Inhibition of Recombinant Human Immunodeficiency Virus Type 1 Replication by a Site-Specific Recombinase

C. CLAY FLOWERS, CLIVE WOFFENDIN, JERZY PETRYNIAK, SHIAOLAN YANG,
AND GARY J. NABEL*

*Departments of Internal Medicine and Biological Chemistry, Howard Hughes Medical Institute,
University of Michigan Medical Center, Ann Arbor, Michigan 48109-0650*

Received 23 September 1996/Accepted 19 December 1996

Current molecular genetic strategies to inhibit productive human immunodeficiency virus type 1 (HIV-1) replication have involved the generation of gene products which provide intracellular inhibition of essential virally encoded proteins or RNA structures. A molecular strategy to excise proviral DNA from HIV-1-infected cells and render these cells virus free would provide an attractive direct antiviral strategy, providing a mechanism to remove viral genes from infected cells. The potential of such a molecular genetic intervention was examined by using the Cre-loxP recombination system. A recombinant HIV-1 clone, designated HIV^{lox}, that contains loxP within a nonessential U3 region of the long terminal repeats was synthesized. The loxP motif was maintained during replication of HIV^{lox} in CEM cells, as demonstrated by reverse transcriptase PCR analyses of genomic RNA isolated from virions. Two different types of HIV-1-permissive cells, CEM cells and 293 cells expressing the CD4 glycoprotein, were transformed with a Cre expression vector which was shown to encode Cre DNA binding and recombinase activities. HIV^{lox} infection of CEM or CD4⁺ 293 cells expressing Cre resulted in a substantial reduction in virus replication compared to control cells, and evidence for the presence of the expected excision product was found. Site-specific excision of HIV-1 can therefore be achieved by using this model system with acute infection. These studies represent one step toward the development of a novel antiviral strategy for the treatment of AIDS.

Human immunodeficiency virus type 1 (HIV-1) replication is initiated through interaction of the viral envelope glycoprotein gp120 with its host cell receptor CD4 (11). Fusion of viral and cellular membranes results in release of the nucleocapsid into the cytoplasm (30). Positive-stranded genomic RNA serves as template for synthesis of a cDNA by HIV-1 reverse transcriptase (RT) (10). Following translocation of the cDNA into the nucleus, the viral integrase mediates insertion of the cDNA into cellular chromatin to produce the HIV-1 provirus (14). Establishment of the provirus is an obligatory step in HIV-1 replication and serves to maintain viral sequences within the infected cell and to transmit these sequences to daughter cells after cellular division. The long terminal repeat (LTR) of proviral DNA directs the expression of viral genes, including those encoding components of the virion (6). HIV-1 particles are assembled at the plasma membrane, followed by budding and proteolytic cleavage of viral structural proteins by the HIV-1 protease to produce mature virions (10).

Antivirals against HIV-1 have been targeted to virally encoded enzymes such as RT, integrase, and protease. Compounds which inhibit RT or integrase may prevent the establishment of proviral DNA, while inhibitors of the viral protease block the maturation of virus particles. Chemical agents targeted to virally encoded enzymes may provide only transient inhibition of virus replication due to the rapid generation of drug-resistant HIV-1 quasispecies (32).

An alternative strategy has been the use of antiviral genes that are delivered to uninfected cells as either RNA or DNA and provide intracellular protection from HIV-1 infection (12).

Antiviral genes include those encoding antisense molecules, ribozymes, transdominant proteins, and intracellular antibodies. The current antiviral strategies, both chemical and genetic, target steps in virus replication leading to integration of proviral DNA or target steps following the establishment of the integrated provirus; however, a strategy to target proviral DNA directly has not been reported. One such strategy takes advantage of the Cre-loxP recombination system of bacteriophage P1, which has been utilized extensively in the excision of DNA both in vitro and in vivo (1, 4, 8, 13, 15, 16, 27, 28, 33, 36–40). A modified Cre protein recognizing sequences within each LTR of proviral DNA could mediate excision of viral coding sequences, resulting in the elimination of the intact provirus.

The Cre-loxP recombination system of bacteriophage P1 catalyzes site-specific recombination in vitro (2, 4), in eukaryotic cells (5, 8, 13, 36–40), and in transgenic animals (15, 16, 27, 28, 33). Only two components are required to mediate recombination; the 38-kDa Cre recombinase and the 34-bp loxP site (1, 19, 20, 22). Cre can mediate either inter- or intramolecular recombination upon recognition of two loxP sites on either linear or supercoiled DNA (2, 4). The loxP site consists of two 13-bp inverted repeats flanking an asymmetric 8-bp spacer (19). Cre excises DNA that is flanked by two loxP sites in direct orientation, producing one circular and one linear DNA molecule. When confronted with two loxP sites arranged in opposite orientation, Cre mediates an inversion of the intervening DNA (2).

Excisive recombination has been used to construct recombinant molecules in cell-free systems (23) and for tissue-specific or inducible gene expression in the transgenic mouse (15, 16, 27, 28, 33). Cre-mediated integrative recombination between two DNA molecules each possessing a loxP motif has been used in vitro to insert mutations specifically into the genomes of viruses (34, 41) and to integrate plasmid DNA into cellular chromatin in a site-specific manner (8, 40). The spec-

* Corresponding author. Mailing address: Howard Hughes Medical Institute, University of Michigan Medical Center, Departments of Internal Medicine and Biological Chemistry, 1150 W. Medical Center Dr., 4520 MSRB I, Ann Arbor, MI 48109-0650. Phone: (313) 647-4798. Fax: (313) 647-4730. E-mail: gnabel@umich.edu.

ificity and efficiency of Cre-mediated recombination and the potential to devise a novel antiviral genetic approach to HIV-1 based on Cre recombinase prompted the determination of whether Cre could provide protection to HIV-1-permissive cells when challenged with a recombinant HIV-1 containing wild-type *loxP*. The results reported in this study demonstrate that expression of Cre provides intracellular inhibition of replication of a recombinant HIV-1 containing *loxP* and indicate that a modified Cre recognizing viral sequences within the LTR has the potential to be an effective antiviral against HIV-1 infection.

MATERIALS AND METHODS

Plasmids. PCR of phage P1 DNA was used to amplify a DNA fragment containing the Cre open reading frame (ORF) (42). The sense primer (5'-TTT TTTAAGCTTCACCATGGCCAATTTACTGACCGTACACCAA-3') introduced *HindIII* and *NcoI* restriction sites and a Kozak sequence for translation initiation (26). The antisense primer (5'-TTTTTTGAGCTCTAGACATCGCC ATCTTCCAGCAG-3') added one amino acid at the carboxy terminus that was followed by a termination codon. The Cre ORF was cloned into the *HindIII* and *XbaI* restriction sites of vector pRc/RSV (Invitrogen) to generate the expression plasmid pRSVCre. The Cre ORF was cloned into pGEX-KT (17) by digestion of pRSVCre with *NcoI* and *XbaI*, releasing the insert DNA, by filling in of both termini of insert DNA with Klenow fragment of DNA polymerase I and by ligation of insert DNA with pGEX-KT vector DNA digested with *BamHI* and *EcoRI* and made blunt by treatment with Klenow fragment.

Generation of recombinant HIV-1. To generate HIV-1 that contains a *loxP* site (HIV^{lox}), a 46-bp oligonucleotide (5'-CCGGAATAACTTCGTATAATGTATG CTATACGAGTTATCCCGGGT3'; the *loxP* sequence is underlined) was inserted into plasmid pNL4-3, which contains the 5' LTR and coding sequences of HIV^{NY5/BRU} (3). The oligonucleotide was cloned into a unique *BspEI* site at position 308 of HIV^{NY5/BRU}. Wild-type or HIV^{lox} was generated by transfection of 293 embryonic kidney epithelial cells with pNL4-3 insert DNA that was ligated end-to-end to restore the 3' LTR. Transfected 293 cells were cocultivated with CEM cells, and infectious virus was further amplified by infection of CEM cells. Titers of virus stocks were determined by limiting dilution, using MAGI cells (25). Virus replication was monitored by the levels of RT activity in infected cell culture supernatants (35). The presence of the *loxP*-containing oligonucleotide within viral genomic RNA was confirmed by RT-PCR. Virus particles were collected by centrifugation of infectious cell culture supernatant, and genomic RNA was isolated by using Trizol (GIBCO BRL). Purified RNA was incubated with RNase-free DNase I for 1 h at 37°C. The first-strand cDNA was synthesized by using oligo(dT) and Superscript II reverse transcriptase (GIBCO BRL) for 1 h at 49°C. Virus-specific primers 1A (5'-ATTGGCAGAACTACACACCAG-3') and 1B (5'-CCAGGCTCAGATCTGGTCTAA-3') were labeled with [γ -³²P]ATP (Amersham), using T4 polynucleotide kinase, and were used for PCR amplification of viral sequences that flank the insertion of the *loxP* oligonucleotide. For *SmaI* restriction digestion of RT-PCR products, 20 U of the enzyme was added directly to the PCR mixture.

Detection of Cre-mediated HIV^{lox} excision product. Standard conditions for PCR were used for analyses of HIV^{lox}-infected cell lysates. Oligonucleotide primers 2A (5'-AATGACTTACAAGGAGCTGTAGATCTTAGCCA-3') and 2B (5'-AGCCGAGTCTCGCTCGAGAGATCTCTCTGGC-3') were radiolabeled with T4 polynucleotide kinase and used in a 50- μ l PCR with 0.25 μ M or 1/10 total primer concentration consisting of labeled oligonucleotide. Radiolabeled RT-PCR products were separated through a 4% nondenaturing polyacrylamide gel. The gel was dried and exposed to Kodak X-Omat film.

Electrophoretic mobility shift assays (EMSA). The preparation of nuclear extracts and transfection of 293 cells were conducted essentially as described previously (29). Cre binding activity was analyzed in transiently transfected 293 cells by adjusting nuclear extracts to 50 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, and 33 mM NaCl, buffer conditions optimal for Cre binding (20). A double-stranded oligonucleotide probe containing the *loxP* site was labeled with [γ -³²P]ATP and incubated with 1 μ g of nuclear extract. Protein-DNA complexes were analyzed by electrophoresis through a 4% nondenaturing polyacrylamide gel.

Generation of cell lines expressing Cre. CEM cells were permanently transformed with pRSVCre or control vector DNA by electroporation. Ten micrograms of linear plasmid DNA and 2×10^6 cells in 0.4-cm cuvettes were pulsed with a Bio-Rad Gene pulser with settings of 300 V and 960 μ F. Twenty-four hours after electroporation, geneticin (G418; Sigma) was added to cell culture medium at a final concentration of 0.8 mg/ml (active concentration). Cells were maintained under antibiotic selection until used for challenge experiments with HIV-1.

In vitro recombination assay. Cre-mediated recombination was assayed in vitro, using purified Cre or nuclear extracts from pRSVCre stably transformed CEM cells. Target DNA for excisional recombination was λ SHLOX (Novagen) genomic DNA. Various amounts of purified Cre or nuclear extracts were added to 100 ng of λ SHLOX DNA in Cre reaction buffer (50 mM Tris-Cl [pH 7.5], 33

FIG. 1. Generation of recombinant HIV^{NY5/BRU} containing *loxP* sites within the LTR. (A) The structure of HIV^{lox} provirus is shown schematically. Rectangles represent the LTRs, a single line denotes the *gag*, *pol*, and *env* coding regions, and wavy lines represent cellular DNA. An oligonucleotide containing the 34-bp *loxP* site and a *SmaI* restriction site was inserted into clone pNL4-3 (25) (at a *BspEI* site [nucleotide 308]) localized within the U3 region of the LTR. Arrows below the viral genome denote the positions of oligonucleotide primers used in PCR of HIV^{lox}-infected cell lysates (Fig. 5C) or RT-PCR. (B) RT-PCR of wild-type (lanes 1 to 5) or HIV^{lox} (lanes 6 to 10) genomic RNA demonstrates that viral RNA containing the *loxP* site is packaged into virions. Genomic RNA was purified from extracellular virions and was used as the template in RT-PCRs with [γ -³²P]ATP-labeled primers 1A and 1B (see Materials and Methods) (lanes 1 to 4 and 6 to 9). For positive controls, *gag*-specific primers were used in RT-PCR, which results in the amplification of a 297-bp DNA fragment (lanes 5 and 10). Negative controls included reactions conducted in the absence of RT (lanes 1 and 6) or in the presence of RNase A (lanes 2 and 7). Radiolabeled RT-PCR products were untreated or were subjected to digestion with *SmaI* (lanes 4 and 9) and were analyzed on a nondenaturing 3.5% polyacrylamide gel; the gel was dried and exposed to Kodak X-Omat film.

mM NaCl, 5 mM spermidine, 10 mM MgCl₂, 0.5 mg of bovine serum albumin per ml) and incubated at 37°C for 1 h. Reaction mixtures were extracted with phenol-chloroform (19:1), and the DNA was precipitated with ethanol and used to transform *Escherichia coli*.

RESULTS

Generation of a replication-competent HIV^{NY5/BRU} clone containing *loxP* sites. A modified Cre recognizing sequences within the LTR of HIV-1 could target proviral DNA for excisional recombination and potentially eliminate intact proviral DNA from infected cells. In this study, we sought first to establish whether native Cre could inhibit virus replication of a recombinant HIV-1 containing the *loxP* binding motif in each LTR. HIV^{NY5/BRU} DNA was modified by insertion of an oligonucleotide containing the *loxP* motif into the U3 region of HIV^{NY5/BRU}, generating the recombinant HIV-1 designated HIV^{lox} (Fig. 1A).

To demonstrate that the *loxP* site was maintained during virus replication, viral genomic RNA was assayed for the presence of the *loxP* site by RT-PCR. Genomic RNA was isolated from extracellular particles harvested from supernatants of CEM cells infected with either HIV^{NY5/BRU} or HIV^{lox}. RT-

FIG. 2. DNA binding and recombinase activities of Cre expressed in 293 cells, in *E. coli*, and in stably transfected CEM cells. (A) EMSA was used to detect Cre binding activity in nuclear extracts prepared from transiently transfected 293 cells. Radiolabeled probe containing the 34-bp *loxP* motif was incubated with 1 μ g of nuclear extract prepared from cells transfected with pRSVCre (lanes 2 to 8) or transfected with pRSVVec (lane 1). The specificity of Cre binding was demonstrated by the addition of 1-, 10-, and 100-fold molar amounts of unlabeled *loxP* probe (lanes 3 to 5, respectively) or with unlabeled nonspecific probe containing the binding site for the transcription factor Sp1. (B) Purified Cre was assayed for recombination by scoring the number of ampicillin-resistant colonies produced by excision of the 3.2-kbp pSHLOX plasmid from the 43-kbp λ SHLOX vector (Novagen). Increasing amounts of purified Cre were incubated with 100 ng of λ SHLOX DNA; 5 μ l of each reaction was used to transform *E. coli*. Negative controls included the substitution of Cre with reaction buffer or the addition of 250 nmol of GST. (C) The level of Cre recombinase activity present in CEM cells stably transfected with pRSVCre (hatched bar) was determined by in vitro excision assay. In parallel reactions, equivalent amounts of nuclear extract from CEM cells stably transfected with pRSVVec were untreated or incubated with reaction buffer or 100 fmol of purified GST (closed bars). (D) LTR^{lox}-dependent gene expression is unaffected by Cre or by insertion of *loxP* into the LTR. For the analysis of basal transcription of LTR^{lox}, the pHIV^{lox}-CAT reporter plasmid was cotransfected with either 1 μ g of pRSVVec control plasmid DNA (left) or 1 μ g of pRSVCre expression plasmid DNA (left) as indicated. Induction of LTR^{lox}-dependent gene expression by NF- κ B was analyzed by cotransfection of pHIV^{lox}-CAT DNA with 1 μ g of pRSVp65 expression plasmid DNA and either 1 μ g of pRSVVec or pRSVCre plasmid DNAs. These results represent the averages of three independent experiments. LTR-dependent gene expression is unaffected by insertion of *loxP* (right). 293 cells were transfected with 1 μ g of either HIV-CAT or HIV^{lox}-CAT. CAT activity represents the average of three independent transfections.

PCR of HIV^{lox} genomic RNA revealed a single PCR product of 455 bp (Fig. 1B, lane 8), a size consistent with the presence of the 46-bp oligonucleotide containing the *loxP* site. Additionally, the 455-bp fragment was digested by *Sma*I to produce fragments of 273 and 183 bp (Fig. 1B, lane 9), a digestion pattern consistent with the presence of a *Sma*I site within the inserted oligonucleotide. In contrast, a fragment of 409 bp was observed for RT-PCR of wild-type virus genomic RNA (Fig. 1B, lane 3); this fragment was not susceptible to digestion with *Sma*I (Fig. 1B, lane 4). Taken together, these data demonstrate that the major population of HIV^{lox} particles in infectious supernatants contain the *loxP* motif and that virus lacking the *loxP* site was not detectable. No PCR products were found in the negative controls, which included the substitution of RT with reaction buffer (Fig. 1B, lanes 1 and 6) or the addition of RNase A prior to the RT-PCRs (Fig. 1B, lanes 2 and 7). Positive controls for RT-PCR included primers specific for the *gag* gene of HIV-1 (Fig. 1B, lanes 5 and 10).

Confirmation of Cre DNA binding and recombinase activities in HIV-1-permissive cells. For expression of Cre in mammalian cells, the phage-encoded start site for translation was replaced with a consensus Kozak sequence (26), and the modified Cre ORF was cloned downstream of the Rous sarcoma virus LTR such that transcription of the Cre gene would be directed by the viral enhancer. The resulting plasmid, pRSVCre, was used to transiently transfect 293 cells. Nuclear extracts were prepared 48 h after transfection, and EMSA were conducted to detect Cre binding to radiolabeled oligonucleotide containing the *loxP* site (Fig. 2A). Previous studies of Cre DNA binding revealed that the *loxP* motif contains two binding sites for Cre (31). When nuclear extracts from pRSVCre-transfected 293 cells were incubated with radiolabeled *loxP*-containing oligonucleotide, two bands with mobilities slower than that of free probe were observed (Fig. 2A, lane 2). The upper band results from the binding of two Cre polypeptides to the probe (Fig. 2A, Dimer), while the lower band results from the bind-

ing of a single Cre polypeptide. A single band was observed when an oligonucleotide containing one Cre binding site was used in EMSA (data not shown). The specificity of Cre binding was demonstrated in assays using unlabeled *loxP* oligonucleotide as the competitor (Fig. 2A, lanes 3 to 5). Cre binding was nearly abolished in the presence of 100-fold-excess competitor *loxP* oligonucleotide (Fig. 2A, lane 5). In contrast, Cre binding was unaffected when unlabeled oligonucleotide containing the binding site for the transcription factor Sp1 was used as the competitor (Fig. 2A, lanes 6 to 8).

Recombinase activity was demonstrated by using an in vitro assay in which purified Cre (Novagen) was incubated with substrate consisting of the 43-kbp λ SHLOX vector (Novagen). Recombination was monitored by Cre-mediated excision of the 3.2-kbp plasmid, pSHLOX, that is contained within the λ SHLOX backbone and is flanked by *loxP* sites. Excision of pSHLOX from lambda vector DNA produces a free, circular plasmid that confers ampicillin resistance following transformation of *E. coli*; therefore, the number of ampicillin-resistant colonies reflects the level of recombinase activity. When 31.1 fmol of purified Cre was incubated with λ SHLOX DNA, 149 ampicillin-resistant colonies were scored (Fig. 2B). Increases in the amount of Cre added to the excision assay resulted in a linear increase in recombinase activity. The absolute requirement for Cre to mediate recombination in this in vitro assay was demonstrated by substitution of Cre protein with either reaction buffer or 250 fmol of glutathione *S*-transferase (GST); in each case, no ampicillin-resistant colonies were observed.

Demonstration of recombinase activity in CEM cells stably transformed with pRSVCre. CEM cells, which are permissive for HIV-1 replication, were used to generate cell lines that express Cre. Cell lines stably transfected with pRSVCre (CEM-Cre) or plasmid vector DNA (CEM-Vec) were selected for resistance to G418. To demonstrate that CEM-Cre cells express functional Cre, nuclear extracts were tested for recombinase activity by using the in vitro excision assay described above. To quantitate levels of recombinase activity present in 40 μ g of nuclear extract prepared from CEM-Cre cells, a standard curve was generated by performing excision assays in parallel with increasing amounts of purified Cre added to 40 μ g of nuclear extract prepared from pRSVVec-transformed cells. The level of recombinase activity detected in CEM-Cre nuclear extracts falls (Fig. 2C) within a range of recombinase activity observed when 1.0 to 10.0 fmol of purified Cre (Novagen) was incubated with pRSVVec nuclear extracts. No recombinase activity was detected in untreated CEM-Vec nuclear extracts or when reaction buffer or GST was added to the control nuclear extracts.

To eliminate the possibility that Cre could transcriptionally repress LTR-dependent gene expression, the capacity of the HIV^{lox} LTR to regulate expression of the chloramphenicol acetyltransferase (CAT) reporter gene in the presence of Cre was analyzed by transient transfection of Jurkat T leukemia cells. Essentially, no differences in the levels of CAT activity were observed when the reporter plasmid HIV^{lox}-CAT was cotransfected with pRSVCre DNA compared to the levels of CAT activity observed with pRSVVec control DNA (Fig. 2D, left), demonstrating that Cre does not affect basal transcriptional activity of the HIV^{lox} LTR. The HIV^{lox} LTR was slightly more responsive to transactivation by the NF- κ B transcription factor p65 in the presence of Cre, as demonstrated by the ~2-fold induction of CAT activity observed when p65 expression plasmid DNA was cotransfected with HIV^{lox}-CAT and pRSVCre or pRSVVec DNA (Fig. 2D, left), showing that Cre does not inhibit LTR-dependent transcription. To eliminate the possibility that insertion of the *loxP* motif into the HIV-

FIG. 3. Inhibition of HIV^{lox} replication in cells transiently transfected with pRSVCre. 293 cells stably expressing CD4 were transfected at 30% confluence in a six-well plate by the calcium phosphate procedure with 1 μ g of either pRSVCre or pRSVVec. Forty-eight hours posttransfection, cells were infected with HIV^{lox} at a multiplicity of infection of 0.05 (A) or with HIV^{NY5/BRU} at a multiplicity of infection of 0.03 (B). RT is expressed as counts per minute per milliliter of infected cell culture supernatant. In general, standard deviations for individual points in the RT assay were $\leq 10\%$. Results are representative of two independent experiments.

LTR could effect enhancer activity, cells were transiently transfected with equivalent amounts of HIV-CAT and HIV^{lox}-CAT DNAs, and the levels of LTR-dependent gene expression were determined by monitoring CAT activity. No significant difference was detected in CAT activity between cells transfected with HIV-CAT and HIV^{lox}-CAT (Fig. 2D, right).

Challenge of pRSVCre transfected CD4⁺ 293 cells with HIV. 293 cells expressing cell surface CD4 were transfected with 1 μ g (Fig. 3) of either pRSVCre or vector DNA. These cells are highly (routinely >50%) transfectable and were used as a screen to determine the efficacy of the Cre expression plasmid in providing an antiviral effect against HIV^{lox}. Forty-eight hours after transfection, the cells were infected with HIV^{lox} at a multiplicity of infection of 0.05. Infected cell culture supernatants harvested from 293 cells transfected with 1 μ g of pRSVVec showed maximum RT activity at 8 days postinfection (Fig. 3A). In contrast, at this time point for cells transfected with 1 μ g of pRSVCre and infected with HIV^{lox}, the level of RT activity was reduced significantly (Fig. 3A). In a separate experiment, 293 cells transfected with pRSVCre and infected with HIV^{NY5/BRU} showed a slight increase in RT levels relative to pRSVVec (Fig. 3B), demonstrating that the inhibition of HIV^{lox} replication by Cre-expressing cells was dependent on the presence of the *loxP* motif within HIV^{lox}. In these cells, inhibition of viral replication was not expected to be complete, since the transient transfection does not provide Cre gene expression in all CD4⁺ 293 cells.

HIV-1 replication in CEM-Cre cells. To determine whether Cre could mediate protection in HIV-infectable T-leukemia cell lines, the CEM cells which expressed Cre were further analyzed. Fluorescent flow cytometric analyses were conducted to determine the relative levels of cell surface CD4 of CEM-Cre, CEM-Vec, and wild-type CEM cells. For all three cell lines, an increase in fluorescence was observed when cells were stained with anti-CD4 antibody compared to the isotype control (Fig. 4A). In addition, the degrees of fluorescence observed with anti-CD4 antibody were approximately equivalent for all cell lines, demonstrating that CD4 is expressed on the surface of CEM-Cre cells at levels comparable to those of wild-type CEM cells.

FIG. 4. Analysis of CEM cells stably transfected with pRSVCre for expression of cell surface CD4 and for the capacity to support HIV-1 replication. (A) Wild-type CEM, CEM-Vec, or CEM-Cre cell lines were analyzed for cell surface expression of CD4 by fluorescent flow cytometric analysis. Cells were incubated with murine anti-CD4 monoclonal antibody conjugated with fluorescein isothiocyanate (filled curve) or with nonspecific fluorescein isothiocyanate-conjugated monoclonal antibody of the same isotype. (B) CEM-Cre or CEM-Vec cells were infected with HIV^{111B} at a multiplicity of infection of 0.001, and virus replication was monitored by RT activity present in extracellular media, with standard deviations of $\leq 10\%$ in general. By the paired *t* test, this difference, though small, was statistically significant ($t = 2.05$, 95% confidence limit). Results are representative of two independent experiments and different multiplicities of infection.

The capacity of CEM-Cre cells to support replication of HIV-1 was assessed by infection of these cells with the lymphocytotropic strain HIV-1^{111B}. Virus replication was monitored by the levels of RT activity in culture supernatants. Infection of CEM-Cre cells or the control CEM-Vec cells showed similar kinetics of replication, with peak RT levels at 11 days postinfection. Infection of CEM-Cre cells resulted in a twofold-higher level of RT activity at day 11 compared to the control CEM-Vec cells (Fig. 4B); while this increase is not significant, these data demonstrate that no nonspecific suppression of virus replication occurs in CEM-Cre cells.

Challenge of CEM-Cre cells with HIV^{lox}. To determine whether cells constitutively expressing Cre could inhibit productive replication of HIV-1 containing *loxP* sites, CEM-Cre cells were challenged with HIV^{lox}. CEM-Cre, CEM-Vec, or CEM cells were infected with either parental HIV^{NY5/BRU} (Fig. 5A) or HIV^{lox} (Fig. 5B). The kinetics of HIV^{NY5/BRU} replication in CEM-Vec, CEM-Cre, and CEM cells were similar in all three cell lines (Fig. 5A), demonstrating that the capacity of CEM cells to support HIV^{NY5/BRU} was not inhibited by Cre expression, as with HIV^{111B} (Fig. 4B). Replication of HIV^{lox} in the control cell lines, CEM and CEM-Vec, was readily observed at a multiplicity of infection of either 0.002 or 0.012 (Fig. 5B). When RT activity was maximal in these infected cultures, syncytia were observed, demonstrating that increases in RT activity corresponded with cytopathological effects. In contrast, CEM-Cre cells infected with HIV^{lox} at either multiplicity of infection (Fig. 5B) showed reduced virus replication compared to infection of control cells in these experiments. At a multiplicity of infection of 0.012 (Fig. 5B, right), RT activity observed for HIV^{lox} infection of CEM-Cre cells was reduced ninefold at day 11 compared to infection of

the control cell lines with HIV^{lox}. In addition, no cytopathological effects were observed during the 2 weeks of HIV^{lox} infection of CEM-Cre cells. When HIV^{lox} infection was conducted at the higher multiplicity of infection (Fig. 5B, right), the protective effect observed for CEM-Cre cells was observed for 2 weeks, whereas at the lower multiplicity of infection (Fig. 5B, left), protection was observed throughout the course of the experiment.

PCR analyses of total cell lysates prepared from CEM-Cre or CEM-Vec cells infected with HIV^{lox} were conducted to detect the product of Cre-mediated excision of HIV^{lox} proviral DNA. Excision of the DNA between the *loxP* motifs of HIV^{lox} (Fig. 1A) is predicted to generate a covalently closed circular molecule of 9,076 bp that contains a single LTR. PCR primers 2A and 2B (Fig. 1A) map within viral coding sequences near the 5' and 3' LTRs, respectively; these primers are in opposite orientation so that PCR is specific for circularized proviral DNA and should produce a DNA fragment of 782 bp. A circular DNA with a single LTR could potentially arise also as an abortive product during acute retroviral infection (10, 24). Analyses of PCR products labeled with [γ -³²P]ATP on a non-denaturing polyacrylamide gel revealed this PCR product amplified from extracts of CEM-Cre cells infected with HIV^{lox} (Fig. 5C, lane 2) at ~ 16 -fold higher levels than control infected cells (Fig. 5C, lane 1). This result suggested that the inhibition of HIV^{lox} replication observed in infected CEM-Cre cells is due to Cre-mediated recombination of HIV^{lox} proviral DNA, resulting in the generation of circular DNA molecules containing a single LTR.

The product of Cre-mediated excision of HIV^{lox} proviral DNA is a circular DNA molecule containing a single LTR. To ascertain the level of LTR-dependent gene expression from a

FIG. 5. Challenge of Cre-expressing cells with HIV^{lox}. CEM cells stably transfected with pRSVCre, pRSVVec, or wild-type CEM cells were infected with HIV^{NY5/BRU} at a multiplicity of infection of 0.008 (A) or with HIV^{lox} at a multiplicity of infection of either 0.002 (B, left) or 0.012 (B, right). Virus replication was monitored by the levels of RT activity in culture supernatants and is representative of three separate experiments with standard deviations of $\leq 10\%$ in general. Results are representative of three independent experiments at different multiplicities of infection. (C) PCR analysis of infected cell lysates harvested 14 days after infection of either CEM-Cre or CEM-Vec cells with HIV^{lox}. Radiolabeled oligonucleotides designated 2A and 2B (Fig. 1A; see Materials and Methods) were used in PCR amplification of circular proviral DNA generated from excision of HIV^{lox} proviral DNA. PCR products were analyzed on a 4% nondenaturing polyacrylamide gel, and the labeled DNA fragments were visualized following autoradiography. Radiolabeled molecular size standards are indicated in base pairs. (D) Comparison of LTR-dependent transcription of circular DNA molecules containing one or two LTRs. Cloned viral DNAs containing either one or two LTRs were released from plasmid DNA by restriction digestion, and the linear viral DNAs were made circular by ligation with T4 DNA ligase. CD4⁺ 293 cells were transfected with 0.1 μ g of circularized DNA, and the levels of RT were monitored every 24 h for 4 days after transfection. The levels of RT represent the averages of three independent transfections.

circular DNA molecule containing a single LTR, CD4⁺ 293 cells were transfected with equivalent amounts of circularized DNA containing either one or two LTRs. The levels of RT observed in supernatants of cells transfected with DNA containing two LTRs were maximal at 4 days after transfection and were significantly greater than the levels of RT observed in supernatants of cells transfected with circular DNA containing only one LTR (Fig. 5D). The amount of virus detected by the RT assay in supernatants from cells transfected with DNA containing one LTR is below the level of detection required for replication-competent virus. These results indicate that circular molecules containing a single LTR are much less efficient in viral gene expression and viral replication.

DISCUSSION

Antiviral agents directed against HIV-1 inhibit the activities of gene products involved in virus replication (10). For example, soluble forms of the CD4 receptor inhibit virus adsorption. Antisense oligonucleotides and ribozymes target genomic viral RNA and prevent the synthesis of proviral DNA by RT (12). Inhibitors of RT prevent synthesis of proviral DNA, and compounds that inhibit viral integrase function to prevent insertion of proviral DNA into cellular chromatin (32). Transdominant mutants of the viral regulatory proteins Tat or Rev inhibit the production of viral structural proteins by impeding the func-

tion of their wild-type counterparts (12). These strategies to inhibit virus replication target either viral RNAs or proteins but do not affect the integrated proviral DNA. In addition, it is necessary to produce large quantities of the antiviral gene product in a sustained fashion to continually inhibit viral replication. A potential genetic strategy for targeting proviral DNA utilizing a modified Cre recombinase that recognizes sequences within the LTR of HIV-1 and excises proviral DNA from the infected cell could be envisioned.

The binding of Cre to its natural target locus, *loxP*, has been well characterized. The 34-bp *loxP* site (22) contains two contiguous binding sites for Cre (31); each binding site contains one inverted repeat and 4 bp of the spacer region (19). Following binding to *loxP*, Cre mediates site-specific recombination through a pathway that includes (i) synapsis of two recombining *loxP* sites, (ii) cleavage of the DNA backbone within the spacer region to generate 6-bp 5' protruding termini, (iii) pairing of single-stranded termini between partner *loxP* sites followed by strand exchange, and (iv) ligation of the DNA backbone to generate the products of recombination (4, 20). Factors known to be important for efficient recombination are the length of the spacer and homology between the spacer regions of two *loxP* partners in recombination (21). The identification of functional *loxP*-like motifs in yeast chromatin has demonstrated that Cre is able to recognize and recombine using motifs with considerable divergence from the *loxP* site

(37). Also, of the *loxP*-like motifs identified, those most efficiently able to mediate recombination possessed the sequence TATA that is located within the inverted repeat and adjacent to the spacer region of *loxP* (37). These results defined sequences within *loxP* that are important for Cre-mediated recombination and, at the same time, demonstrate that the sequence of a functional *loxP* site is not absolute in terms of the consensus *loxP* sequence.

The domains of Cre that mediate DNA binding and recombinase activities are not well defined. A 25-kDa carboxy-terminal fragment of Cre is sufficient for DNA binding to *loxP* but binds less efficiently than wild-type polypeptide and does not possess recombinase activity (18). Mutational analysis of Cre has revealed at least four residues that affect Cre binding activity (43). Substitution mutations of residues 87, 91, 288, and 294 reduce but do not abolish Cre binding activity. Mutations of Cre that completely abolish DNA binding activity have not been identified. In contrast, three residues (His-369, Arg-399, and Tyr-433) of Cre that are absolutely essential for recombinase activity (43) and are conserved in other members of the recombinase family, including the yeast FLP and lambda Int recombinases (7), have been identified.

Before attempting to modify the recognition properties of Cre, it was necessary to determine whether Cre could recognize and excise proviral DNA containing wild-type *loxP* motifs in each LTR and result in significant inhibition of virus replication. In this report, it was demonstrated that expression of Cre can effectively inhibit replication of a recombinant HIV-1 clone containing *loxP*. The degree of inhibition of virus replication depended on the levels of Cre expression. Transient expression of Cre in HIV-1-permissive 293 cells showed a moderate inhibition of virus replication. This is likely a consequence of less than 100% transfection efficiency, with cells not expressing Cre providing a reservoir for productive infection. This notion was supported by the greater degree of protection provided by Cre expressed constitutively in permanently transformed CEM cells.

Although not demonstrated directly, the mechanism of Cre-mediated inhibition is likely due to excision of proviral DNA from cellular chromatin. The product of Cre-mediated excision of HIV^{lox} proviral DNA would be a circular DNA molecule containing a single LTR. Single-LTR-containing circles have been detected previously as a product of HIV-1 replication (24). This product is the result of unintegrated proviral DNA and is believed to be a nonfunctional product produced during retrovirus replication (10). Despite the detection of a small amount of this product in vector control CEM cells, PCR analyses of HIV^{lox}-infected cell lysates revealed a substantially higher amount of this HIV^{lox} excision product in CEM-Cre cells (Fig. 5C). These data suggest that the expected excision product, which could have been derived from either unintegrated or integrated provirus, is detectable at increased levels in Cre-protected cells after challenge with HIV^{lox}.

Recently, Choulaka et al. (9) described a Moloney murine leukemia virus vector that has *loxP* and a transgene inserted into the U3 region of the LTRs. Following integration of proviral DNA, Cre-mediated excision resulted in the deletion of vector sequences with a single LTR and the transduced gene remaining integrated within cellular chromatin. While the strategy described by Choulaka et al. (9) involves excision of a stably integrated proviral sequence, no studies have yet addressed the question of whether Cre can affect viral replication in an acute infection or whether the approach is applicable to lentiviruses. The findings reported here demonstrate that the efficiency of Cre-mediated excision of proviral DNA is sufficient to inhibit HIV-1 replication during acute infection and

therefore may provide an alternative molecular genetic approach to HIV-1 disease. Overall, this study supports the idea that a modified Cre could provide a reagent for therapy against HIV-1 infection. This antiviral strategy differs from current methodologies in that it has the potential to excise the viral genome from cells. Cre has been shown to mediate recombination between sites whose sequence diverges from canonical *loxP*. Therefore, a degree of flexibility in DNA recognition and recombination exists within the Cre molecule; however, the binding specificities of large DNA binding proteins such as Cre have not been reported. Whether Cre can be altered to recognize binding sites other than *loxP* is not clear but most certainly requires a more detailed understanding of the binding and recombination functions of this polypeptide. That Cre has been used successfully in transgenic animals suggests that expression of this recombinase is not cytotoxic in vivo. These studies suggest that site-specific recombinases can potentially contribute to genetic targeting of HIV and may facilitate gene correction strategies in other human diseases. The anti-proviral DNA strategy described in this report requires circumventing several obstacles before it is applicable for the interdiction of HIV infection; nevertheless, the potential for this model system in the treatment of AIDS warrants further investigation.

ACKNOWLEDGMENTS

We thank David Friedman (Department of Microbiology, University of Michigan) for bacteriophage P1 genomic DNA. We also thank Donna Gschwend for secretarial assistance and Nancy Barrett for preparation of figures.

This work was supported in part by grant RO1 AI36207 (G.J.N.) and NRSA training grant AI 07413 (S.Y.) from the National Institutes of Health.

REFERENCES

1. Abremski, K., and R. Hoess. 1984. Bacteriophage P1 site-specific recombination. *J. Biol. Chem.* **259**:1509–1514.
2. Abremski, K., R. Hoess, and N. Sternberg. 1983. Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. *Cell* **32**:1301–1311.
3. 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.
4. Adams, D. E., J. B. Bliska, and N. R. Cozzarelli. 1992. Cre-lox recombination in *Escherichia coli* cells. *Mol. Biol.* **226**:661–673.
5. Anton, M., and F. L. Graham. 1995. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J. Virol.* **69**:4600–4606.
6. Antoni, B. A., S. B. Stein, and A. B. Rabson. 1994. Regulation of human immunodeficiency virus infection: implications for pathogenesis. *Adv. Virus Res.* **43**:53–145.
7. Argos, P., A. Landy, K. Abremski, J. Egan, E. Haggard-Ljungquist, R. H. Hoess, M. L. Kahn, B. Kalionis, S. V. L. Narayana, L. S. Pierson III, N. Sternberg, and J. M. Leong. 1986. The integrase family of site-specific recombinases: regional similarities and global diversity. *EMBO J.* **5**:433–440.
8. Baubonis, W., and B. Sauer. 1993. Genomic targeting with purified Cre recombinase. *Nucleic Acids Res.* **21**:2025–2029.
9. Choulaka, A., V. Guyot, and J. Nicolas. 1996. Transfer of single gene-containing long terminal repeats into the genome of mammalian cells by a retroviral vector carrying the *cre* gene and the *loxP* site. *J. Virol.* **70**:1792–1798.
10. Coffin, J. M. 1996. Retroviridae and their replication, p. 1437–1500. In B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), *Fields virology*. Lippincott-Raven Publishers, Philadelphia, Pa.
11. Dalglish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**:763–766.
12. Dropulic, B., and K. Jeang. 1994. Gene therapy for human immunodeficiency virus infection: genetic antiviral strategies and targets for intervention. *Hum. Gene Ther.* **5**:927–939.
13. Fukushige, S., and B. Sauer. 1992. Genomic targeting with a positive-selection *lox* integration vector allows highly reproducible gene expression in

- mammalian cells. *Proc. Natl. Acad. Sci. USA* **89**:7905–7909.
14. Goff, S. P. 1992. Genetics of retroviral integration. *Annu. Rev. Genet.* **26**: 527–544.
 15. Gu, H., J. D. Marth, P. C. Orban, H. Mossmann, and K. Rajewsky. 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265**:103–106.
 16. Gu, H., Y. Zou, and K. Rajewsky. 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through cre-*loxP*-mediated gene targeting. *Cell* **73**:1155–1164.
 17. Hakes, D. J., and J. E. Dixon. 1992. New vectors for high level expression of recombinant proteins in bacteria. *Anal. Biochem.* **202**:293–298.
 18. Hoess, R., K. Abremski, S. Irwin, M. Kendall, and A. Mack. 1990. DNA specificity of the Cre recombinase resides in the 25 kDa carboxyl domain of the protein. *J. Mol. Biol.* **216**:873–882.
 19. Hoess, R. H., and K. Abremski. 1984. Interaction of the bacteriophage P1 recombinase cre with the recombining site *loxP*. *Proc. Natl. Acad. Sci. USA* **81**:1026–1029.
 20. Hoess, R. H., and K. Abremski. 1985. Mechanism of strand cleavage and exchange in the cre-*lox* site-specific recombination system. *J. Mol. Biol.* **181**:351–362.
 21. Hoess, R. H., A. Wierzbicki, and K. Abremski. 1986. The role of the *loxP* spacer region in P1 site-specific recombination. *Nucleic Acids Res.* **14**:2287–2300.
 22. Hoess, R. H., M. Ziese, and N. Sternberg. 1982. P1 site-specific recombination: nucleotide sequence of the recombining sites. *Proc. Natl. Acad. Sci. USA* **79**:3398–3402.
 23. Holt, C. L., and G. S. May. 1993. A novel phage gamma replacement cre-*lox* vector that has automatic subcloning capabilities. *Gene* **133**:95–97.
 24. Jurriaans, S., A. deRonde, J. Dekker, J. Goudsmit, and M. Cornelissen. 1992. Analysis of human immunodeficiency virus type 1 LTR-LTR junctions in peripheral blood mononuclear cells of infected individuals. *J. Gen. Virol.* **73**:1537–1541.
 25. 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.
 26. Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**:283–292.
 27. Kuhn, R., F. Schwenk, M. Aguet, and K. Rajewsky. 1995. Inducible gene targeting in mice. *Science* **269**:1427–1428.
 28. Lakso, M., B. Sauer, B. Mosinger, Jr., E. J. Lee, R. W. Manning, S. H. Yu, K. L. Mulder, and H. Westphal. 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**:6232–6236.
 29. Leung, K., and G. J. Nabel. 1988. HTLV-I transactivator induces interleukin-2 receptor expression through an NF- κ B-like factor. *Nature* **333**:776–778.
 30. Lifson, J. D., M. B. Feinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engleman. 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature* **323**:725–728.
 31. Mack, A., B. Sauer, K. Abremski, and R. Hoess. 1992. Stoichiometry of the Cre recombinase bound to the *lox* recombining site. *Nucleic Acids Res.* **20**:4451–4455.
 32. Mitsuyasu, H., and R. Yarochan. 1994. Development of antiretroviral therapy for AIDS and related disorders, p. 721–742. *In* S. Broder, T. C. Merigan, and D. Bolognesi (ed.), *Textbook of AIDS medicine*. Williams & Wilkins, Co., Baltimore, Md.
 33. Orban, P. C., D. Chui, and J. D. Marth. 1992. Tissue- and site-specific DNA recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**:6861–6865.
 34. Peakman, T. C., R. A. Harris, and D. R. Gewert. 1992. Highly efficient generation of recombinant baculoviruses by enzymatically mediated site-specific *in vitro* recombination. *Nucleic Acids Res.* **20**:495–500.
 35. Potts, B. J. 1990. Mini reverse transcriptase assay, p. 103–106. *In* A. Aldovini and B. D. Walker (ed.), *Techniques in HIV research*. Stockton Press, New York, N.Y.
 36. Sauer, B. 1987. Functional expression of the cre-*lox* site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**:2087–2096.
 37. Sauer, B. 1992. Identification of cryptic *lox* sites in the yeast genome by selection for cre-mediated chromosome translocations that confer multiple drug resistance. *J. Mol. Biol.* **223**:911–928.
 38. Sauer, B., and N. Henderson. 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. USA* **85**:5166–5170.
 39. Sauer, B., and N. Henderson. 1989. Cre-stimulated recombination at *loxP*-containing DNA sequences placed into the mammalian genome. *Nucleic Acids Res.* **17**:147–161.
 40. Sauer, B., and N. Henderson. 1990. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biol.* **2**:441–449.
 41. Sauer, B., M. Whealy, A. Robbins, and L. Enquist. 1987. Site-specific insertion of DNA into a pseudorabies virus vector. *Proc. Natl. Acad. Sci. USA* **84**:9108–9112.
 42. Sternberg, N., B. Sauer, R. Hoess, and K. Abremski. 1986. Bacteriophage P1 Cre gene and its regulatory region evidence for multiple promoters and for regulation by DNA methylation. *J. Mol. Biol.* **187**:197–212.
 43. Wierzbicki, A., M. Kendall, K. Abremski, and R. Hoess. 1987. A mutational analysis of the bacteriophage P1 recombinase Cre. *J. Mol. Biol.* **195**:785–794.