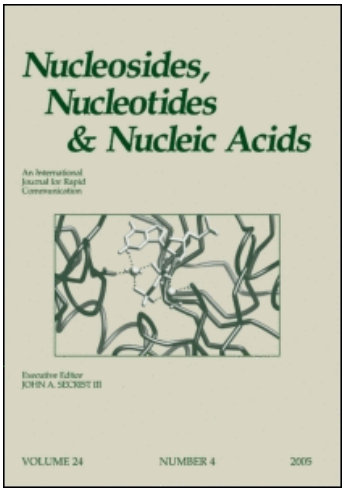

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INHIBITION OF HIV-1 REPLICATION BY THE CRE-LOXP HAMMERHEAD RIBOZYME

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ABSTRACT

Antiviral strategies to suppress productive human immunodeficiency virus type 1 (HIV-1) replication have included the generation of gene products that provide intracellular inhibition of an essential viral protein or RNA. The potential of such a molecular genetic intervention was examined by using the Cre/loxP recombination system. In this study, we constructed a loxP-casstte vector (LTR-ribozyme) and a Cre recombinase expression vector (LTR-Cre). The transcription of the ribozyme and Cre genes was designed to be driven from the LTR promoter. These vectors were transiently transfected into COS cells along with the viral expression vector, and inhibited the expression of viral protein in COS cells. These data further support the potential of this system as a therapeutic agent for HIV-1.

INTRODUCTION

An alternative strategy for the management of human immunodeficiency virus type1 (HIV-1) infection has been the use of antiviral genes that are delivered to uninfected cells as either RNA or DNA and provide intercellular protection from the virus. Several strategies targeting HIV-1 gene expression have been shown to beeffective in inhibiting virus replication, and these include intracellular expression

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of transdominant proteins, antisense molecules, ribozymes, and intracellular antibodies. The potential of such a molecular genetic intervention was examined by using the Cre-loxP recombination system (1,2). We describe here the function of an HIV-1 dependent ribozyme expression vector, with which the site-specific excision of loxP sequences can be achieved by using the Cre-loxP system as a molecular switch in an acute infection.

RESULTS AND DISCUSSION

In our previous study, the cDNA inserted between the loxP sequence required promoter activation to synthesize the Cre protein by recombination, so we examined its expression (3). The gene encoding luciferase was co-transfected with the Cre expression vector (pBS185) (4–6) into COS cells, and after two days, the luciferase activity was measured. We confirmed that it functioned as a plasmid DNA from the cDNA inserted between the loxP sequence in the Cre protein, and that its quantity depended on promoter activation. In addition to the loxP cassette vector, we also analyzed lox-Rz, a recombinant ribozyme vector targeted against the LTR U5 portion of the HIV-1_{NL4-3} strain. We also constructed a plasmid in which the HIV-1 LTR promoter drives the expression of the Cre protein. Plasmids expressing lox-Rz, LTR-Cre, the luciferase gene, and other parts of the HIV-1 genome, except env and nef, were transfected into COS cells. After two days, the luciferase activities in these cells were measured (Fig. 1).

As a result, transiently expressed lox-Rz and LTR-Cre were found to inhibit the HIV-1 expression by the luciferase assay.

Furthermore, plasmids containing the Cre/loxP system and expressing the HIV-1 gene were transfected into COS cells. After two days, the amounts of HIV-1 gag p24 antigen in these cells were measured. The LTR-Cre/lox-Rz system strongly suppressed the production of gag p24 antigen (Fig. 2).

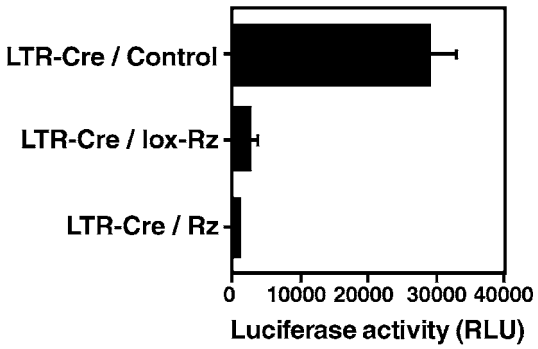

Figure 1. Inhibition of luciferase activity by the ribozyme expressed from the Cre/loxP system after Cre recombinase introduction (LTR-Cre). The lox-Rz expressed the strongest inhibition of the luciferase activity in COS cells.

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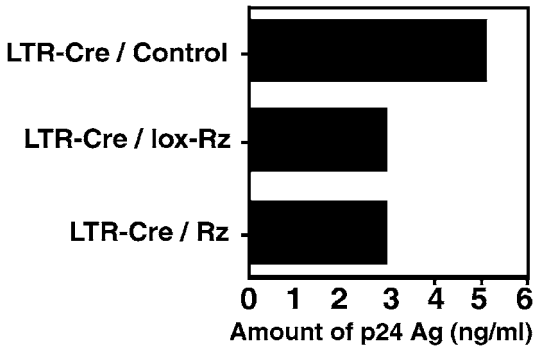

Figure 2. Inhibition of HIV-1 gag p24 antigen production. The lox-Rz showed significant suppression of the HIV-1 gag p24 antigen expression in COS cells.

Our results suggest that the LTR-Cre/lox-Rz recombination system could be useful in the establishment of an effective genetic therapy against HIV-1 infection.

EXPERIMENTAL

Construction of Plasmids

The ribozyme expression vector and the loxP cassette vector, pBS246 (GIBCO BRL), were digested with KpnI, phenol/chloroform extracted, ethanol precipitated, and ligated to the KpnI sites (lox-Rz). The plasmid containing the Cre gene, pBS185 (GIBCO BRL), was digested with SstI and NarI. The termini of the resultant insert DNA were blunted with T4 DNA polymerase. This insert DNA was ligated with the LTR-luc vector DNA, which had been digested with HindIII and SalI and made blunt by treatment with T4 DNA polymerase.

Intracellular Recombination of lox-Rz

COS cells were grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10%-heat-inactivated fetal bovine serum (FBS), 50 units/ml penicillin, and 50 units/ml streptomycin in a moist atmosphere with 5% CO_2 at 37°C. The cultures, in 24-well plates (1×10^4 cells/1 ml/well), were washed with PBS(-), and 500 μl of serum free D-MEM were added to each well. The ribozyme expression vector lox-Rz was cotransfected with a 10-fold (by weight) excess of the Cre expression plasmid LTR-Cre and a 0.1-fold portion (by weight) of the plasmid expressing the luciferase gene and other parts of the HIV-1 genome (except *env* and *nef*), pNL-luc (0.1 μg of lox-Rz and 1 μg of LTR-Cre and 0.01 μg of pNL-luc) using 3 μl of the FuGENE^{TM} 6 transfection reagent (Roche Diagnostics K.K.). DNA-FuGENE^{TM} 6 complexes were added to the cells, which were then incubated at 37°C. At 4 h posttransfection, 500 μl of D-MEM containing 20% FBS were

added to each cell culture. After 48 h, the cells were washed twice with PBS(-), 200 μl of cell lysis solution (PicaGene®) were added, and the mixtures were incubated for 15 min. The lysates were harvested and centrifuged at 12,000 rpm for 3 min. In the luciferase assay, 10 μl of cell lysate were used. To measure the relative light units (RLU), a luminometer (LUMAT LB 9507; EG&G BERTHOLD) was used.

Anti-HIV-activity of lox-Rz. COS cells were incubated in D-MEM at 37°C and in a 5% CO_2 atmosphere for 24 hours. The cultures, in 60mm dishes (3×10^5 cells/3 ml), were washed with PBS(-), and 1μg of lox-Rz was cotransfected with 1 μg of LTR-Cre using 3 μl of the FuGENE™ 6 transfection reagent. After 24 h, 0.5 μg of pNL4-3 complexed with 1 μl of the FuGENE™ 6 transfection reagent was added, and the cells were cultured for 24 more h. Anti-HIV activity was determined by measuring the amount of p24 antigen in the supernatant, using an HIV-1 p24 ELISA.

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