV-1. Mutant infectivity in semipermissive and permissive cells as quantified by 50% tissue culture infectious doses, however, was only 0.0006 to 0.005% of that of WT. Since the frequencies of mutant DNA recombination in these lines ranged from 0.023 to <0.093% of the WT, we conclude that productive replication in the absence of integrase function most likely required the illegitimate integration of HIV-1 into host chromosomes by cellular DNA recombination enzymes.

Retroviruses carry two enzymes, reverse transcriptase (RT) and integrase (IN), which function early in the viral life cycle. Soon after infection, RT converts genomic RNA into linear double-stranded cDNA. This DNA, which contains a copy of the viral long terminal repeat (LTR) at each end, is the substrate for IN-mediated DNA recombination. IN initially processes the 3′ ends of the cDNA adjacent to phylogenetically conserved CA dinucleotides and then inserts these cleaved ends into a target DNA site in a cell chromosome. The *cis*-acting end regions important for integration define the viral attachment (*att*) sites, which are comprised of U3 and U5 sequences in the upstream and downstream LTRs, respectively. (For a recent review of retroviral integration, see reference 6.)

In addition to the linear DNA product of reverse transcription, various types of circular DNA form in retroviral-infected cells. Whereas some of these result from IN-mediated autointegration of the viral cDNA into itself, others result from host-mediated enzyme activities. One type of host-mediated circle, which contains a single copy of the LTR, most likely forms by homologous recombination between the LTRs. A second type contains two tandem LTRs and probably forms by ligation of the two cDNA ends. Host-mediated DNA circles are considered to be dead end products of reverse transcription (6).

Although IN function is believed to be essential for retroviral growth (6), IN and *att* site mutations can disrupt the viral life cycle at distinct steps. Whereas some mutations (class I) block replication specifically at the integration step, others (class II) cause pleiotropic defects. Cells infected with integration-specific class I mutants contain higher levels of unintegrated DNA circles than do wild-type (WT)-infected cells (reviewed in reference 14), and class I human immunodeficiency virus type 1 (HIV-1) IN mutants display 12 to 19% of WT activity in the multinuclear activation of galactosidase indicator (MAGI) assay (2, 15, 60). This single-round infection assay requires de novo synthesis of the viral Tat protein and its subsequent *trans*-activation of an integrated β-galactosidase (β-Gal) gene in HeLa-derived CD4-LTR/β-Gal cells (15, 27, 31). Class I IN mutants recombine with host cell chromosomes about 10^{-4} as frequently as the WT, but this low level of integration is apparently mediated by host enzyme activities and not viral IN (19, 30). Thus, although IN mutant viruses have not been shown to support productive retroviral infections, they can apparently support some transient level of gene expression in HIV-1-infected cells (2, 8, 15, 53, 60). Single-round class I IN mutants carrying the gene for firefly luciferase (Luc) in the viral *nef* position, however, displayed only about 0.2% of WT activity in infected human rhabdomyosarcoma (RD) cells (34). Thus, the level of transient gene expression from unintegrated HIV-1 DNA might depend on the identity of the infected cell (8) and/or the particular indicator assay.

To further examine the influence of target cell type, we

* Corresponding author. Mailing address: Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. Phone: (617) 632-4361. Fax: (617) 632-3113. E-mail: alan_engelman@dfci.harvard.edu.

† Present address: Department of Pathology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, 162-8640, Japan.

determined levels of WT and class I IN mutant gene expression in four different T-cell lines by using single-round HIV-1 carrying the gene for chloramphenicol acetyltransferase (CAT). Mutant CAT activity ranged from <0.03 to 0.3% of WT activity, indicating an approximately 10-fold influence of cell type on gene expression from unintegrated DNA relative to WT-integrated proviral DNA. Based on this, we reexamined replication capacities of class I and class II mutant HIV-1 in a variety of transformed and primary CD4-positive human cells. Unexpectedly, certain T-cell lines supported productive class I mutant replication under conditions that restricted class II mutant growth. Remarkably, class I mutant HIV-1 could be continually passaged in permissive cells without reversion to an integration-competent genotype. Our results highlight contributions of cell type to gene expression in the absence of IN function, suggesting that unintegrated DNA may contribute to HIV-1 pathogenesis and the development of AIDS in certain settings.

MATERIALS AND METHODS

Plasmids. Single-round CAT expression vectors carrying either WT (20) or IN mutant D116A (41) were previously described. Plasmid pHI.Luc was built by amplifying the Luc gene from pGL3-Basic (Promega Corp., Madison, Wis.) and ligating digested DNA to *Bam*HI-*Not*I-digested pHI.libGFP, a single-round vector similar to previously described pHIvec2.GFP (22). HIV-1 vectors carrying the gene for puromycin acetyltransferase (*pac*) were built by amplifying *pac* from pPur (Clontech, Palo Alto, Calif.) and ligating digested DNA to either *Bam*HI-*Xho*I-digested pHVSL3P (42) or *Bam*HI-*Not*I-digested pHI.libGFP. Plasmid pRL-β-actin, which expresses the gene for *Renilla* luciferase (R-Luc) from the β-actin promoter (55), was kindly provided by Marianne Sweetser (University of Washington, Seattle).

Gag-Pol was expressed from a separate plasmid when using *pac* expression vectors. For this, WT IN was expressed from CMVΔP1ΔenvpAvpu/vpr (38), and a D116A derivative was built by ligating the *pol*-containing *Pfl*MI fragment from pHXBH10ΔenvCAT(D116A) (41) with *Pfl*MI-digested CMVΔP1ΔenvpAvpu/vpr. Plasmids pSVIII-env (20) and pHCMV-G (62), which express the HIV-1 and vesicular stomatitis virus G (VSV-G) glycoproteins, respectively, were previously described. Rev was expressed from a separate plasmid as previously described (22, 42) when using VSV-G.

WT pNL4-3 (1) and mutants D116N (15), K156E/K159E (24), and 0A/0B (5) were previously described. Overlapping PCR (15) was used to incorporate the codon for D64N into pNL43(D116N), yielding double mutant pNL43(N/N). Mutants E152Q and 1-212 were similarly built by PCR. To facilitate the combination of IN mutations, pUC(A/P) was built by introducing unique *Age*I and *Pfl*MI sites into the pUC19 polylinker. The *Age*I-*Pfl*MI fragments from N/N and E152Q were subcloned into pUC(A/P), and then the *Bsm*I-*Pfl*MI fragment from pUC(E152Q) was swapped for the corresponding fragment in pUC(N/N), yielding pUC(NNQ). The *Age*I-*Pfl*MI fragment from pUC(NNQ) was reintroduced into WT pNL43/*Xma*I (5), yielding pNLX(NNQ). The *Age*I-*Pfl*MI fragment from pNLX(NNQ) was placed into pNLX(0A/0B), yielding pNLX(NNQ.LTR). CCR5-tropic N/N was made by placing the *Eco*RI-*Bam*HI fragment from pNL(AD8) (17) into pNLX(N/N). The presence of IN and *att* site mutations, as well as the absence of off-site changes, was confirmed by sequencing PCR-generated DNAs.

Cells, viruses, and infections. 293T cells (39) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. HeLa-CD4 cells (27) were grown in DMEM containing 0.1 mg of G418 sulfate per ml, and CD4-LTR/β-Gal cells (27) were grown in DMEM containing 0.1 mg of G418 sulfate and 0.05 mg of hygromycin B per ml. T-cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum. Peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) from HIV-1-seronegative donors were purified by Ficoll-Paque density gradient centrifugation. PBMC grown in RPMI were stimulated for 3 days with 1 μg of phytohemagglutinin (Sigma, St. Louis, Mo.)/ml. One day prior to infection, interleukin 2 (Chiron Corp., St. Louis, Mo.) was added to the final concentration of 100 U/ml. Enriched preparations of MDM cultured in macrophage-driving media were purified by adherence to plastic essentially as previously described (10).

Virus stocks were prepared by transfecting 293T cells with DNA in the presence of calcium phosphate (47). Whereas CAT viruses were prepared as previously described using two plasmids (20), *pac* viruses required three or four different plasmids depending on the identity of the viral envelope (22, 42). Transfected cell supernatants were tested for Mg^{2+}-dependent ^{32}P-RT activity as previously described (15) and for p24 levels by enzyme-linked immunosorbent assay as recommended by the manufacturer (NEN Life Science Products, Boston, Mass.). Virus stocks were filtered through 0.45-μm-diameter pores prior to infection. Viruses were pretreated with DNase I as previously described (11) to degrade residual plasmid when infected cells were lysed for DNA extraction.

T cells (2×10^6) infected with 10^7 cpm of RT activity (10^7 RT-cpm) of HIV-1 CAT in 0.5 ml for 16 h were washed twice with serum-free RPMI and cultured for an additional 44 h in 5 ml of RPMI. Cells were counted, lysed in 20 μl of 0.25 M Tris-HCl (pH 7.6) per 10^6 cells by freeze-thaw, and processed for in vitro CAT assays essentially as previously described (20). Twofold dilutions of lysate were tested in CAT assays, and the percent of acetylated chloramphenicol was determined by scintillation counter or phosphorimager scanning (Molecular Dynamics, Sunnyvale, Calif.). Nonspecific CAT activities from cells mock infected with envelope-lacking viruses were subtracted from WT and D116A values. Percent acetylation was graphed against lysate volume, and standard curves were generated to determine the volume of lysate required for 50% acetylation of added substrate. This volume was then converted to the corresponding level of input virus (in RT-cpm).

Titers of WT and D116A CAT were determined using the MAGI assay as previously described (15, 27). For CAT activity, 1.1×10^6 CD4-LTR/β-Gal or HeLa-CD4 cells seeded in 10-cm-diameter plates were infected the following day with 1.2×10^8 RT-cpm (4 ml) for 16 h. Cells washed twice in serum-free DMEM were then cultured for 32 h in 10 ml of DMEM. Cells were trypsinized, lysed in 100 μl of 0.25 M Tris-HCl (pH 7.6) per 10^6 cells by freeze-thaw, and processed for in vitro CAT assays as described above.

Unless otherwise specified, T cells (2×10^6) infected with 2×10^6 RT-cpm of WT or mutant NL4-3 in 0.5 ml for 18 h were washed three times with serum-free RPMI and resuspended in 5 ml of RPMI. Cultures were split at regular intervals, and aliquots of supernatants were saved for RT assays. For determination of 50% tissue culture infectious dose ($TCID_{50}$), cells infected with serial 10- or 3-fold dilutions of virus were plated in 24-well plates at 10^5 cells/well and split at regular intervals for 3 to 4 weeks, and aliquots of the media were saved for RT assays. Whereas MT-4- and C8166-containing plates were scored by cytopathic effect, 174xCEM, CEM-12D7, and Jurkat cells were scored positive when RT activity was threefold over mock-infected values. $TCID_{50}$ was calculated according to the method of Spearman and Karber (29). Each determination was performed at least twice.

Frequency of IN mutant DNA recombination. Toxic concentrations of puromycin dihydrochloride (Sigma) were determined for the following cell lines as previously described (12): Jurkat, 10 μg/ml; CEM-12D7 and MT-4, 0.5 μg/ml; C8166, 0.4375 μg/ml; 174xCEM, 0.375 μg/ml. Cells (2×10^6) infected with 2×10^6 RT-cpm of WT or D116A *pac* in 0.5 ml for 18 h were washed three times with serum-free RPMI, drug was added 3 days postinfection (dpi), and 6 dpi, cells were serially diluted into 96-well plates in the presence of 20% conditioned media. Cells were fed at regular intervals, and single-cell colonies were counted 2 to 3 weeks postseeding. Mutant DNA recombination frequency was calculated by dividing the number of mutant puromycin-resistant colonies by the number of WT colonies. Each determination was performed at least twice.

Cell fractionation, Southern blotting, and PCR. For cloning passed virus, cells were fractionated by the Hirt method as previously described (18). Supernatant DNA (4 to 10 μl) was amplified by *Pfu* polymerase (Stratagene, La Jolla, Calif.) using *Age*I- and *Pfl*MI-tagged (15) or LTR-specific primers (5, 11) in buffer supplied by the manufacturer. Reaction mixtures heated at 96°C for 1 min were cycled 20 to 25 times by denaturing at 96°C for 20 s, annealing at 58°C for 45 s, and extending at 72°C for 4 min, followed by a final 10-min extension at 72°C. Fragments purified from polyacrylamide gels (47) were either sequenced directly or digested with *Age*I and *Pfl*MI and ligated to *Age*I-*Pfl*MI-digested pNL4-3 prior to sequencing.

For Southern blotting and inverse PCR, cytoplasmic DNA was recovered as previously described (11) after lysing 3.0×10^7 MT-4 cells in 1 ml of buffer K (20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 150 mM KCl, 1 mM dithiothreitol, 20 μg of aprotinin/ml, 0.025% [wt/vol] digitonin). Nuclei resuspended in 2 ml of buffer P1 (Qiagen Inc., Valencia, Calif.) were lysed as recommended by the manufacturer. The supernatant obtained after centrifugation was applied to an equilibrated Qiagen column, and DNA was recovered as recommended by the manufacturer. The genomic DNA pellet was resuspended in 6 ml of buffer G (6 M guanidine-HCl, 0.1 M sodium acetate [pH 5.5], 5% Tween 20, 0.5% Triton X-100) by heating at 56°C for several hours. Ethanol was layered onto the suspension, and

DNA was recovered by spooling onto glass essentially as previously described (4).

Southern blotting was performed as previously described (11), and levels of unintegrated DNA were quantified by a phosphorimager (Molecular Dynamics). Integration was detected by inverse PCR essentially as previously described (31). For this, genomic DNA (2 to 15 μg) digested with *Hin*dIII was recovered following extraction with phenol-chloroform and precipitation with ethanol. DNA (500 ng) was ligated overnight in 50 μl at 16°C with 1 U of T4 DNA ligase (New England Biolabs, Beverly, Mass.). Following heat inactivation (65°C for 10 min), DNA was recovered by precipitation with ethanol and was resuspended in 10 μl of H_2O, and 2 μl was amplified using nested PCR. The first round (50 μl) contained 0.5 μM (each) primers AE459 (11) and AE452 (5) and 2 U of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, Calif.) in buffer recommended by the manufacturer. Following heating at 95°C for 2 min, reactions were cycled 20 times by denaturing (95°C for 15 s), annealing (58°C for 1 min), and extending (72°C for 45 s), followed by a 7-min extension at 72°C. DNA (2 μl) transferred to a second PCR (25 μl) was amplified as in the first round by using 1 U of DNA polymerase, primers AE322 and AE347, and 200,000 cpm of 5′-end-labeled AE347 (5). Reaction products were detected by autoradiography following polyacrylamide gel electrophoresis.

Determination of HIV-1 promoter strength by transfection. T cells (3×10^6) were transfected with 1 μg each of pEGFP-C1 (Clontech), pRL-β-actin (55), and pHI.Luc by using LipofectAMINE 2000 (Life Technologies, Inc., Rockville, Md.). For this, DNA in 100 μl of serum-free RPMI was mixed with 100 μl of serum-free RPMI containing 10.3 μl of Lipofectamine, and 45 min later, this mixture was added to cells in a final volume of 1 ml serum-free RPMI. Following 4 h at 37°C, RPMI (4 ml) was added. Cells were counted, washed, and lysed in 20 μl of Passive Lysis Buffer (Promega Corp.) per 10^6 cells 48 h posttransfection. Lysed cells were centrifuged at 20,630 × *g* for 15 min at 4°C, and 5 μl of supernatant was analyzed using the DualLuciferase Reporter Assay System (Promega Corp.) as instructed by the manufacturer. Luminescence was detected using a EG&G Berthold Microplate LB 96V Luminometer. Relative HIV-1 promoter strength was calculated by normalizing the level of pHI.Luc-driven Luc activity to the level of pRL-β-actin-driven R-Luc activity from a total of six independent transfections.

RESULTS

Cell type-dependent HIV-1 gene expression in single-round infection assays. Previous studies reported that levels of HIV-1 class I IN mutant gene expression varied over a wide range, from approximately 0.2 to 19% of WT expression, using two different infection assays (2, 15, 34, 60). Because of this, we evaluated levels of WT and class I mutant gene expression across numerous cell lines using a single infection strategy. For this, single-round HIV-1 carrying the bacterial CAT gene in the viral *nef* position and either WT or class I IN mutant D116A (2, 7, 14) was pseudotyped with the HIV-1 envelope as previously described (20, 42). Four different CD4-positive T-cell lines, C8166 (45), MT-4 (35), Jurkat (59), and CEM-12D7 (43), each of which was previously used to study HIV-1 IN mutant replication (7, 15, 16, 28, 50, 53), were infected with equal RT-cpm of virus. Cells were lysed 60 h postinfection, and the level of virus inoculum that yielded 50% acetylation in in vitro CAT assays was determined. By this method, a lower RT-cpm value equated to a more efficient infection.

C8166 was the most permissive cell line (Table 1). Compared to C8166, about 7-, 30-, and 40-fold more WT virus was required to achieve 50% acetylation using MT-4, Jurkat, and CEM-12D7 cells, respectively (Table 1). A similar hierarchy of cell line activity was observed in the absence of IN function. C8166 was still the most permissive cell line, but CEM-12D7 cells were now more permissive than Jurkat cells: compared to C8166, about 2-, 24-, and >97-fold more D116A virus was required to achieve equal CAT activities using MT-4, CEM-12D7, and Jurkat cells, respectively (Table 1). Although

TABLE 1. Levels of HIV-1 gene expression in single-round infections

Cell line	Expt	CAT activity (RT-cpm)[a]		
		WT	D116A	WT/D116A (%)
C8166	Expt 1	7.3×10^3	9.3×10^6	0.08
	Expt 2	5.4×10^3	5.1×10^6	0.11
	Avg			0.10
MT-4	Expt 1	4.0×10^4	1.9×10^7	0.21
	Expt 2	4.3×10^4	1.1×10^7	0.39
	Avg			0.30
CEM-12D7	Expt 1	3.7×10^5	1.6×10^8	0.23
	Expt 2	1.5×10^5	1.9×10^8	0.08
	Avg			0.16
Jurkat	Expt 1	1.7×10^5	$>7.0 \times 10^8$	<0.02
	Expt 2	2.0×10^5	$>7.0 \times 10^8$	<0.03
	Avg			<0.03

[a] Amount of input virus required for 50% acetylation of added chloramphenicol.

D116A was more active in C8166 cells than in the other lines, the level of D116A activity relative to WT activity was greatest in MT-4 cells (Table 1). Compared to this relative level of CAT activity, D116A was about 2-, 3-, and >10-fold less active in CEM-12D7, C8166, and Jurkat cells, respectively (Table 1). Based on these differences in both absolute and relative levels of CAT activity in these four cell lines, we decided to reevaluate the replication capacity of integration-defective HIV-1 in a variety of CD4-positive cell types.

Cell type-dependent replication in absence of IN function. We used four different HIV-1 mutants, three class I and one class II, in preliminary assays for viral spread. In order to suppress the frequency of reversion back to WT during productive replication, we analyzed mutants that differed from the WT by more than 1 nucleotide. Since our previously described class I IN active site mutant D116N (15) carried just a single base change, we incorporated a second active-site substitution, D64N, yielding double mutant N/N (Fig. 1). The other two

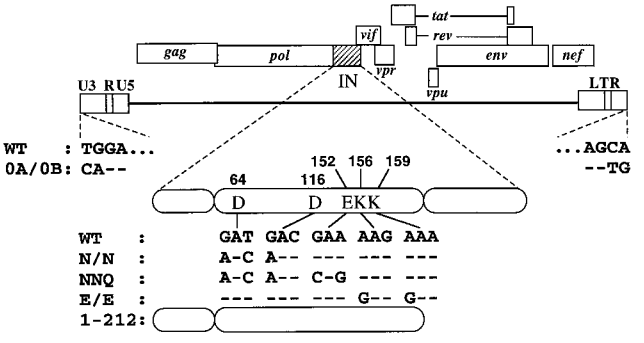

FIG. 1. IN and *att* site mutations. Shown beneath the genetic map of HIV-1 are the plus-strand termini of proviral U3 and U5 and a diagram of IN highlighting active-site (Asp-64, Asp-116, and Glu-152) and *att* DNA binding (Lys-156 and Lys-159) residues. The nucleotide changes in mutants 0A/0B, N/N, NNQ, and E/E are shown beneath the corresponding WT sequence. The size of the 1-212 deletion mutant is shown relative to the 288-residue WT protein. LTR, long terminal repeat.

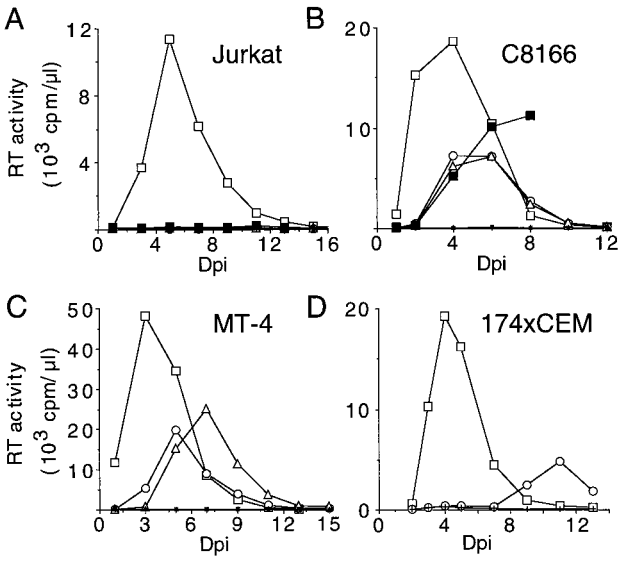

FIG. 2. Replication kinetics of WT and mutant HIV-1 in various T-cell lines. Cells infected with the indicated viruses were monitored for RT activity at the indicated time points. Jurkat (A) and C8166 (B) cells were infected with WT (□), N/N (○), E/E (△), 1-212 (×), 0A/0B (■), or mock-treated supernatant (+). (C) MT-4 cells were infected with WT (□), N/N (○), E/E (△), 1-212 (×), or virus-free supernatant (+). (D) 174xCEM cells were infected with WT (□), N/N (○), or mock-treated supernatant (+).

class I mutants were previously described. One, E/E, disrupted two lysines important for IN-*att* DNA binding (24), and the other, 0A/0B (5), was an *att* site mutant that disrupted the invariant CA dinucleotides in both U3 and U5 (Fig. 1). Since deletion mutants of IN fall into the class II category (14), we engineered three stop codons after Glu-212 in IN, yielding C-terminal deletion mutant 1-212 (Fig. 1).

Viruses produced from transfected 293T cells were normalized for RT activity, and cells (2×10^6 cells) were infected with 10^7 RT-cpm (about 1.5 μg of p24) of WT or mutant HIV-1. Jurkat (Fig. 2A) and CEM-12D7 (not shown) cells supported peak WT replication 5 and 7 dpi, respectively, under these conditions. Neither of these cell lines supported detectable levels of mutant replication over prolonged observation periods: Jurkat and CEM-12D7 cells infected with 0A/0B were monitored for 1 month, and Jurkat cells infected with N/N and 1-212 were monitored for 2 months (Fig. 2A and data not shown). C8166 cells supported peak WT replication 4 dpi (Fig. 2B). Unexpectedly, C8166 cells also supported the replication of N/N, E/E, and 0A/0B (Fig. 2B). Replication of 1-212, however, was not detected under these conditions. MT-4 cells also supported replication of N/N and E/E but not that of 1-212 (Fig. 2C). We note that MT-4 cells infected with as much as 3.0×10^7 RT-cpm did not support detectable 1-212 replication over 1 month of observation. Thus, MT-4 and C8166 cells supported class I mutant replication under conditions that restricted class II mutant growth. In contrast, both classes of IN mutants were restricted from replicating in Jurkat and CEM-12D7 cells. Indistinguishable from the effects of WT, we note that virtually all MT-4 and C8166 cells were lysed as a result of class I mutant viral replication. Since class I mutant virus treated with DNase I prior to infection replicated identically to untreated samples (data not shown), we conclude that plasmid carryover from transfection played no role in the observed mutant viral replication phenotypes.

Since both C8166 and MT-4 cells were originally transformed with human T-cell leukemia virus type 1 (HTLV-1), we considered the possibility that preexisting HTLV-1 IN might complement the class I HIV-1 integration defects in these cells. This seemed unlikely because 0A/0B, which grew similarly to IN mutants N/N and E/E (Fig. 2B), contained changes in the parts of the *att* sites that are conserved among all retroviruses (5, 6, 14) (Fig. 1). That is, it seemed unlikely that 0A/0B cDNA could be a viable substrate for any retroviral IN protein. Consistent with this interpretation, 0A/0B preintegration complexes isolated from infected C8166 cells did not show evidence for in vivo 3′ processing by IN or detectable levels of DNA strand transfer activity in in vitro integration reaction mixtures (H. Chen and A. Engelman, unpublished data). However, to further rule out the possibility of IN complementation in MT-4 and C8166 cells, other HTLV-negative human T-cell lines and primary cell types were screened for their ability to support mutant virus replication. Whereas neither H9 (40), Molt-4 Clone 8 (26), PBMC, nor MDM supported a detectable level of class I mutant growth (data not shown), N/N replicated in 174xCEM cells (Fig. 2D), a T-B cell hybrid (46) that to the best of our knowledge does not contain a preexisting retroviral IN protein.

To investigate mutant replication further, supernatant collected from MT-4 cells at the peak of N/N growth (9 dpi with 10^6 RT-cpm) was passed onto fresh MT-4 and Jurkat cells. Whereas Jurkat cells infected with 10^7 RT-cpm of this virus did not support a detectable level of replication, MT-4 cells infected with either 10^6 or 10^7 RT-cpm supported efficient HIV-1 growth (data not shown). MT-4 cells undergoing this second round of infection were lysed by Hirt extraction (21), and HIV-1 in the cell supernatant was molecularly cloned and sequenced. All of the clones analyzed (six total) retained both substitutions in the active site of the IN enzyme. Two of these clones were sequenced across their entire IN regions, and each sequence was identical to that of N/N (one had an A→G transition that did not alter the encoded amino acid). Thus, both the starting genotype and cell type-dependent replication phenotype of N/N produced in 293T cells were maintained after infection and HIV-1 replication in MT-4 cells.

Definition of permissive, semipermissive, and nonpermissive T-cell lines. Although the previous results showed that N/N could be passed onto fresh MT-4 cells, a past study reported self-limiting replication of integration-defective HIV-1 (8). To test if the replication described here might also be self-limiting, we repeatedly passaged class I mutant HIV-1 in MT-4 and 174xCEM cells. To further limit the possibility of reversion during extensive tissue culture, the remaining IN active site residue in N/N, Glu-152, was replaced with Gln. The resulting NNQ mutant was then combined with the four *att* sites changes in 0A/0B, yielding the multiple-mutatant NNQ.LTR. NNQ.LTR would minimally require 7 nucleotide changes to revert back to WT (Fig. 1).

As expected, NNQ.LTR replicated in C8166 (data not shown), MT-4 (Fig. 3A) and 174xCEM (Fig. 3G) cells, but not in either CEM-12D7 or Jurkat cells. Also as expected, the

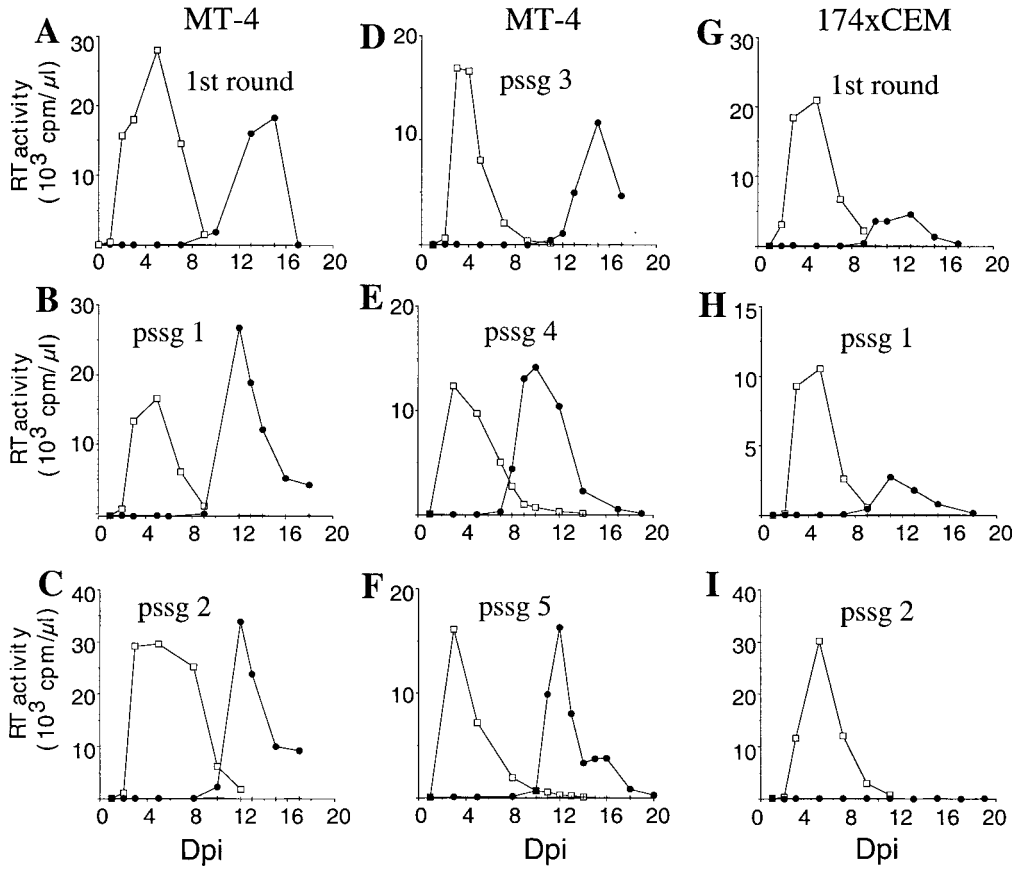

FIG. 3. Serial passage (pssg) of WT and NNQ.LTR in MT-4 and 174xCEM cells. (A) MT-4 cells infected with WT (□), NNQ.LTR (●), or mock supernatant (+) were monitored for RT activity at the indicated time points. (B) Virus recovered at the days of peak WT and NNQ.LTR replication in panel A were used to inoculate fresh MT-4 cells (passage 1). (C to F) WT and NNQ.LTR replication upon passages 2 through 5, respectively. (G) 174xCEM cells were infected as described for panel A. (H and I) Passages 1 and 2, respectively, of WT and NNQ.LTR onto fresh 174xCEM cells.

supernatant from infected MT-4 cells initiated a second round of replication when passed onto fresh MT-4 cells (Fig. 3B) but not Jurkat cells (not shown). NNQ.LTR growth peaked 12 days after passage, which was similar to its replication profile in the initial round of infection (Fig. 3A and B). At 12 dpi, the 5-ml culture contained approximately 1.4×10^8 total RT-cpm, or about 70-fold more NNQ.LTR than was used to initiate the second round of infection (Fig. 3B). Thus, NNQ.LTR initiated a productive HIV-1 infection, and the yield of progeny virus was similar to that of the WT under these conditions (Fig. 3B). NNQ.LTR maintained this approximately 8-day delay in WT levels of productivity following five serial passages in MT-4 cells (Fig. 3A to F), and as was the case for virus collected after the first passage, Jurkat cells infected with fifth-round NNQ.LTR did not support a detectable level of mutant viral replication. MT-4 cells undergoing the sixth round of infection were lysed by Hirt extraction, and HIV-1 DNA in the Hirt supernatant was amplified by PCR using primers specific for IN and the U3 and U5 *att* sites. DNA sequence analysis revealed that all of the IN and *att* site mutations were maintained, proving that class I integration-defective HIV-1 replicated over several months of serial passage in MT-4 cells without acquiring an integration-competent phenotype (Fig. 3A to F) or genotype.

In contrast to the results with MT-4, NNQ.LTR recovered from infected 174xCEM cells replicated for only one serial passage (Fig. 3G to I). We therefore conclude that NNQ.LTR replication in 174xCEM cells was self-limiting. Based on the ability of class I integration-defective HIV-1 to continuously replicate upon repeated passage, we propose the following classification of CD4-positive human T-cell lines and primary cells: permissive, semipermissive, and nonpermissive. Whereas MT-4 cells are permissive, 174xCEM cells are semipermissive and Jurkat cells, CEM-12D7 cells, H9 cells, Molt-4 Clone 8 cells, PBMC, and MDM are nonpermissive (Table 2). Al-

TABLE 2. Summary of permissive, semipermissive, and nonpermissive T-cell lines and primary cells as defined in text

Definition	T-cell line
Permissive	C8166, MT-4
Semipermissive	174×CEM
Nonpermissive	Jurkat, CEM-12D7, H9, Molt-4 Clone 8, PBMC, MDM

TABLE 3. WT and NNQ.LTR $TCID_{50}$s using different T-cell lines

Cell line	Expt	$TCID_{50}$ (ng of p24)		
		WT	NNQ.LTR	NNQ.LTR/WT
C8166	Expt 1	1.6×10^{-4}	15	9.4×10^{4}
	Expt 2	0.7×10^{-4}	17	2.4×10^{5}
	Avg			1.7×10^{5}
MT-4	Expt 1	1.1×10^{-4}	6.4	5.8×10^{4}
	Expt 2	1.1×10^{-4}	6.4	5.8×10^{4}
	Avg			5.8×10^{4}
174×CEM	Expt 1	4.5×10^{-3}	69	1.5×10^{4}
	Expt 2	4.9×10^{-3}	120	2.5×10^{4}
	Avg			2.0×10^{4}
CEM-12D7	Expt 1	1.5×10^{-3}	>1,500	$>1.0 \times 10^{6}$
	Expt 2	1.4×10^{-3}	>1,500	$>1.1 \times 10^{6}$
	Avg			$>1.1 \times 10^{6}$
Jurkat	Expt 1	1.5×10^{-3}	>1,500	$>1.0 \times 10^{6}$
	Expt 2	9.4×10^{-4}	1,500[a]	1.6×10^{6}
	Avg			$\geq 1.3 \times 10^{6}$

[a] Thirteen wells yielded RT-cpm values that ranged from 5 to 10% of WT values in this experiment. The resulting viruses, however, did not support detectable levels of RT activity when assayed in fresh Jurkat cells.

though C8166 cells were not analyzed by serial passage, we speculate that they are permissive based on WT and class I mutant CAT activities (Table 1) and $TCID_{50}$ values (see below). We note that Jurkat cells infected with class I mutant viruses derived from transfected 293T cells occasionally yielded detectable levels of RT activity 20 to 30 dpi. However, fresh Jurkat cells infected with these supernatants did not support detectable RT production over prolonged observation periods. Thus, although Jurkat cells can support some transient gene expression under these conditions, we characterize them as nonpermissive because the resulting virus was noninfectious in fresh Jurkat cells.

WT and class I mutant infectivities by $TCID_{50}$. Although we have shown that HIV-1 can replicate in certain cell lines in the absence of IN function, our experiments until now were performed using relatively high levels of input virus. To further investigate class I mutant replication, we next quantitated WT and NNQ.LTR infectivities under conditions that required viral spread. For this, WT and NNQ.LTR $TCID_{50}$ values were determined by end-point dilution on five different T-cell lines. Each cell type was infected with dilutions of WT or NNQ.LTR, cells were then extensively washed prior to seeding in 24-well plates, and 2 to 4 weeks postinfection, the viral dilution that yielded replication in 50% of the wells was calculated as previously described (29). Under these conditions, most infections were initiated by one virus particle.

Whereas about 0.12, 0.11, 4.7, 1.5, and 1.2 pg of WT p24 were sufficient to infect 50% of C8166, MT-4, 174xCEM, CEM-12D7, and Jurkat cells, respectively, about 16, 6.4, 95, >1,500, and ≥1,500 ng of NNQ.LTR p24, respectively, were required (Table 3). Two important conclusions can be gleaned from these results. First, NNQ.LTR initiated bona fide spreading and productive HIV-1 replication in permissive and semipermissive T-cell lines. Second, spreading infection assays are extremely insensitive to large differences in titer and infectivity. For example, WT was 4 to 5 orders of magnitude more infectious than NNQ.LTR in permissive and semipermissive cells (Table 3). Yet, these large differences in $TCID_{50}$ equated to only about a 1-week delay in peak viral growth (Fig. 3).

Class I mutant replication occurs in absence of normal integration. Our results until now demonstrated that class I IN mutants initiated productive, spreading infections in certain T-cell lines. We next directed our attention to the mechanism of mutant virus replication. Class I mutants recombine with chromosomes about 0.01% as frequently as WT HIV-1, but integration in this case is apparently promoted by host enzyme activities and not IN (19, 30). Thus, two possibilities we considered for mutant viral replication were gene expression from unintegrated DNA templates and/or transcription from illegitimately integrated proviruses. To begin to distinguish between these possibilities, WT and N/N-infected MT-4 cells were fractionated and analyzed for HIV-1 DNA content. Cytoplasmic DNA was recovered following cell lysis in isotonic buffer, and nuclei were lysed using alkali. Whereas unintegrated nuclear DNA was recovered using Qiagen columns, the genomic DNA pellet was recovered essentially as previously described (4). Unintegrated DNA was detected by Southern blotting, and integration was analyzed by inverse PCR (31) as follows.

Although local chromosomal hotspots exist for retroviral integration (61), HIV-1 for the most part integrates randomly throughout the human genome (9). Inverse PCR uses restriction enzyme digestion and intramolecular ligation to detect the frequency and distribution of genomic *Hin*dIII sites adjacent to a population of integrated proviruses (Fig. 4A) (31). In addition to a ladder of DNA products reflecting the randomness of genomic integration, two different internal HIV-1 fragments are detected using this strategy. All DNA forms yield a 1,102-bp fragment, but only host-mediated two LTR-containing circles yield a 261-bp product (Fig. 4A).

Consistent with previous reports (2, 15, 30, 60), cells infected with N/N contained a higher level of host-mediated circles than WT-infected cells at 1 dpi and DNA synthesis in class II-infected cells was undetectable (Fig. 4C). In the WT infection, the level of unintegrated DNA increased dramatically from 1 to 2 dpi, in parallel with HIV-1 production (Fig. 4B and C). Replication was initially detected in N/N-infected cells 2 dpi, and HIV-1 levels increased over time roughly in parallel with the level of unintegrated DNA (Fig. 4B and C). At their replication peaks (7 dpi for N/N, 2 dpi for WT), N/N-infected cells contained approximately 70% as much unintegrated DNA (Fig. 4C) and yielded about half as much virus (Fig. 4B) as WT-infected cells. As expected, a large population of integrated proviruses was detected in the genomic DNA of WT-infected cells (Fig. 4D, lanes 3 and 4). In contrast, this DNA ladder was virtually absent from the N/N infection: the only readily detectable *Hin*dIII fragments in this case were attributed to amplification of either plasmid-related sequences (lane 2) or cofractionating two LTR circles (lanes 5 to 9). We therefore conclude that class I IN mutant viral replication produced near-normal levels of unintegrated HIV-1 DNA and progeny virions without yielding the normal level of integrated provirus. Although these results are consistent with the model that N/N replicated from unintegrated DNA, we note that a minor population of novel bands was detected in N/N-infected genomic DNA upon long autoradiographic exposure. Although difficult

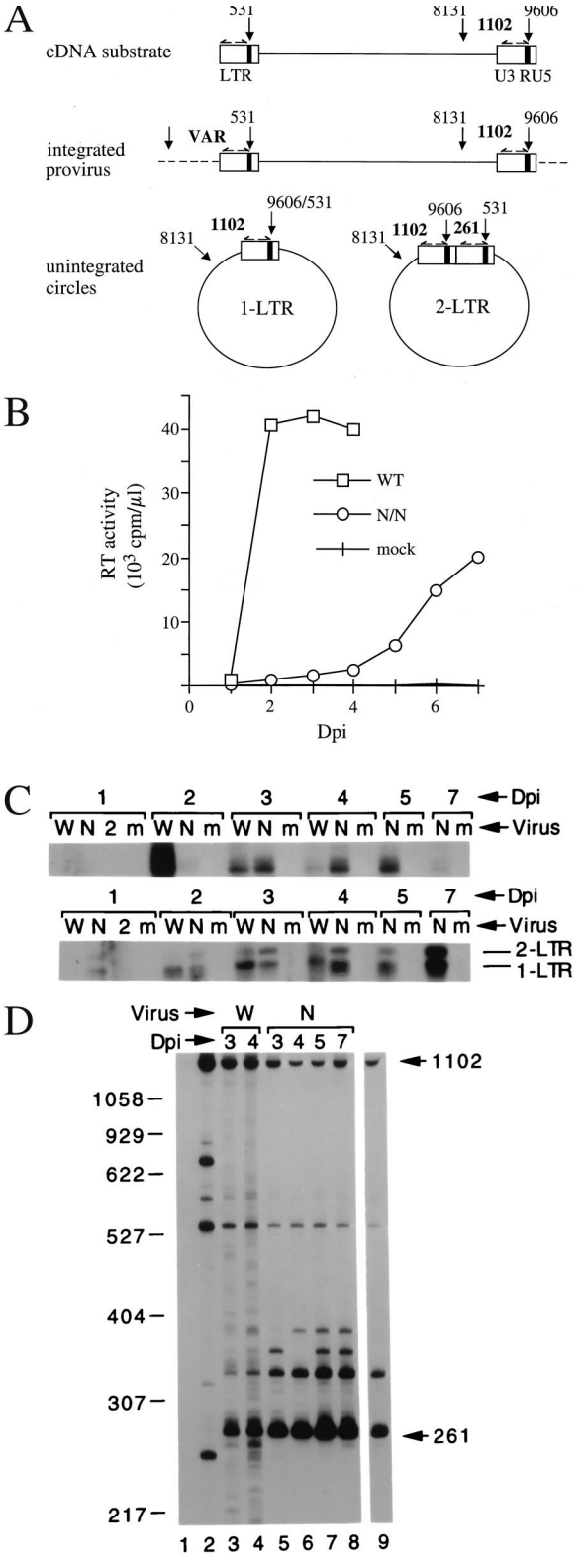

FIG. 4. N/N replicates in MT-4 cells in the absence of normal proviral formation. (A) Inverse PCR strategy. Shown are unintegrated linear cDNA, integrated provirus, and host-mediated DNA circles. The *Hin*dIII sites at positions 531, 8131, and 9606 in the NL4-3 strain of HIV-1 are marked by vertical arrows, and the locations of LTR-specific PCR primers are indicated by horizontal arrows. The sizes of inverse PCR products (bold) are indicated in base pairs. VAR, the variable inverse PCR product whose length is determined by the location of an adjacent genomic *Hin*dIII site. A variable-length ladder of DNA products reflects relative frequency and distribution of retroviral integration (31). Dashed line, host genomic DNA. (B) WT and N/N replication in MT-4 cells (10^8) infected with 10^9 RT-cpm for 2 h at 37°C. RT activity was measured at the indicated times. (C) Cytoplasmic (above) and nuclear (below) unintegrated DNA from WT (W)-, N/N (N)-, 1-212 (2)-, and mock (m)-infected cells lysed at the indicated times. The migration positions of circular DNA containing either one LTR (1-LTR) or two LTRs (2-LTR) are indicated. Linear DNA was most likely absent from nuclear extracts due to alkaline lysis (3). Unintegrated DNA levels were quantified by adding the signals from both panels. (D) Inverse PCR. Lane 1, genomic DNA from mock-infected cells; lane 2, 1 ng of plasmid pNL4-3 was mixed with 10 μg of mock-infected DNA prior to *Hin*dIII digestion; lanes 3 to 8, genomic DNA prepared from the indicated infections; lane 9, 10 μg of mock-infected DNA was mixed with Hirt supernatant from approximately 5×10^4 N/N-infected MT-4 cells. The migration positions of the internal 1,102- and 261-bp products are indicated to the right of the gel. Some of the inverse PCR products detected in infected-cell lysates resulted from either nonspecific amplification of plasmid-related sequences (approximately 530-bp product in lane 2) or amplification of 2-LTR circles in the genomic DNA fractions (lane 9). Each of the four intense bands that migrated between 261 and approximately 400 bp in lanes 5 through 9 were attributed to 2-LTR circles (lane 9 and data not shown). The migration positions of molecular mass standards are indicated on the left (in base pairs). LTR, long terminal repeat.

to precisely quantitate by inverse PCR, we speculate that this low level of integration represents the approximate 10^{-4} illegitimate recombination frequency previously reported for class I IN mutant viruses (19, 30). We therefore considered the possibility that the frequency of class I mutant DNA recombination within a given cell line might be an important determinant of its permissivity. To investigate this hypothesis, we quantitated the level of D116A integration in MT-4, C8166, 174xCEM, CEM-12D7, and Jurkat cells.

Permissive and nonpermissive cell lines support similar mutation recombination frequencies. Single-round HIV-1 carrying the *pac* gene in place of *nef* and either WT or D116A IN were pseudotyped with either the HIV-1 or VSV-G glycoprotein essentially as previously described (42). Cells infected with equal RT-cpm of envelope-matched viruses were extensively washed, incubated with predetermined levels of puromycin for 3 days, and then serially diluted into 96-well plates in the presence of drug. After 2 to 3 weeks, puromycin-resistant colonies were counted and mutant DNA recombination frequency was calculated as colony number per RT-cpm of input D116A versus WT.

Unexpectedly, nonpermissive CEM-12D7 and Jurkat cells supported more WT *pac* colonies than either MT-4 or 174xCEM cells (Table 4). This was most likely due to differences in plating efficiencies among cell lines. For example, in the absence of drug, about 20 MT-4 cells and 150 174xCEM cells were required per well for cell growth. Jurkat cells, in contrast, tolerated densities as low as one cell per well without drug. Despite this limitation, precise DNA recombination frequencies were determined for four of the five cell lines. MT-4 was the only cell line that did not support a detectable level of D116A *pac*-resistant colonies (Table 4).

The level at which a particular cell line supported D116A integration did not correlate with its permissivity. For example, nonpermissive CEM-12D7 and permissive C8166 cells sup-

TABLE 4. IN mutant DNA recombination frequencies across cell lines

Cell line	Expt	No. of colonies[a]			
		WT	D116A	Mock	D116A/WT
C8166	Expt 1	3.6×10^4	10	<0.63	2.8×10^{-4}
	Expt 2	2.3×10^4	3.8	<0.4	1.7×10^{-4}
	Avg				2.3×10^{-4}
MT-4	Expt 1	6.6×10^2	<0.5	<0.5	$<7.6 \times 10^{-4}$
	Expt 2	1.4×10^3	<1.5	<1.5	$<1.1 \times 10^{-3}$
	Avg				$<9.3 \times 10^{-4}$
174×CEM	Expt 1	2.1×10^3	0.4	<0.4	1.9×10^{-4}
	Expt 2	8.4×10^2	0.48	<0.48	5.7×10^{-4}
	Avg				3.8×10^{-4}
CEM-12D7	Expt 1	3.1×10^4	21	<0.25	6.8×10^{-4}
	Expt 2	6.0×10^3	8.2	<0.25	1.4×10^{-3}
	Avg				1.0×10^{-3}
Jurkat	Expt 1	4.4×10^4	15	<0.63	3.4×10^{-4}
	Expt 2	8.3×10^4	11	<0.63	1.3×10^{-4}
	Avg				2.4×10^{-4}

[a] Number of colonies per 2×10^6 RT-cpm of input virus.

ported the highest and lowest recombination frequencies, respectively (Table 4). We note that we did not investigate the mechanism of D116A integration here. However, a previous sequence analysis of 11 proviruses derived from three different class I IN mutant viruses revealed little evidence for IN-mediated DNA recombination (19). Integration normally yields a 5-bp target site duplication flanking the HIV-1 provirus (57, 58), and 10 of 11 sequenced mutants lacked this characteristic. Although one mutant displayed a 5-bp duplication, it contained thymidine substituted for the invariant adenine residue at the U3 *att* terminus that normally joins to target DNA (6), suggesting that recombination enzymes other than IN most likely played a critical role in its integration (19). Based on those results, we speculate that host enzymes were responsible for most if not all of the D116A *pac* recombination events that occurred in C8166, 174xCEM, CEM-12D7, and Jurkat cells.

DISCUSSION

Although it is widely believed that IN function is absolutely required for productive retroviral replication, previous reports differed on the extent to which unintegrated DNA could serve as a template for gene expression and synthesis of HIV-1 proteins (2, 8, 15, 44, 53, 60). We previously noted that this was in part influenced by the nature of the viral mutation: whereas class I IN mutations blocked HIV-1 replication specifically at the integration step, class II mutations caused more pleiotropic damage, including defects in virion morphogenesis and reverse transcription (14). Because of this pleiotropy, class II mutants displayed only 0.1 to 0.8% of WT titers in the MAGI assay under conditions where class I mutants yielded 12 to 19% (2, 14, 15). In addition to this mutational influence, the identity of the target cell also appeared to affect the level of gene expression in the absence of IN function. In this case, self-limiting replication of integration-defective HIV-1 was detected in primary MDM but not PBMC (8). In the present study, we focused primarily on this influence of target cell type on gene expression. Unexpectedly, we found that a subset of transformed T-cell lines supported productive HIV-1 replication in the absence of IN-mediated DNA recombination.

Cell type and assay type-dependent gene expression in the absence of IN function. Although 12 to 19% of WT titers were previously observed using the MAGI assay (2, 15, 60), class I IN mutants carrying the Luc gene in the place of *nef* displayed only about 0.2% of WT activity in RD cells (34). To investigate this apparent cell type difference, we determined levels of WT and class I mutant HIV-1 required for 50% CAT activity in four different T-cell lines. Large differences in HIV-1 activity were detected regardless of IN function. That is, about 40-, 30-, and 7-fold more WT virus was required for CEM-12D7, Jurkat, and MT-4 cells, respectively, than for C8166 cells (Table 1). These results are consistent with previous reports that certain T-cell lines are more permissive than others in their ability to support HIV-1 replication (43, 52). In addition to these differences, however, we detected an approximately 10-fold effect of cell type on levels of D116A CAT activity relative to WT activity (Table 1). These levels, which ranged from <0.03% for Jurkat cells to 0.3% for MT-4 cells, are in-line with the previous 0.2% level reported for single-round Luc viruses (34). Not surprisingly, we conclude that the identity of the indicator gene in the viral *nef* position does not influence the level of gene expression from class I IN mutant viruses in transient-infection assays. Thus, we were perplexed as to why class I mutant titers were as high as 19% of the WT titers in the MAGI assay (2).

Since the MAGI assay uses HeLa-derived CD4-LTR/β-Gal cells (27), we tested whether HeLa cells might present an especially favorable environment for gene expression from unintegrated DNA. For this, we determined levels of WT and D116A required for 50% CAT activity using two related cell lines, HeLa-CD4 and CD4-LTR/β-Gal (27), and compared these values to the relative MAGI titers of the viruses. Whereas approximately 2.4×10^5 and 3.0×10^5 RT-cpm of WT CAT were required in HeLa-CD4 and CD4-LTR/β-Gal cells, respectively, about 1.2×10^8 and 1.1×10^8 RT-cpm of D116A CAT were required, respectively. Thus, D116A supported about 0.2 and 0.27% of WT CAT activity in HeLa-CD4 and CD4-LTR/β-Gal cells, respectively. We therefore conclude that the relative level of class I IN mutant gene expression in HeLa cells was similar to those of other cell types when the indicator gene was in the viral *nef* position (Table 1). Yet, as expected, D116A CAT yielded about 10% as many blue-staining cells as WT CAT in the MAGI assay (data not shown). Thus, the indicator assay itself can apparently influence the outcome of class I mutant gene expression relative to WT as much as 37-fold in a single cell type. Since the Tat protein responsible for indicator gene function in both assays was expressed from the incoming mutant viral genome (15, 31), we conclude that the activation of integrated β-Gal is more sensitive than the activation of viral CAT or Luc for detecting gene expression from unintegrated HIV-1 DNA.

Cell-type dependent HIV-1 replication without IN function. Based on our result that cell type affected the relative level of D116A gene expression as much as 10-fold (Table 1), we re-evaluated class I and class II mutant replication kinetics in a variety of CD4-positive cells (Table 2). Our results for the first

time show that IN function is not required for bona fide spreading and productive retroviral replication (Fig. 2 and 3). Similar to previous observations with the MAGI assay (2, 15, 60), we found that only class I mutants, and not class II, functioned in viral replication assays (Fig. 2). It is important, however, to highlight two limitations of class I mutant viral replication. First, mutant infectivity in permissive and semipermissive cell lines as quantified by $TCID_{50}$ was 20,000- to 170,000-fold less efficient than that of the WT (Table 3). Second, mutant replication was not detected in either primary PBMC or MDM. Thus, despite finding that as little as 6.4 ng of class I mutant p24 was sufficient to initiate replication in 50% of MT-4 cells (Table 3), we stress that IN remains an important target for the development of antiviral drugs.

This work highlights the importance of paying attention to target cell type when determining mutant viral replication phenotypes. Permissive MT-4 (28, 53) and C8166 (7) cells were previously used to study HIV-1 IN mutant viral replication. Stevenson et al. (53) studied deletions of IN, which are predicted to behave as class II mutants (14). Thus, the lack of mutant viral replication in that study was probably due to class II pleiotropy. Whereas the IN analyzed by Lafemina et al. contained the E152Q class I mutation, it also contained a second change, V151D (28). The effects of V151D alone on MT-4-dependent replication were not addressed in that study, so it is possible that the double mutant behaved differently than a true class I mutant. Cannon et al. (7) used 50 ng of class I D116A to infect 5×10^5 C8166 cells, which, based on our data, should have replicated (Fig. 2 and Table 3). Since infectivity was scored by syncytium formation 3 to 4 dpi, we speculate that those cell cultures were terminated prior to mutant replication (Fig. 2). D116A replication also went undetected in H9 cells despite multiple weeks of observation (7). Consistent with that result, we determined here that H9 was a nonpermissive T-cell line (Table 2).

Cara et al. detected self-limiting replication of integration-defective HIV-1 in primary MDM (8). The mutation analyzed in that study was an IN deletion which, as mentioned, is predicted to behave as class II. Yet, we were unable to detect class I mutant HIV-1 replication in MDM despite using relatively high levels of virus and cells derived from multiple blood donors (Table 2). It is possible that viral strain differences or cell culture techniques contributed to these different results. We note that our results are consistent with previous reports that MDM do not support detectable levels of class I IN mutant replication (17, 60). We propose that future investigations aimed at determining deleterious effects of IN and *att* site mutations on HIV-1 replication should avoid permissive and semipermissive target cell lines. On the other hand, infecting MT-4 cells with $\geq$50 ng of p24, for example, should help determine whether mutants identified as replication-defective in nonpermissive cells are of the class I or class II phenotype (Fig. 2 and Table 3).

Mechanism of class I IN mutant replication in permissive cell lines. Since MT-4 and C8166 cells were originally transformed with HTLV-1, we considered the possibility that preexisting HTLV-1 IN might complement class I HIV-1 integration defects in these cells and in doing so form the basis for cell line permissivity. Our finding that 174xCEM cells also supported mutant viral replication (Fig. 2D), however, ruled out the necessity for preexisting retroviral IN protein. However, 174xCEM cells were notably less permissive than either MT-4 or C8166 cells (Fig. 2 and 3). Thus, might the preexisting HTLV-1 genome somehow contribute to class I IN mutant viral replication? We feel that the HTLV-1 Tax protein, which is expressed in both MT-4 and C8166 cells (23) and is a potent *trans*-activator of the HIV-1 promoter (51), plays a role in permissivity (see below). However, our finding that N/N replicated in MT-4 cells without forming the normal level of integrated provirus (Fig. 4) together with the results that MT-4 and C8166 cells supported frequencies of D116A *pac* recombination that did not differ significantly from nonpermissive Jurkat and CEM-12D7 cells (Table 4), demonstrates that replication occurred in permissive cell lines independent of an HTLV-1 or cellular function(s) that restored functional integration to class I IN mutant HIV-1.

Other parameters of cell line permissivity. (i) Viral entry. We therefore considered other parameters that might contribute to cell line permissivity. Since we observed as much as a 40-fold difference in WT HIV-1 CAT activity across cell lines (Table 1), we focused our attention on functions that influenced permissivity independent of integration. Srivastava et al. (52) previously highlighted rate and efficiency of virus entry as major determinants of permissiveness: whereas 30 min was sufficient to allow 50% of WT HIV-1 to enter C8166 cells, about 4 h was required for Jurkat cells (52). To minimize the effects of these different entry rates, we allowed viral infections to proceed for 16 to 18 h. Under these conditions, we infer that virtually all and between 90 and 95% of potentially infectious virus entered C8166 and Jurkat cells, respectively. We therefore speculate that virus entry did not significantly limit the first round of HIV-1 replication. Although CEM-12D7 cells were not directly tested, we note that, similar to Jurkat cells, 4 h was required for 50% entry into A3.01 cells (52). Since CEM-12D7 cells were derived from A3.01 cells (43), we speculate that entry did not limit the first round of replication in CEM-12D7 cells. Consistent with this interpretation, we determined by fluorescence-activated cell sorting that the levels of HIV-1 receptors CD4 and CXCR4 on the surface of CEM-12D7 cells were equal to or greater than the levels of these proteins on Jurkat, 174xCEM, C8166, and MT-4 cells (data not shown). Despite controlling for entry in first-round infections, however, we speculate that limited entry most likely played an important role in restricting the spread of class I mutant HIV-1 throughout nonpermissive cell cultures.

(ii) Transcription from the HIV-1 promoter. Both MT-4 and C8166 cells express the HTLV-1 Tax protein (23). Since Tax is a strong *trans*-activator of the HIV-1 promoter (51), we investigated promoter activity in the different cell lines. For this, cells were cotransfected with two different reporter gene constructs. Whereas one drove Tat-dependent Luc expression from the HIV-1 promoter, the other construct expressed the gene for R-Luc under the control of the β-actin promoter (55). Since β-actin is a constitutively expressed gene, its expression is used to normalize levels of heterologous gene expression in a variety of cell types and experimental conditions (reviewed in reference 54). Thus, to assess the relative strength of the HIV-1 promoter across cell lines, HIV-1-driven Luc activity was normalized to β-actin-driven R-Luc activity in different transfected cell extracts.

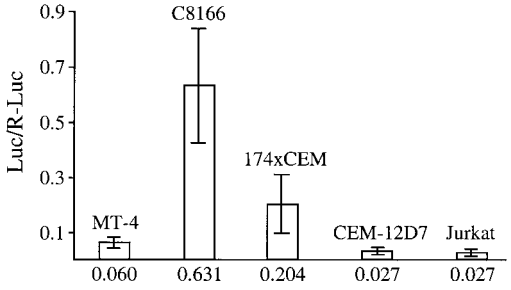

FIG. 5. Cell type-dependent HIV-1 promoter activity. The activity of the HIV-1 promoter relative to β-actin is shown for the indicated cell lines. The average activity following six independent transfections is noted beneath each bar. Error bars represent standard deviation from the mean.

TABLE 5. Comparison of WT and class I IN mutant activities across cell lines

Cell line	Relative CAT activity[a]	Integration[b]	$TCID_{50}$[c]
C8166	0.10	0.023	0.0006
MT-4	0.30	<0.093	0.0017
174×CEM	ND[d]	0.038	0.005
CEM-12D7	0.16	0.10	<0.00008
Jurkat	<0.03	0.024	≤0.00009

[a] Average percentages from Table 1.
[b] From Table 3. Averages are expressed as follows: (D116A *pac*/WT *pac*) × 100%.
[c] From Table 2. Averages are expressed as follows: (NNQ.LTR $TCID_{50}$/WT $TCID_{50}$) × 100%.
[d] ND, not determined.

The results show that nonpermissive CEM-12D7 and Jurkat cells supported similar low levels of HIV-1 gene expression (Fig. 5). In comparison, MT-4, 174xCEM, and C8166 cells supported about 2.2-, 7.6-, and 23-fold more activity, respectively (Fig. 5). We note that HIV-1 transcription levels in CEM-12D7, Jurkat, MT-4, and C8166 cells (Fig. 5) correlated roughly with the levels of WT virus required for 50% CAT activity with these cells (Table 1). This result is consistent with our conclusion that entry did not play a major role in restricting the initial round of mutant viral replication. The results are also consistent with the interpretation that efficient transcription from the HIV-1 promoter played a role in permissivity. We note that the B-lymphoblastoid parent of 174xCEM, 721.174 (46), was transformed with Epstein-Barr virus (13), and that various Epstein-Barr virus proteins can up-regulate the HIV-1 promoter (25, 33, 48). Thus, we propose that a discerning feature of permissive and semipermissive cells is their expression of heterologous viral proteins that enhance HIV-1 transcription.

C8166 cells supported about 10-fold higher levels of HIV-1 transcription than did MT-4 cells (Fig. 5). Similarly, about sevenfold more WT CAT virus was required for MT-4 cells than for C8166 cells (Table 1). Yet, both cell types were equally infected with the WT as measured by $TCID_{50}$s (Table 3). Thus, we conclude that MT-4 cells are more efficient than C8166 cells at fulfilling a step(s) in the HIV-1 cycle that occurs after transcription and translation but prior to the next round of virus entry. Based on this, we speculate that MT-4 cells are particularly efficient at assembling and releasing HIV-1 particles. Similar to the results reported here, we note that other studies reported that MT-4 cells supported the replication of HIV-1 mutants that failed to replicate in less-permissive Jurkat (41) and CEM-12D7 (36) cells.

(iii) Gene expression from unintegrated DNA versus integrated proviruses. What function(s) might contribute to productive class I IN mutant replication? To address this, we compiled in Table 5 a comparison of mutant and WT activities as measured by CAT activity (Table 1), $TCID_{50}$ (Table 3), and DNA recombination frequency (Table 4). In C8166 cells, for example, D116A integrated about 0.023% as frequently as WT, and D116A CAT was about 0.1% as active as WT CAT (Table 5). We therefore conclude that integration could have accounted for as much as 23% of the total CAT activity in D116A-infected C8166 cells. Similarly, we conclude that D116A integration accounted for as much as 31 and 62% of the CAT activity in MT-4 and CEM-12D7 cells, respectively. Based on our results with CD4-LTR/β-Gal cells, however, we note that the activity of D116A relative to that of the WT varied as much as 37-fold in the same cell type. It would therefore appear that unintegrated DNA can express the majority of the Tat protein in cells transiently infected with class I mutants.

In contrast to concluding that unintegrated DNA contributed at least in part to class I mutant gene expression during transient infections, we note that the relative $TCID_{50}$s of NNQ.LTR and WT in 174xCEM, C8166, MT-4, Jurkat, and CEM-12D7 cells were about 8-, 38-, 55-, 267-, and 1,250-fold lower, respectively, than the frequencies of D116A integration in these cell lines (Table 5). Based on this, we conclude that DNA recombination most likely played an important role in class I IN mutant viral replication. In other words, although class I IN mutants supported productive HIV-1 replication in the absence of IN function, they most likely required the integration of their genomes into cell chromosomes to achieve this result. Although integration may be necessary, we note that it alone is not sufficient for mutant viral replication (Table 5). We therefore conclude that the ability of certain T-cell lines to support productive class I IN mutant viral replication depends on a number of permissivity factors, most of which, including virus entry, assembly/release, and transcription, would appear to influence the efficiency of HIV-1 replication independent of integration.

Unintegrated HIV-1 DNA and AIDS dementia. Large levels of unintegrated HIV-1 DNA have been detected in the brains of some patients with AIDS dementia (37, 56). Although the extent to which this DNA might serve as a template for gene expression and subsequent protein production has not been directly tested, our finding that different T-cell lines affected the level of gene expression from unintegrated DNA as much as 10-fold suggests that certain in vivo cell types may also have the ability to preferentially express unintegrated forms of HIV-1. Since secreted Tat protein has been implicated in the development of AIDS dementia (32, 49) and our results are consistent with the interpretation that Tat can be expressed from unintegrated DNA, we propose that it will be worthwhile to assess the activity of class I IN mutant HIV-1 relative to WT in infected human neuronal cell cultures in vitro. The results of

these experiments may shed light on whether gene expression from unintegrated DNA plays a role in HIV-1 pathogenesis and the development of AIDS dementia in vivo.

ACKNOWLEDGMENTS

We thank H. Göttlinger, W. Marasco, J. Sodroski, and M. T. Sweetser for plasmid DNA, H. Chen for help with Southern blotting, K.-T. Jeang for stimulating discussion in the early phase of this project, H. Göttlinger, J. Sodroski, and Å. Ohagen for valuable discussion, and Å. Ohagen for critical review of the manuscript.

This work was supported by NIH grant AI45313 (A.E.), the G. Harold and Leila Y. Mathers Foundation (A.E.), and the Japanese Foundation for AIDS Prevention (N.N.).

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Ansari-Lari, M. L., L. A. Donehower, and R. A. Gibbs.** 1995. Analysis of human immunodeficiency virus type 1 integrase mutants. Virology **211:**332–335.
3. **Arad, U.** 1998. Modified Hirt procedure for rapid purification of extrachromosomal DNA from mammalian cells. BioTechniques **24:**760–762.
4. **Bowtell, D. D. L.** 1987. Rapid isolation of eukaryotic DNA. Anal. Biochem. **162:**463–465.
5. **Brown, H. E. V., H. Chen, and A. Engelman.** 1999. Structure-based mutagenesis of the human immunodeficiency virus type 1 DNA attachment site: effects on integration and cDNA synthesis. J. Virol. **73:**9011–9020.
6. **Brown, P. O.** 1997. Integration, p. 161–203. *In* J. M. Coffin, S. H. Hughes, and H. E. Varmus (ed.), Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
7. **Cannon, P. M., W. Wilson, E. Byles, S. M. Kingsman, and A. J. Kingsman.** 1994. Human immunodeficiency virus type 1 integrase: effect on viral replication of mutations at highly conserved residues. J. Virol. **68:**4768–4775.
8. **Cara, A., F. Guarnaccia, M. S. Reitz, Jr., R. C. Gallo, and F. Lori.** 1995. Self-limiting, cell-type dependent replication of an integrase-defective human immunodeficiency virus type 1 mutant in human primary macrophages but not T lymphocytes. Virology **208:**242–248.
9. **Carteau, S., C. Hoffmann, and F. Bushman.** 1998. Chromosome structure and human immunodeficiency virus type 1 cDNA integration: centromeric alphoid repeats are a disfavored target. J. Virol. **72:**4005–4014.
10. **Cayabyab, M., G. B. Karlsson, B. A. Etemad-Moghadam, W. Hofmann, T. Steenbeke, H. Matilda, J. W. Fanton, M. K. Axthelm, N. L. Letvin, and J. G. Sodroski.** 1999. Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXBc2). J. Virol. **73:**976–984.
11. **Chen, H., S.-Q. Wei, and A. Engelman.** 1999. Multiple integrase functions are required to form the native structure of the human immunodeficiency virus type 1 intasome. J. Biol. Chem. **274:**17358–17364.
12. **de la Luna, S., and J. Ortin.** 1992. pac gene as efficient dominant marker and reporter gene in mammalian cells. Methods Enzymol. **216:**376–385.
13. **DeMars, R., C. C. Chang, S. Shaw, P. J. Reitnauer, and P. M. Sondel.** 1984. Homozygous deletions that simultaneously eliminate expressions of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T cells to mutant cells that have decreased expression of class II antigens. Hum. Immunol. **11:**77–97.
14. **Engelman, A.** 1999. In vivo analysis of retroviral integrase structure and function. Adv. Virus Res. **52:**411–416.
15. **Engelman, A., G. Englund, J. M. Orenstein, M. A. Martin, and R. Craigie.** 1995. Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. J. Virol. **69:**2729–2736.
16. **Engelman, A., Y. Liu, H. Chen, M. Farzan, and F. Dyda.** 1997. Structure-based mutagenesis of the catalytic domain of human immunodeficiency virus type 1 integrase. J. Virol. **71:**3507–3514.
17. **Englund, G., T. S. Theodore, E. Freed, A. Engelman, and M. A. Martin.** 1995. Integration is required for productive infection of monocyte-derived macrophage by human immunodeficiency virus type 1. J. Virol. **69:**3216–3219.
18. **Freed, E. O., and M. A. Martin.** 1996. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. J. Virol. **70:**341–351.
19. **Gaur, M., and A. D. Leavitt.** 1998. Mutations in the human immunodeficiency virus type 1 integrase D, D(35)E motif do not eliminate provirus formation. J. Virol. **72:**4678–4685.
20. **Helseth, E., M. Kowalski, D. Gabuzda, U. Olshevsky, W. Haseltine, and J. Sodroski.** 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J. Virol. **64:**2416–2420.
21. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
22. **Hofmann, W., D. Schubert, J. LaBonte, L. Munson, S. Gibson, J. Scammell, P. Ferrigno, and J. Sodroski.** 1999. Species-specific, postentry barriers to primate immunodeficiency virus infection. J. Virol. **73:**10020–10028.
23. **Jeang, K.-T., D. Derse, M. Matocha, and O. Sharma.** 1997. Expression status of Tax protein in human T-cell leukemia virus type 1-transformed MT-4 cells: recall of MT-4 cells distributed by the NIH AIDS Research and Reference Reagent Program. J. Virol. **71:**6277–6278.
24. **Jenkins, T. M., D. Esposito, A. Engelman, and R. Craigie.** 1997. Critical contacts between HIV-1 integrase and viral DNA identified by structure-based analysis and photo-crosslinking. EMBO J. **16:**6849–6859.
25. **Kenney, S., J. Kamine, D. Markovitz, R. Fenrick, and J. Pagano.** 1988. An Epstein-Barr virus immediate-early gene product trans-activates gene expression from the human immunodeficiency virus long terminal repeat. Proc. Natl. Acad. Sci. USA **85:**1652–1656.
26. **Kikukawa, R., Y. Koyanagi, S. Harada, N. Kobayashi, M. Hatanaka, and N. Yamamoto.** 1984. Differential susceptibility to the acquired immunodeficiency syndrome retrovirus in cloned cells of human leukemia T-cell line Molt-4. J. Virol. **57:**1159–1162.
27. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:**2232–2239.
28. **Lafemina, R. L., C. L. Schneider, H. L. Robbins, P. L. Callahan, K. LeGrow, E. Roth, W. A. Schleif, and E. A. Emini.** 1992. Requirement of active human immunodeficiency virus type 1 integrase enzyme for productive infection of human T-lymphoid cells. J. Virol. **66:**7414–7419.
29. **Lane, J. R.** 1999. Quantitative HIV culture, p. 11–15. *In* N. L. Michael and J. H. Kim (ed.), Methods in molecular medicine, vol. 17: HIV protocols. Humana Press, Totowa, N.J.
30. **Leavitt, A. D., G. Robles, N. Alesandro, and H. E. Varmus.** 1996. Human immunodeficiency virus type 1 integrase mutants retain in vitro integrase activity yet fail to integrate DNA efficiently during infection. J. Virol. **70:**721–728.
31. **Lewis, P., M. Hensel, and M. Emerman.** 1992. Human immunodeficiency virus infection of cells arrested in the cell cycle. EMBO J. **11:**3053–3058.
32. **Liu, Y., M. Jones, C. M. Hingtgen, G. Bu, N. Laribee, R. E. Tanzi, R. D. Moir, A. Nath, and J. J. He.** 2000. Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. Nat. Med. **6:**1380–1387.
33. **Mallon, R., J. Borkowski, R. Albin, S. Pepitoni, J. Schwertz, and E. Kieff.** 1990. The Epstein-Barr virus BZLF1 gene product activates the human immunodeficiency virus type 1 5′ long terminal repeat. J. Virol. **64:**6282–6285.
34. **Masuda, T., V. Planelles, P. Krogstad, and I. S. Y. Chen.** 1995. Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 *att* site: unusual phenotype of mutants in the zinc finger-like domain. J. Virol. **69:**6687–6696.
35. **Miyoshi, I., H. Taguchi, I. Kubonishi, S. Yoshimoto, Y. Ohrauki, Y. Shiraishi, and T. Akagi.** 1982. Type-C virus-producing cell lines derived from adult T-cell leukemia. Jpn. J. Cancer Res. **28:**219–228.
36. **Murakami, T., and E. O. Freed.** 2000. The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions. Proc. Natl. Acad. Sci. USA **97:**343–348.
37. **Pang, S., Y. Koyanagi, S. Miles, C. Wiley, H. V. Vinters, and I. S. Y. Chen.** 1990. High levels of unintegrated DNA in brain tissue of AIDS dementia patients. Nature **343:**85–89.
38. **Parolin, C., B. Taddeo, G. Palu, and J. Sodroski.** 1996. Use of cis- and trans-acting viral regulatory sequences to improve expression of human immunodeficiency virus vectors in human lymphocytes. Virology **222:**415–422.
39. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90:**8392–8396.
40. **Popvic, M., M. G. Sarnagadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497–500.
41. **Reil, H., A. A. Bukovsky, H. R. Gelderblom, and H. G. Göttlinger.** 1998. Efficient HIV-1 replication can occur in the absence of the viral matrix protein. EMBO J. **17:**2699–2708.
42. **Richardson, J. H., W. Hofmann, J. G. Sodroski, and W. A. Marasco.** 1998. Intrabody-mediated knockout of the high-affinity IL-2 receptor in primary human T cells using a bicistronic lentivirus vector. Gene Ther. **5:**635–644.
43. **Ross, E. K., A. J. Buckler-White, A. Rabson, G. Englund, and M. A. Martin.** 1991. Contribution of NF-κB and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. J. Virol. **65:**4350–4358.
44. **Sakai, H., M. Kawamura, J.-I. Sakuragi, S. Sakuragi, R. Shibata, A. Ishimoto, N. Ono, S. Ueda, and A. Adachi.** 1993. Integration is essential for efficient gene expression of human immunodeficiency virus type 1. J. Virol. **67:**1169–1174.

45. **Salahuddin, S. Z., P. D. Markham, F. Wong-Staal, G. Franchini, V. S. Kalyanaraman, and R. C. Gallo.** 1983. Restricted expression of human T-cell leukemia-lymphoma virus (HTLV) in transformed human umbilical cord blood lymphocytes. Virology **129:**51–64.
46. **Salter, R. D., D. N. Howell, and P. Cresswell.** 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. Immunogenetics **21:** 235–246.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Scala, G., I. Quinto, M. R. Ruocco, M. Mallardo, C. Ambrosini, B. Squitieri, P. Tassone, and S. Venuta.** 1993. Epstein-Barr virus nuclear antigen 2 transactivates the long terminal repeat of human immunodeficiency virus type 1. J. Virol. **67:**2853–2861.
49. **Shi, B., J. Raina, A. Lorenzo, J. Busciglio, and D. Gabuzda.** 1998. Neuronal apoptosis induced by HIV-1 Tat protein and TNF-alpha: potentiation of neurotoxicity mediated by oxidative stress and implications for HIV-1 dementia. J. Neurovirol. **4:**281–290.
50. **Shin, C., B. Taddeo, W. A. Haseltine, and C. M. Farnet.** 1994. Genetic analysis of the human immunodeficiency virus type 1 integrase protein. J. Virol. **68:**1633–1642.
51. **Siekevitz, M., S. F. Josephs, M. Dukovitch, N. Peffer, F. Wong-Staal, and W. C. Greene.** 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-1. Science **238:**1575–1578.
52. **Srivastava, K. K., R. Fernandez-Larsson, D. M. Zinkus, and H. L. Robinson.** 1991. Human immunodeficiency virus type 1 NL4-3 replication in four T-cell lines: rate and efficiency of entry, a major determinant of permissiveness. J. Virol. **65:**3900–3902.
53. **Stevenson, M., S. Haggerty, C. A. Lamonica, C. M. Meier, S. K. Welch, and A. K. Wasiak.** 1990. Integration is not necessary for expression of human immunodeficiency virus type 1 protein products. J. Virol. **64:**2421–2425.
54. **Suzuki, T., P. J. Higgins, and D. R. Crawford.** 2000. Control selection for RNA quantitation. BioTechniques **29:**332–337.
55. **Sweetser, M. T., T. Hoey, Y.-L. Sun, W. M. Weaver, G. A. Price, and C. B. Wilson.** 1998. The roles of nuclear factor of activated T cells and ying-yang 1 in activation-induced expression of the interferon-gamma promoter in T cells. J. Biol. Chem. **273:**34775–34783.
56. **Teo, I., C. Veryard, H. Barnes, S. F. An, M. Jones, P. L. Lantos, P. Luthert, and S. Shuanak.** 1997. Circular forms of unintegrated human immunodeficiency virus type 1 DNA and high levels of viral protein expression: association with dementia and multinucleated giant cells in the brains of patients with AIDS. J. Virol. **71:**2928–2933.
57. **Vincent, K. A., D. York-Higgins, M. Quiroga, and P. O. Brown.** 1990. Host sequences flanking the HIV provirus. Nucleic Acids Res. **18:**6045–6067.
58. **Vink, C., M. Groenink, Y. Elgersma, R. A. M. Fouchier, M. Tersmette, and R. H. A. Plasterk.** 1990. Analysis of the junctions between human immunodeficiency virus type 1 proviral DNA and human DNA. J. Virol. **64:**5626–5627.
59. **Weiss, A., R. L. Wiskocil, and J. D. Stobo.** 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pretranslational level. J. Immunol. **133:**123–128.
60. **Wiskerchen, M., and M. A. Muesing.** 1995. Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct gene expression from unintegrated viral DNA templates, and sustain propagation in primary cells. J. Virol. **69:**376–386.
61. **Withers-Ward, E. S., Y. Kitamura, J. P. Barnes, and J. M. Coffin.** 1994. Distribution of targets for avian retrovirus DNA integration in vivo. Genes Dev. **8:**1473–1487.
62. **Yee, J.-K., A. Miyanohara, P. LaPort, K. Bouic, J. C. Burns, and T. Friedmann.** 1994. A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary lymphocytes. Proc. Natl. Acad. Sci. USA **91:**9564–9568.