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Construction and use of a replication-competent human immunodeficiency virus (HIV-1) that expresses the chloramphenicol acetyltransferase enzyme

(marked viruses/replication studies/drug screening)

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ABSTRACT **The construction and properties of an infectious human immunodeficiency virus (HIV) that expresses the bacterial gene chloramphenicol acetyltransferase are described. This virus can be used *in vitro* to screen for drugs that inhibit HIV infection. The marked virus may also be used to trace the routes of infection from the site of inoculation in animal experiments.**

The human immunodeficiency virus type 1 (HIV-1), a human retrovirus, is the causative agent of acquired immunodeficiency syndrome (AIDS) (1–4).

A detailed picture of the pathogenesis of HIV-1 has not been constructed. It is possible that the routes of infection in tissues might be traced in chimpanzees, the only species other than humans known to be infected by the virus. One of the problems for such work is that it is often difficult to detect virus in infected tissues, as very little viral protein or RNA is made. A replication-competent HIV virus that expressed an enzyme not normally present in animal tissues might facilitate this work.

The genomes of HIV and closely related viruses possess several coding regions in addition to the *gag, pol,* and *env* genes characteristic of all retroviruses. Most of these reading frames encode products that are essential for the viral life cycle. The *tat* (5–8) and *rev* (also called *trs*) (9–11) genes encode small regulatory proteins critical for HIV replication and gene expression. The *vif* gene encodes a 23-kDa protein important for virus infectivity (12, 13). However, the *nef* gene, located at the 3′ end of the viral genome immediately following the *env* gene and overlapping the 3′ long terminal repeat, encodes a 27-kDa protein which has been determined not to be required for either the infectivity or cytopathicity of the virus in cultured human T cells (14, 15). As this open reading frame is relatively long, overlaps no other known HIV genes, and is clearly nonessential, at least *in vitro*, it was considered to be an excellent candidate for replacement with a foreign gene.

MATERIALS AND METHODS

Cell Lines and Viruses. The HIV proviral clone pHXBc2 was kindly supplied by R. C. Gallo and F. Wong-Staal (16). The Jurkat cell line is a T4$^+$ human malignant T-lymphoblastic line provided to us by the laboratory of Cox Terhorst (Dana–Farber Cancer Institute). C8166 cells are a human T-cell lymphotropic virus type 1-transformed human lymphocyte line expressing very high levels of the T4 marker (17).

DNA Transfections. Jurkat cells were transfected by a DEAE-dextran technique as described (18), using 10^7 cells per transfection. Afterwards, the cells were resuspended in 15 ml of RPMI 1640 medium plus 15% fetal bovine serum. All cells were subsequently given a complete medium change daily throughout the course of the experiment.

HeLa cells were seeded at a density of 10^6 cells per 100-mm plate the day before transfection. Cells were transfected the next day by a calcium phosphate coprecipitation technique as described (18). Cells were collected for assay 48 hr later.

Chloramphenicol Acetyltransferase (CAT) Assays. Cells were spun down, washed once with phosphate-buffered saline (PBS), and resuspended in a small volume of 200 mM Tris·HCl (pH 7.5). Lysates were then analyzed for CAT activity as described (18).

Reverse Transcriptase Assays. For each assay, 1 ml of culture medium was collected and centrifuged for 1 hr at 15,000 × *g* to pellet virions. Pellets were resuspended in 10 μl of 50 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.25 M KCl/20% (vol/vol) glycerol and assayed as described (19).

HIV p24 Assay. Culture medium (1 ml) was centrifuged for 1 hr at 15,000 × *g* to pellet virions. Pellets were resuspended in 100 μl of assay buffer containing 0.5% Triton X-100 and assayed using a commercially prepared HIV p24 radioimmune assay kit (New England Nuclear, NEK-040) according to the manufacturer's directions.

Immunoprecipitation. Cells were washed once with PBS and resuspended in 2.5 ml of cysteine-free RPMI 1640 medium plus 10% fetal bovine serum supplemented with 50 μCi of [^{35}S]cysteine per ml (1 Ci = 37 GBq). Cells were labeled overnight and then harvested, washed with PBS, and lysed in 0.5 ml of 0.05 M Tris·HCl, pH 7.0/0.15 M NaCl, containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Immunoprecipitation with AIDS patient antiserum and polyacrylamide gel electrophoresis were performed as described (20).

RESULTS

The first step toward the replacement of *nef* sequences with a marker gene was the removal of nonessential sequences in the 3′ end of the genome. The starting plasmid used for these experiments, pHXBc2 *Xba* I, was an infectious proviral clone, pHXBc2 (16), containing an artificially introduced restriction enzyme cleavage site near the 3′ end of the gene encoding the envelope transmembrane protein (21). This mutation introduced a unique *Xba* I site as well as a termination signal at a position 17 codons before the natural termination signal of the envelope glycoprotein (Fig. 1). Preliminary experiments had indicated that the presence of this restriction site did not alter the kinetics of virus replication in Jurkat cells as compared to the pHXBc2 parental virus (data not shown).

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Abbreviations: HIV-1, human immunodeficiency virus type 1; CAT, chloramphenicol acetyltransferase.

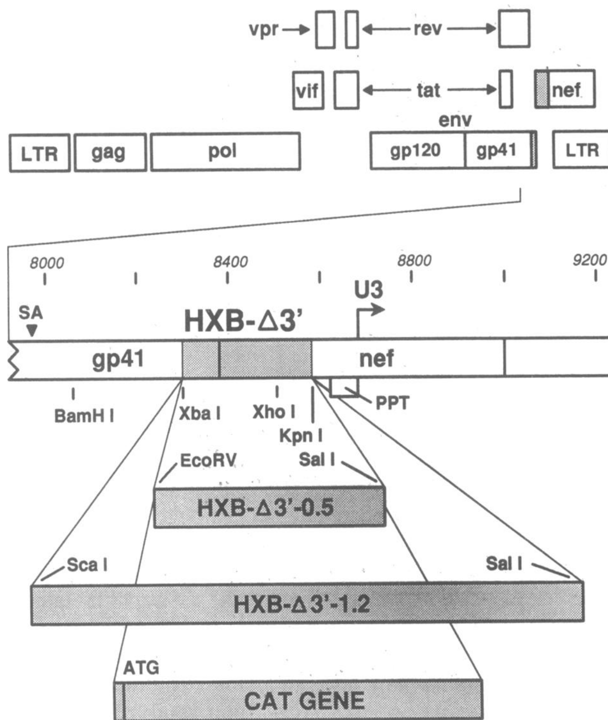

FIG. 1. Diagram of HIV proviral constructions. (*Upper*) Scheme of the parental clone pHXBc2 showing locations of the different reading frames. LTR, long terminal repeat. (*Lower*) Detail of the 3′ end of the genome. pHXB-Δ3′ was constructed by deleting the region between a 12-bp *Xba* I linker insertion into the 3′ end of the *env* gene and a *Kpn* I site located within the *nef* gene. pHXB-Δ3′-0.5 and pHXB-Δ3′-1.2 contain segments of pBR plasmid DNA inserted in place of the region deleted in pHXB-Δ3′. The segment inserted in pHXB-Δ3′-0.5 was derived from the 470-bp pBR fragment located between the unique *Eco*RV/*Sal* I cleavage in that plasmid. The segment in pHXB-Δ3′-1.2 was derived from a 1.17-kb fragment excised using the *Sal* I and *Sca* I sites. pHXB-CAT1 contains an 800-bp cassette excised from the plasmid pU3R-I (18) carrying the coding sequences of the bacterial CAT gene inserted into pHXB-Δ3′ between the *Xba* I and *Kpn* I sites. pHXB-CAT2 (not diagrammed) was prepared by inserting the same cassette into a proviral clone containing a slightly smaller deletion between the *Xba* I site and an *Xho* I site located within the *nef* gene. In each case, appropriate linkers were attached to the inserted segments to make them compatible with the proviral vector.

The sequences in pHXBc2 *Xba* I between the *Xba* I site and a *Kpn* I site located 60 nucleotides 5′ to the beginning of the 3′ long terminal repeat were excised, resulting in a mutant provirus (pHXB-Δ3′). This provirus contains a deletion of 280 nucleotides including the 3′ end of the *env* gene, the *nef* initiation codon, and 220 nucleotides of the *nef* gene.

To investigate possible size constraints for insertion of foreign DNA into the proviral genome upon normal virus function, two DNA segments derived from the plasmid pBR322 were inserted in place of the deleted segment to produce the plasmids pHXB-Δ3′-0.5 and pHXB-Δ3′-1.2 (Fig. 1). Each proviral clone was then transfected into Jurkat cells and monitored daily for signs of HIV infection. Parameters checked included cell number, syncytia formation, and reverse transcriptase activity of the culture supernatants (Table 1).

Virus produced by transfection with pHXBc2 *Xba* I replicated as well as that produced by the wild-type provirus. pHXB-Δ3′-0.5, containing a 470-nucleotide insertion for a net size increase of 190 nucleotides, also produced virus that replicated as well as the wild type. However, virus derived from transfection with pHXB-Δ3′-1.2 containing an insert of 1.17 kilobases for a net size increase of ≈890 base pairs (bp)

Table 1. **Summary of data collected at two time points after transfection of Jurkat cells with the parental provirus plasmid pHXBc2 or one of the deletion mutants derived from it**

Plasmid	Syncytia formation		Cytopathic effect		Reverse transcriptase	
	Day 5	Day 12	Day 5	Day 12	Day 5	Day 12
pHXBc2	+++	+	++	+++	+++	+++
pHXB-Δ3′	+++	+	++	+++	+++	+++
pHXB-Δ3′-0.5	+++	+	++	+++	+++	+++
pHXB-Δ3′-1.2	+	++		++	+	++

were dramatically attenuated for replication. Transfection with this plasmid resulted in the appearance initially of only small numbers of syncytia and very low level production of reverse transcriptase activity. No significant cytopathic effect on the cells was observed well after the time of extensive cell death in the other three cultures (days 4–6). Only at much later times posttransfection (days 10–12) was there some increase in the number of syncytia and amount of reverse transcriptase activity in the pHXB-Δ3′-1.2-transfected culture, coincident with a decline in the numbers of viable cells. Filtered supernatant collected from the pHXB-Δ3′-1.2-transfected culture proved unable to initiate new cycles of infection when added to fresh uninfected Jurkat cells. Supernatants from cultures transfected with the other proviruses used in the experiment (diluted to correct for differences in virus titers as measured by reverse transcriptase activity) all initiated new rounds of infection when added to fresh Jurkat cultures.

These experiments suggested that it might be possible to insert a small functional gene at the *Xba* I site without interfering with virus replication. The gene for CAT was selected as an indicator, as the entire coding sequence is located within a 750-nucleotide-long region. Moreover, a simple sensitive assay for this enzyme activity exists (22) and antisera that recognize the CAT protein are available (23).

Two proviral plasmids that incorporated the coding sequences for the CAT gene were made. A segment of DNA ≈800 nucleotides long that contained the entire coding sequence for the CAT gene as well as the translation initiation and termination signals was excised from the plasmid pU3R-I (18) and inserted into each provirus. For pHXB-CAT1 the CAT gene was inserted between the *Xba* I and *Kpn* I sites in place of the sequences removed in the deleted provirus pHXB-Δ3′. For pHXB-CAT2, a similar insertion was made between the *Xba* I site and the *Xho* I site within the *nef* gene. In both proviruses, the CAT gene is located 3′ to the natural splice acceptor used for production of the *nef* protein (24). The CAT cassette itself carries no promoter or other regulatory elements, so that expression of the gene is entirely dependent on the viral long terminal repeat. The plasmid pHXB-CAT1 has a net size increase of 570 bp over the wild-type pHXBc2 proviral clone and in turn is 110 bp smaller than pHXB-CAT2. Both proviruses were transfected into Jurkat cells.

Fig. 2 shows that the virus produced by the pHXB-CAT1 provirus replicated only slightly slower than the wild-type virus. The course of infection in cells transfected with pHXB-CAT1 appeared to lag behind that in the culture transfected with pHXBc2 by 2–3 days, by whatever parameter was measured. However, by day 8–9 posttransfection, the pHXB-CAT1 culture attained high levels of reverse transcriptase activity and a dramatic cytopathic effect very similar to those seen in the wild-type culture. By contrast, although the culture transfected with pHXB-CAT2 exhibited low levels of reverse transcriptase activity from 3 days posttransfection onward and displayed significant syncytia

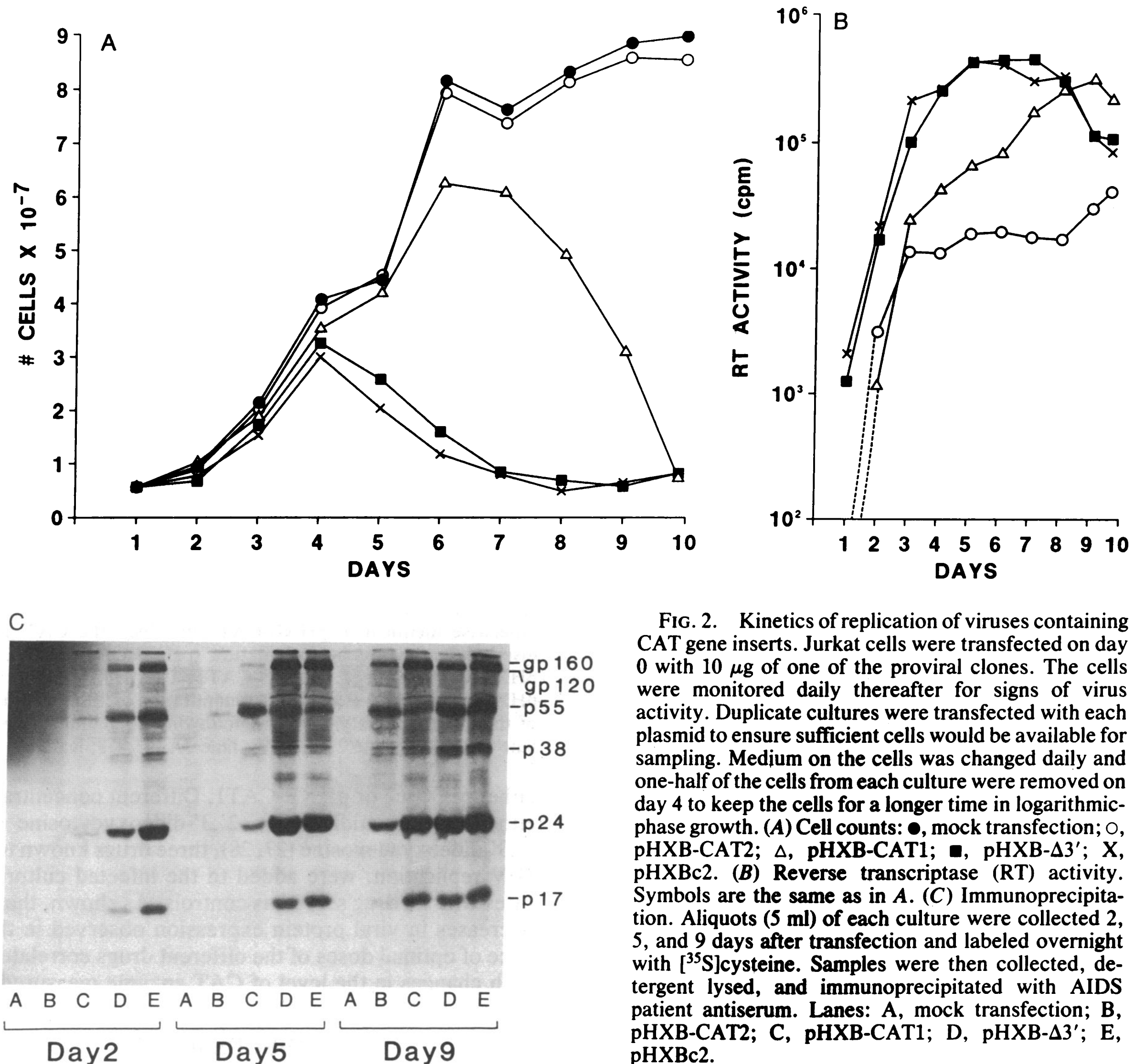

FIG. 2. Kinetics of replication of viruses containing CAT gene inserts. Jurkat cells were transfected on day 0 with 10 μg of one of the proviral clones. The cells were monitored daily thereafter for signs of virus activity. Duplicate cultures were transfected with each plasmid to ensure sufficient cells would be available for sampling. Medium on the cells was changed daily and one-half of the cells from each culture were removed on day 4 to keep the cells for a longer time in logarithmic-phase growth. (A) Cell counts: ●, mock transfection; ○, pHXB-CAT2; △, pHXB-CAT1; ■, pHXB-Δ3′; X, pHXBc2. (B) Reverse transcriptase (RT) activity. Symbols are the same as in A. (C) Immunoprecipitation. Aliquots (5 ml) of each culture were collected 2, 5, and 9 days after transfection and labeled overnight with [^{35}S]cysteine. Samples were then collected, detergent lysed, and immunoprecipitated with AIDS patient antiserum. Lanes: A, mock transfection; B, pHXB-CAT2; C, pHXB-CAT1; D, pHXB-Δ3′; E, pHXBc2.

formation, the infection did not progress normally and a significant cytopathic effect was not observed as late as 10 days posttransfection. Furthermore, filtered supernatant fluid from the culture transfected with pHXB-CAT2 on day 10 did not produce indications of virus infection when applied to fresh Jurkat cells. Corresponding supernatants containing equivalent reverse transcriptase units from cultures transfected with pHXBc2, pHXB-Δ3′, and pHXB-CAT1 all were able to initiate new cycles of infection (data not shown).

These observations are closely mirrored by the results of protein-labeling experiments. On days 2, 5, and 9 posttransfection, aliquots of each culture were metabolically labeled with [^{35}S]cysteine, lysed, and immunoprecipitated with AIDS patient antiserum (Fig. 2C). HIV proteins are strongly evident in aliquots from cultures transfected with either wild-type or pHXB-Δ3′ proviruses even when labeling was initiated only 48 hr posttransfection. The small difference seen in this experiment between the day-2 pHXB-Δ3′ and pHXBc2 lanes was not repeatable. Virus-specific proteins are only weakly evident in lanes representing day-2 lysates from cells transfected with pHXB-CAT1 or pHXB-CAT2 proviruses. However, by day 5 the virus-specific proteins do accumulate in the lysate from the pHXB-CAT1 culture, and by day 9 the pHXB-CAT1 lane is virtually indistinguishable from the pHXBc2 and pHXB-Δ3′ lanes. By contrast, the day-5 lysate from the culture transfected with pHXB-CAT2 is not significantly different from the day-2 lysate. Only in the day-9 lysate can an increase in the intensity of the virus-specific bands in the pHXB-CAT2 culture be seen. Still at this time the amounts of viral proteins detected are well below those observed in the lysates from the other transfected cultures.

To test for expression of the inserted CAT gene, aliquots of Jurkat cells transfected with pHXB-CAT1 were collected daily. Results of CAT assays carried out on these samples are summarized in Fig. 3. CAT activity was easily detectable only 1 day posttransfection and quickly progressed to very high levels as the infection spread through the culture. The slight decline in the level of CAT expression at late times posttransfection presumably reflects the loss in viability of the culture that is occurring by this time.

Expression of the *gag/pol* and envelope proteins of HIV is known to require the presence of the *rev* gene product (9, 10). Evidence also exists that suggests that the *nef* gene product is made independently of *rev*. The mRNA for the *nef* product removes by splicing the known positive and negative cis-acting elements crucial to *rev* regulation (24, 25). To test whether expression of CAT enzyme by pHXB-CAT1 is under *rev* control, we introduced a frameshift at the *Bam*HI site within the provirus that eliminated the ability of the provirus to produce a functional art protein. The pHXBCAT-1 and pHXB/BFS-CAT1 plasmids were then transfected into HeLa cells. HeLa cells, which do not express T4, were used so that reinfection of cells by the nondefective pHXB-

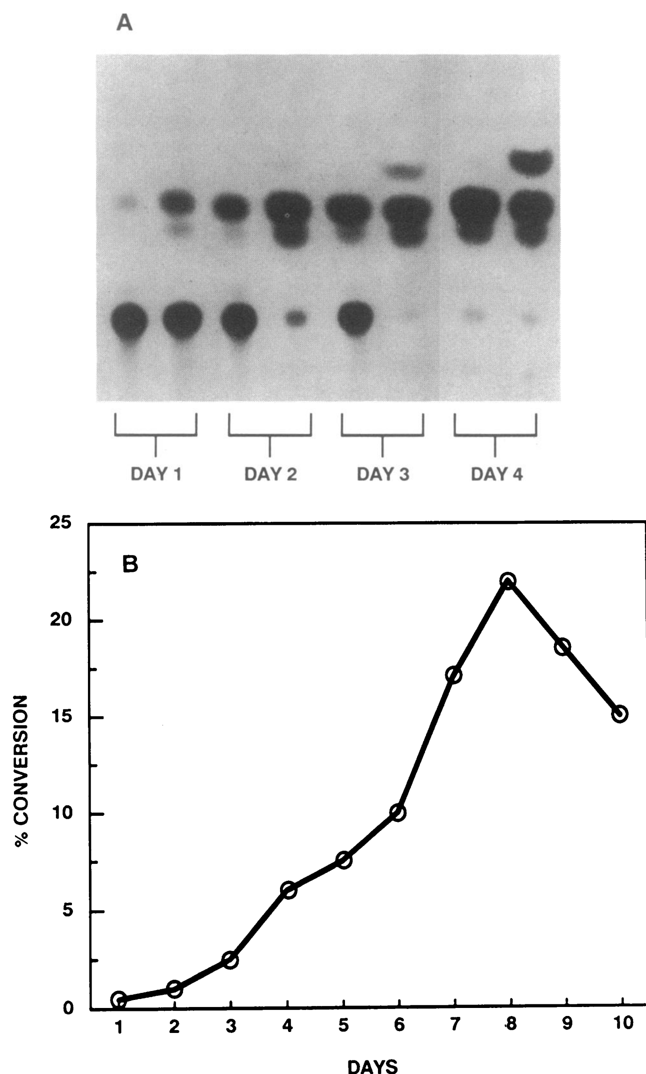

FIG. 3. CAT activity in cultures transfected on day 0 with pHXB-CAT1. One milliliter was collected daily. The cells were washed once with PBS and then resuspended in 150 μl of 200 mM Tris (pH 7.5) for CAT assays. (*A*) Autoradiogram of a CAT assay representing conversions obtained with extracts from days 1–4 in the experiment. Each pair of spots in the autoradiogram represents conversion after 5- and 60-min reaction times. Extracts from day-3 posttransfection onward had to be assayed at 1:10 or 1:100 dilutions to maintain the conversions within the linear range of the assay. (*B*) Changes in the level of CAT enzyme activity recorded over the entire course of the experiment. The scale used for the graph is percent conversion of [^{14}C]chloramphenicol per min by 70 μl of 1:10 dilution of the original samples.

CAT1-derived virus would not occur. CAT activity of the cells was assayed 48 hr later. As shown in Fig. 4, CAT activity in cells transfected with pHXB/BFS-CAT1 was similar to that seen in cells transfected with pHXB-CAT1. Transfection of cells with a provirus containing the CAT gene inserted in the antisense orientation (pHXB-CAT1-anti) produced no CAT activity. Cotransfection of HXB/BFS-CAT1 with a *rev* expressor plasmid, pIIIex*art*, resulted in a decrease in CAT activity by a factor of ≈4. This is consistent with a reported decrease in the ratio of spliced to unspliced HIV mRNAs seen in the presence of *rev*. Therefore, expression of CAT in pHXB-CAT1 is not dependent on the presence of the HIV rev protein.

As a first application of the pHXB-CAT1 provirus, the use of the marked virus as an assay for antiviral drugs was attempted (Fig. 5). Duplicate sets of C8166 cell cultures were infected with equivalent titers of virus stocks derived from

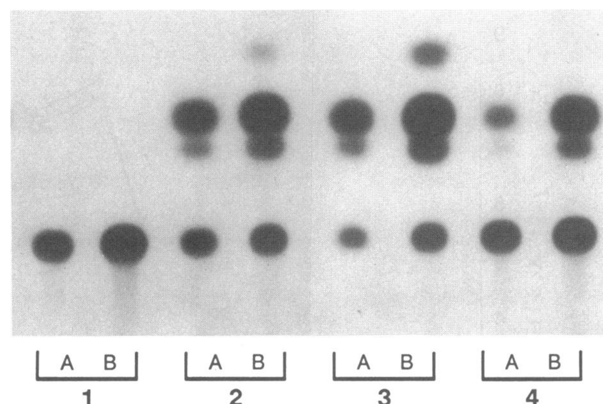

FIG. 4. Plates containing 10^6 HeLa cells were transfected with either pHXB-CAT1 or a *rev*-defective variant, pHXB/BFS-CAT1, with or without a rev expressor pIIIex*art*. Ten micrograms of each proviral plasmid was used, plus 8 μg of pIIIex*art*. Forty-eight hours posttransfection, the cells were scraped into PBS, spun down, and resuspended in 150 μl of 0.25 M Tris·HCl (pH 7.5) for CAT assays. Autoradiogram shows conversions obtained after 5-min (lane A in each pair) and 60-min (lane B in each pair) incubations with 70 μl of each lysate. Control lysate was from transfection of a construction otherwise identical to pHXB-CAT1 in which the CAT gene was inserted in the reverse orientation (pHXB-CAT-anti). Lanes: 1, pHXB-CAT1-anti; 2, pHXB-CAT1; 3, pHXB/BFS-CAT1; 4, pHXB/BFS-CAT1 + pIIIexart. Quantitation of the counts from each of the 5-min assays yielded the following percentage conversions: lane 1A, 0–3%; lane 2A, 35.3%; lane 3A, 31.4%; lane 4A, 8.5%.

either pHXBc2 or pHXB-CAT1. Different concentrations of either azidothymidine (26), 2′,3′-dideoxycytosine (27), or 2′,3′-dideoxyadenosine (27, 28), three drugs known to inhibit HIV replication, were added to the infected cultures. Cultures with no drug served as controls. As shown, the marked decreases in viral protein expression observed in the presence of optimal doses of the different drugs correlate closely with changes in the level of CAT enzyme measured.

DISCUSSION

The experiments presented here demonstrate that a foreign gene can be incorporated into a HIV provirus and successfully expressed without disrupting functions critical for virus replication and cytopathic effect. The major constraint upon DNA inserted into the region of the *nef* gene of the HIV provirus appears to be size. The normal full-length HIV is apparently close to the maximum size permitted for efficient transmission, as proviruses with net-size increases of 700 nucleotides or more compared to the wild type were unable to mount successful infections after exposure of T cells to supernatants from cells transfected with these plasmids. The limitation on the size of the insert may reflect limitations on the size of the virion RNA that can be efficiently packaged into the virus particle. The experiments also show that the HIV-CAT1 virus can be used to measure the effect of antiviral drugs. Use of this virus should permit a rapid, quantitative means of screening antiviral drug activity. The experiments also show that a gene in the position of *nef* can be expressed in the absence of *rev* activity.

Noteworthy is the observation that the provirus pHXB-Δ3′ used for these experiments lacks the coding sequence of the final 17 amino acids of gp41. The carboxyl-terminal sequences of the *env* gene protein are highly conserved amongst HIV-1 isolates and between HIV-1 and HIV-2 and simian immunodeficiency virus (29–31). Nonetheless, viruses that contain envelope proteins lacking these sequences replicate well.

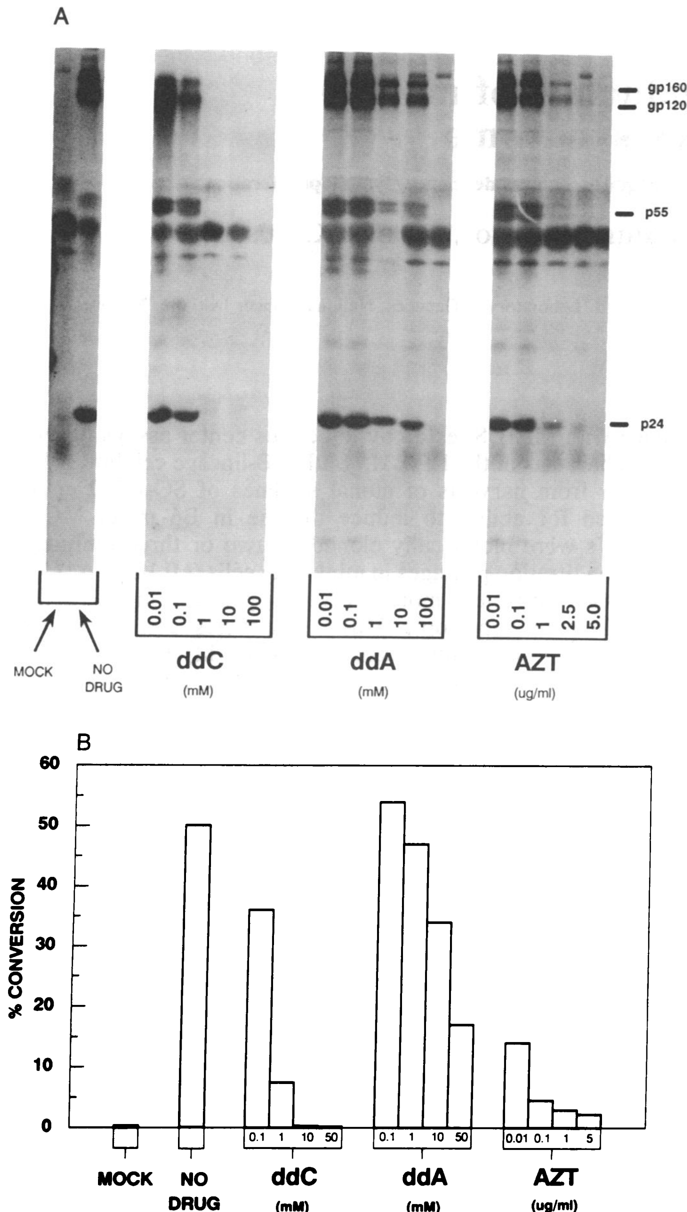

FIG. 5. Inhibition of virus replication in cultures infected with pHXBc2 or pHXB-CAT1-derived viruses by azidothymidine (AZT), 2′,3′-dideoxycytosine (ddC), or 2′,3′-dideoxyadenosine (ddA). C8166 cells were set up in 24-well plates at a density of 2×10^5 cells per ml, 1 ml per well, in various concentrations of drug or with no drug. Duplicate wells were set up for each drug concentration. Reverse transcriptase (2000 units of wild-type pHXBc2-derived virus or 2000 units of pHXB-CAT1-derived virus) was added to each well. Reverse transcriptase activity in the virus stocks was determined as described in the legend to Fig. 2*B*. Cells were then incubated for 1 week with a partial medium change on day 4. Aliquots of the cells and media were collected and assayed on day 7. (*A*) Immunoprecipitations. Cells in wells infected with pHXBc2-derived virus were labeled overnight with [^{35}S]cysteine. Samples were then collected, detergent lysed, and immunoprecipitated with AIDS patient antiserum. (*B*) CAT assays. Cells in wells infected with pHXB-CAT1-derived virus were washed and collected in 85 μl of 0.25 M Tris·HCl (pH 7.5). Scale used in the graph represents percentage conversion of [^{14}C]chloramphenicol in 60 min by 70 μl of each extract.

The construction of an infectious HIV virus that expresses a functional CAT gene should provide a sensitive enzyme marker for studies of infection in animals. At the peak of virus production in Jurkat cells transfected with the HXB-CAT1 provirus, CAT activity could be detected by using as little as 10 μl of the suspension culture, containing only a few thousand cells. The CAT assay appears to be at least as sensitive as the reverse transcriptase assay for detection of the virus activity and does not require concentration of virions. Use of this marked virus should permit rapid analysis of the tissues that are infected as well as measure the level of virus expression in different organs.

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