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A hairpin ribozyme inhibits expression of diverse strains of human immunodeficiency virus type 1

(catalytic RNA/anti-human immunodeficiency virus type 1 therapeutic agent/intracellular immunization)

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ABSTRACT **Ribozymes have enormous potential as antiviral agents. We have previously reported that a hairpin ribozyme expressed under the control of the β-actin promoter that cleaves human immunodeficiency virus type 1 (HIV-1) RNA in the leader sequence can inhibit HIV-1 (pHXB2gpt) expression. For such a ribozyme in a retroviral vector delivery system to be useful in gene therapy for the treatment of HIV-1 infection, it must be able to inhibit the expression of multiple HIV-1 strains. We have now cloned this ribozyme into various regular expression vectors (including retroviral vectors) by using various gene expression control strategies. Here we show by transient transfection that inhibition of expression of diverse strains of HIV-1 can be achieved by this ribozyme expressed in the proper vectors. These data further support the potential of this hairpin ribozyme as a therapeutic agent for HIV-1.**

Ribozyme technology has emerged as a powerful extension of the antisense approach to inhibition of gene expression (1). Intracellular expression of hammerhead-type ribozymes directed against human immunodeficiency virus type 1 (HIV-1) RNA has been extensively studied and shown to confer resistance to HIV-1 infection (2–4). A hairpin ribozyme was originally discovered (7) in the negative-strand satellite RNA of the tobacco ringspot virus. This small RNA genome utilizes cis-cleaving ribozymes during rolling-circle replication (5–10). We have described (11) an engineered form of this hairpin ribozyme that was designed to cleave the 5′ leader sequence of HIV-1 HXB2 clone mRNA at position +111/112 from the cap site. *In vitro* study demonstrated that one ribozyme molecule can cleave multiple target molecules (unpublished result). Expression of this hairpin ribozyme under the control of the β-actin promoter inhibited HIV-1 (HXB2) expression and Tat activity.

One important parameter to ensure the efficacy of the ribozyme against HIV infection is high intracellular expression. This can be achieved by employing strong promoters and promoters whose genes are ubiquitously expressed. Here we have used the human $tRNA^{Val}$ gene and the adenovirus *VA1* gene promoters, both transcribed by RNA polymerase (pol) III, as expression cassettes for the hairpin ribozyme. The small size, high rate of transcription, and universal expression in various tissues of the pol III transcription units make them intriguing candidates for expressing ribozymes against HIV-1. Earlier studies have demonstrated the utility of both pol III, tRNA (12–14) and *VA1* genes (15) for expression of antisense RNA, ribozymes, and RNA decoys. Furthermore, since retroviral vectors are the best available vehicles for gene delivery, we are beginning to explore this system by combining the conventional Moloney murine leukemia virus-based vectors with pol III expression control units.

To use this ribozyme in gene therapy for the treatment of HIV-1 infection, it is important to evaluate the ability of the ribozyme to inhibit the expression of various HIV-1 strains, since HIV-1 is well known for its variability. Although the leader sequence targeted by this ribozyme is conserved among most HIV-1 isolates, it is still possible that differences in surrounding sequences can affect accessibility and ribozyme cleavage. Furthermore, a single base change in the target region of one of a typical North American HIV-1 strains, MN, has been observed. The ribozyme's ability to inhibit MN expression *in vivo* is an important indicator of how mutation might affect ribozyme activity. Our results show that this ribozyme is able to inhibit diverse strains of HIV-1.

MATERIALS AND METHODS

Enzymes and Chemicals. All restriction enzymes used were from Bethesda Research Laboratories or Boehringer Mannheim. The buffers for restriction enzymes were supplied by the manufacturers. T4 DNA ligase and the sequencing kit were obtained from Pharmacia. The *in vitro* transcription kit and relevant enzymes were obtained from Promega. Bovine calf serum, antibiotics (penicillin and streptomycin), L-glutamine, sodium pyruvate, phosphate-buffered saline (PBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO. The HIV-1 p24 antigen detection kit was obtained from Coulter.

Construction of pol III-Driven Ribozyme Plasmids. Unless stated otherwise, all recombinant DNA techniques were performed as described (16). The HIV-1 5′-leader-sequence-specific hairpin ribozyme was cloned as follows. Chemically synthesized double-stranded deoxyribonucleotides corresponding to human $tRNA^{Val}$ promoter (138 bp) and adenovirus *VA1* (104 bp) promoter were cloned into pHR and pdHR (11) plasmids (which contain the active and disabled ribozymes, respectively) upstream of the ribozyme coding sequence at *Eco*RI–*Bam*HI sites (Fig. 1). The resultant plasmids containing the pol III promoters were digested with *Mlu* I and *Pst* I to remove the autocatalytic cassette (11) and replace it with pol III termination sequences (17). The sequence of clones was confirmed by DNA sequencing. The

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Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; Neo, neomycin resistance; pol, polymerase.

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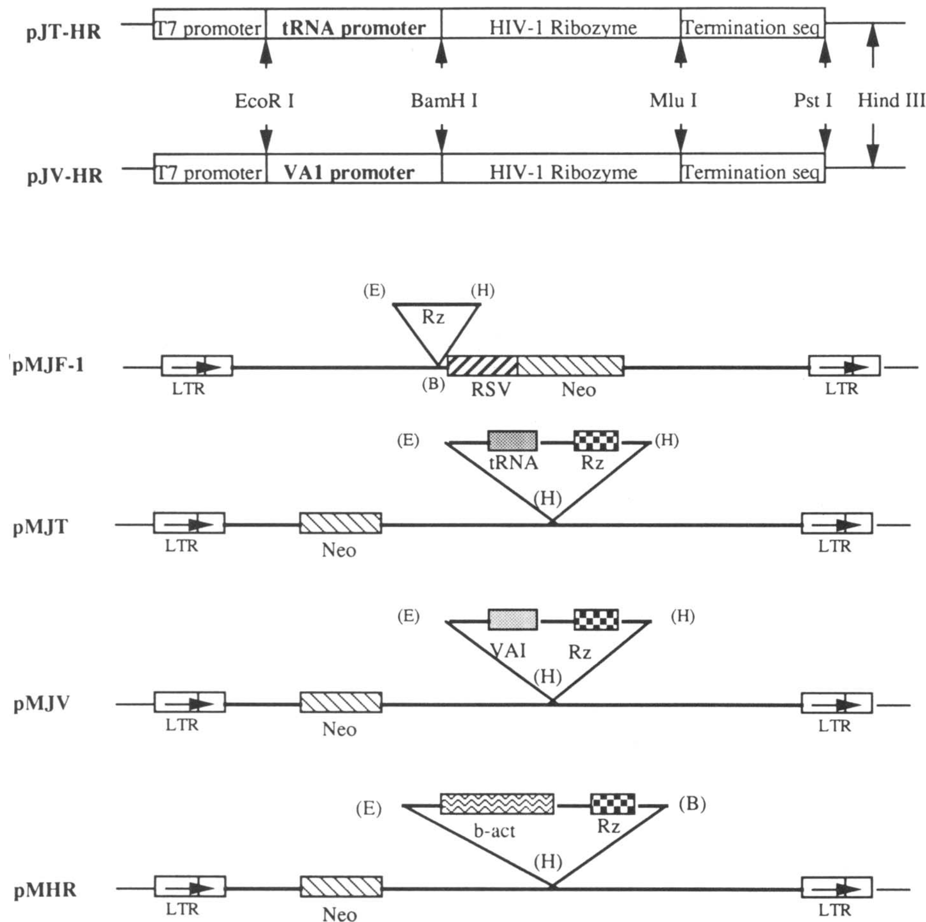

FIG. 1. Schematic representation of the expression and retroviral vector constructions (not drawn to scale). See text for the design of each plasmid. Seq, sequence; Rz, ribozyme; b-act, β-actin; E, *Eco*RI; H, *Hind* III; B, *Bam*HI.

vectors were designated pJT-HR for tRNA promoter and pJV-HR for *VA1* promoter (Fig. 1).

Construction of Retroviral Vectors. The vectors in which the ribozyme was driven by the internal pol III promoters were constructed as follows. The retroviral vector pLNL6 (from Fred Levine of University of California, San Diego) was digested by *Hin*dIII downstream of the neomycin-resistance (Neo) gene (Fig. 1). The linearized vector was further blunt-ended with the Klenow fragment of DNA pol I. The insert containing the tRNA or *VA1* pol III promoter–ribozyme cassette (including the termination signal) was removed from the pJT-HR or pJV-HR plasmid (Fig. 1) by digestion at *Eco*RI and *Hin*dIII sites and blunt-ended with the Klenow fragment. The resulting retroviral vectors were designated pMJT (for tRNA internal promoter) and pMJV (for *VA1* internal promoter, Fig. 1). The retroviral vector with an internal β-actin promoter was constructed using the same vector, with an *Eco*RI–*Bam*HI insert taken from pβ-HR (11). The hairpin autocatalytic cassette (11) is included in the latter insert. The resulting retroviral vector after ligation was named pMHR (Fig. 1). To compare the ribozyme expression from the internal promoters vs. the retroviral long terminal repeat (LTR), another vector (pLRNL-2, from Jiing-Kuan Yee of University of California, San Diego) was used. The ribozyme taken from pHR (11) by *Eco*RI/*Hin*dIII digestion was inserted into the *Bam*HI cloning site immediately upstream of the Rous sarcoma virus promoter that drives the Neo gene (Fig. 1).

Cells and Transfections. HeLa cells were propagated in DMEM containing 10% (vol/vol) fetal bovine serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were grown to ≈70% confluence in a 12-well plate (1×10^5 cells per well) plated out 1 day prior to transfection. Before transfection, the medium was replaced with 2 ml of fresh DMEM containing the same additives. Calcium phosphate-precipitated plasmid DNA was added to the cells. After 24 hr, the medium was removed and the cells were rinsed two or three times with 1× PBS. The cultures were then maintained in 2 ml of DMEM containing 10% fetal bovine serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 1 mM sodium pyruvate.

p24 Antigen Quantification. The Coulter HIV-1 p24 ELISA kit was used to quantitate the core antigen according to the manufacturer's instructions. A_{450} was measured using an ELISA plate reader. Viral protein (p24) concentration in the culture supernatant harvested 40–48 hr after transfection was determined from the absorbance using a standard curve. The values were then reported as percentage of activity or as the p24 protein concentration. The inhibition of expression of HIV-1 p24 was used to determine the effectiveness of the ribozyme as an inhibitor of HIV-1 replication and expression.

Dot-Blot Analysis. Total RNA from HeLa cells transfected with various DNA samples was isolated as described (11). Briefly, the cells were washed twice with ice-cold 1× PBS and then lysed by adding 10 mM EDTA (pH 8.0)/0.5% SDS/0.1 M sodium acetate, pH 5.2/10 mM EDTA was added to the lysed cells. Total RNA was recovered from the cell lysates by a single extraction with water-saturated phenol and ethanol-precipitation. To remove the template DNA, the isolated RNA was subjected to DNase I treatment. The reaction was stopped by adding 10 mM EDTA/0.2% SDS, and the RNA was extracted in phenol/chloroform and then ethanol-precipitated. The recovered total RNA was redissolved in diethyl pyrocarbonate (DEPC)-treated water, and 20 mg was

immobilized on GeneScreen*Plus* membrane (DuPont) by gentle suction with a blotting manifold (Bethesda Research Laboratories). The membrane was then probed with a 5′-end radioactively labeled synthetic DNA probe complementary to the ribozyme RNA or with an *in vitro*-transcribed and internally radiolabeled RNA probe complementary to HIV-1 Rev response element (RRE) RNA. The intensity of the dots was quantified by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Effects of the Ribozyme Driven by pol III Promoters on HIV-1 Expression. We cloned the HIV-1 5′-leader-sequence-specific ribozyme gene into mammalian expression vectors downstream of the human $tRNA^{Val}$ promoter and the adenovirus *VA1* promoter and upstream of the pol III termination signal (ref. 18 and Fig. 1). The expression of the ribozyme in HeLa cells transfected with these plasmids was determined by RNA dot blot analysis. When compared with human β-actin promoter (pol II)-controlled expression (10), the expression of the ribozyme driven by a pol III promoter was 88% higher (data not shown).

To determine the effectiveness of ribozyme-mediated inhibition of HIV expression in a transient assay, an effector plasmid (pHXB2gpt), a reporter plasmid (pC15CAT) (19), and the ribozyme-containing plasmids (pJT-HR and pJV-HR) or pUC19 as the control were cotransfected into HeLa cells by the calcium phosphate method as described (11). For comparison, the same ribozyme driven by the β-actin promoter (11) was used in parallel. Cell lysates were subjected to chloramphenicol acetyltransferase assay, and the levels of HIV-1 p24 antigen (gag gene product) in the culture supernatant were analyzed by ELISA. The results obtained were graphed as the relative percentage of control value (100%) (Fig. 2 *A* and *B*). The HIV-1 ribozyme expressed by pol III promoters inhibited HIV-1 expression significantly (overall 70–95%). In the assay for Tat protein activity (Fig. 2*A*), the ribozyme directed by the $tRNA^{Val}$ promoter yielded >10% greater inhibition than the ribozyme directed by β-actin promoter. For p24 expression (Fig. 2*B*), ribozymes directed by both pol III promoters yielded 15–25% more inhibition than the β-actin promoter-directed ribozyme. As described (11), the effect of the ribozyme-containing pol III promoter plasmids for the inhibition of HIV-1 expression was DNA-dose-dependent (data not shown).

Cleavage and Target Specificity of the Ribozyme. Our previous study showed that inhibition by the ribozyme driven by the β-actin promoter functioned primarily as a catalytic and not an antisense molecule and that its action was dependent on the presence of a target sequence (11). We have now verified a similar function and specificity for the pol III-promoter-driven ribozymes. To determine the contribution of the catalytic activity vs. the antisense activity to the inhibition of HIV-1 expression, a disabled ribozyme (11) with three point mutations (AAA → UGC, positions 22–24) was constructed in pol III promoter-containing plasmids. These mutations rendered the ribozyme inactive but had no effect on its binding to target RNA (11). Wild-type and disabled ribozymes were expressed at the same levels in transfected cells but the disabled ribozyme yielded only ≈10% inhibition of HIV-1 expression (data not shown). Therefore, the inhibition of HIV-1 replication and expression observed is probably contributed mainly by the catalytic property of the ribozyme and not its antisense features. When pTAT was used to replace pHXB2gpt to supply Tat protein in trans (11), neither pJT-HR nor pJV-HR showed inhibition of chloramphenicol acetyltransferase activity. This confirms the fact that the HIV-1 ribozyme inhibits only the expression of messengers containing the target sequence.

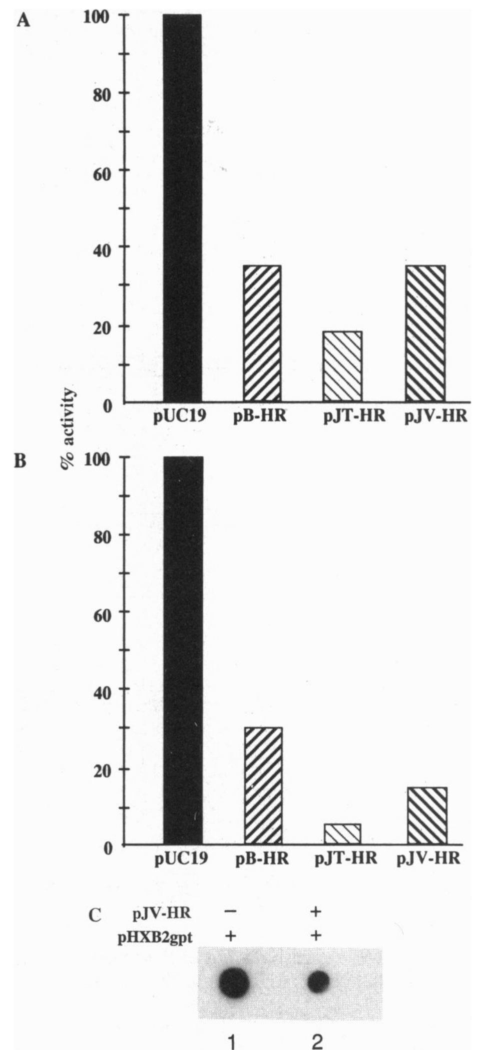

FIG. 2. Effect of the ribozyme on HIV-1 expression in a transient assay. The effector plasmid (pHXB2gpt) and a reporter plasmid (pC15CAT) were triple-transfected into HeLa cells with a ribozyme-containing plasmid pUC19, pβ-HR, pJT-HR, or pJV-HR in a weight (μg) ratio of 1:10 (effector plasmid/ribozyme plasmid). Plasmids used are indicated. (*A*) Ribozyme effect on Tat activity. Transfected HeLa cells were harvested after 48 hr and subjected to chloramphenicol acetyltransferase assay. Radioactivity in the TLC slices was measured using a scintillation counter and results are expressed relative to the control. (*B*) Ribozyme effect on p24 expression. The supernatant was subjected to p24 antigen analysis and the values were represented as relative percentage. (*C*) Total RNA was isolated by the rapid isolation method. Total RNA (20 μg) was dotted and hybridized with a [α-^{32}P]UTP internally labeled HIV-1-specific probe. Lanes: 1, RNA prepared from pUC19/pHXB2gpt-transfected cells; 2, RNA from pJV-HR/pHXB2gpt-transfected cells.

We further determined whether the reduced expression of HIV-1 proteins directly resulted from a reduced amount of HIV-1 mRNA. Equal amounts of total RNA from HeLa cells transfected with pHXB2gpt/pJV-HR and pHXB2gpt/pUC19 at a weight (μg) ratio of 1:10 were subjected to dot blot analysis with HIV-1-specific DNA. The result shown in Fig. 2*C* indicates that the presence of the ribozyme reduced HIV-1 transcripts by 71%. The level of viral protein p24 inhibition observed *in vivo* (80–90%, Fig. 2*B*) was higher than the levels observed via RNA dot blot analysis (71%, Fig. 2*C*), possibly due to the fact that some viral mRNA is only partially degraded and is still detectable by dot blot but not translatable into proteins.

The Leader-Sequence Ribozyme Inhibits Diverse HIV-1 Strains. A necessary feature of any effective therapeutic

agent against AIDS will be its ability to inhibit various HIV-1 strains. We addressed this important issue by using different retroviral vectors containing the ribozyme (Fig. 1) to cotransfect with DNA constructs from a variety of HIV-1 strains. Three strains, MN, SF-2, and Eli, were chosen that exhibit various genetic distances from HXB2, with Eli (a Zairean strain) being the most distant. Interestingly, the leader sequence targeted by the ribozyme under study (TGCCCGTC-TGTTGTGT) is completely conserved among known HIV-1 strains, including the Zairean strain Eli, with the exception of that of MN, which shows a 1-base mutation [(lower case type) TGCCCGTCTGTTaTGT] (20).

As seen in Fig. 3*A*, all three retroviral vectors in which the ribozyme gene is driven by the internal promoters (Fig. 1) show significant inhibition of SF-2 expression (compare pMJT, pMJV, and pMHR to the control bar pLRNL-2). These three internal promoters include our previously reported pol II promoter-driven β-actin (in pMHR) and the pol III promoters of $tRNA^{Val}$ and *VA1* (in pMJT and pMJV) reported here. On the other hand, the retroviral vector pMJF-1 in which the ribozyme gene is driven directly by Moloney murine leukemia virus LTR (Fig. 1) did not inhibit HIV-1 expression (Fig. 3*A*, compare bars pMJF-1 and pLRNL-2). To determine whether the inability of pMJF-1 to inhibit SF-2 is due to the lack of expression of the ribozyme in this vector, RNA dot blot analysis was carried out (Fig. 3*B*). Expression of ribozyme sequences was detected in all retroviral vectors carrying the ribozyme gene including pMJF-1. In contrast, the vector alone (bar pLRNL-2) produced no ribozyme. In fact, pMJF-1, which did not inhibit the HIV-1 expression, is one of the high-expression vectors. A possible explanation for the inability of this vector to inhibit virus expression is that the ribozyme cloning site is several hundred base pairs away from the transcription initiation point. The extra long arm attached at the 5′ end of the ribozyme may interact with cellular nucleic acids or proteins, which affect its accessibility to the substrates.

The effect of pMJT, pMJV, and pMHR on Eli and MN was then investigated. As shown in Fig. 4*A*, a similar extent of inhibition was observed for strains SF-2 and Eli (Figs. 3*A* and

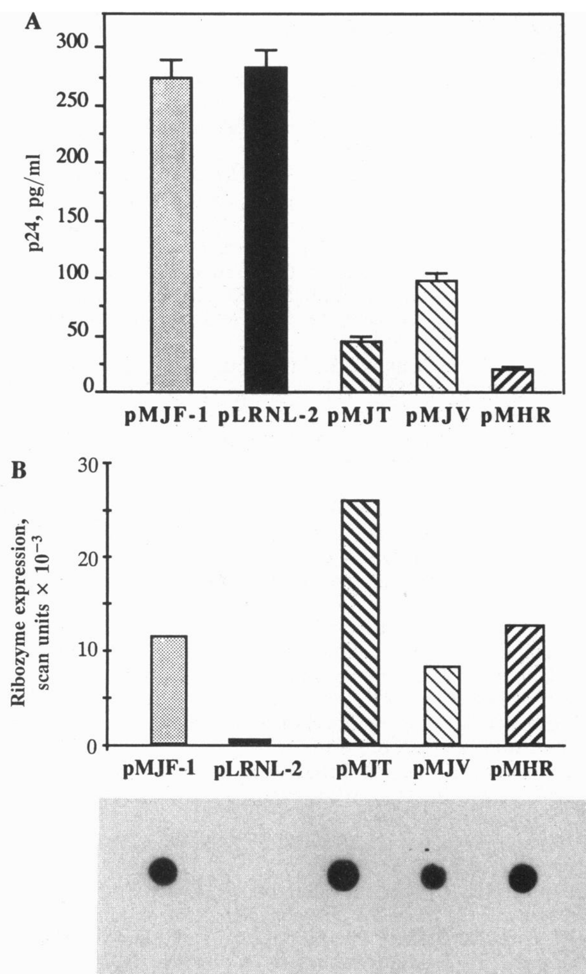

FIG. 3. Effect of the ribozyme on HIV-1 SF-2 expression and the ribozyme expression in retroviral vectors. (*A*) Ribozyme inhibition of SF-2. The effector plasmid (pSF-2) was cotransfected in duplicate or in triplicate into HeLa cells with the ribozyme-containing retroviral vector [0.5:10 effector plasmid/ribozyme vector weight (μg) ratio] pMJF-1, pMJT, pMJV, or pMHR or the retroviral vector without ribozyme gene pLRNL-2. After 48 hr, the cells were harvested and the supernatant was subjected to p24 antigen analysis. (*B*) Ribozyme expression. Total RNA was isolated from HeLa cells transfected with pMJF-1, pLRNL-2 (vector), pMJT, pMJV, or pMHR, dotted on the membrane, and hybridized by 5′-end-radiolabeled ribozyme-specific probe. The intensity of the dots was quantified in scan units by using a PhosphorImager.

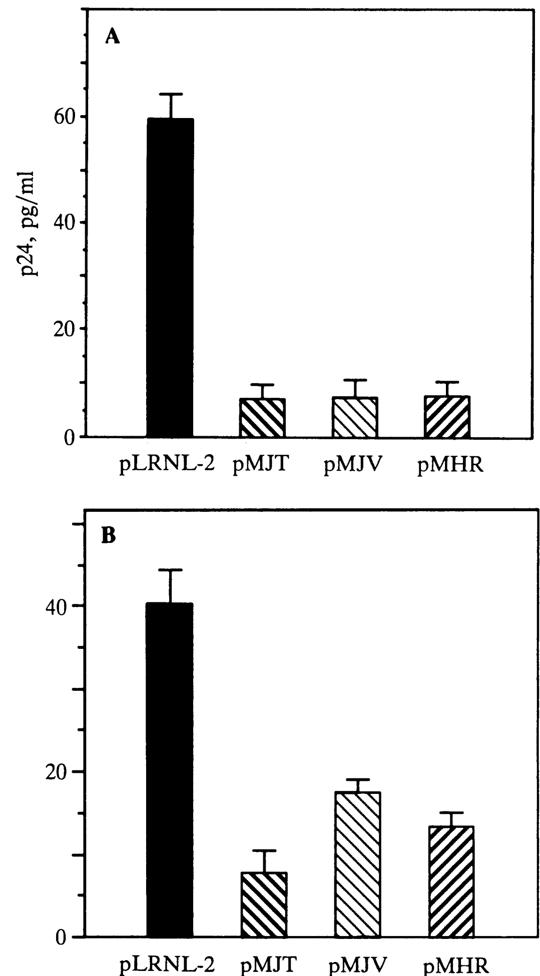

FIG. 4. Effect of ribozyme expressed in retroviral vectors on HIV-1 Eli and MN strains expression in a transient assay. The effector plasmid (pEli or pMN) was cotransfected in duplicate or in triplicate into HeLa cells with the ribozyme-containing retroviral vector pMJT, pMJV, or pMHR or the retroviral vector without ribozyme gene pLRNL-2. (*A*) Ribozyme inhibition of Eli. For HIV-1 pEli, a 0.25:10 effector plasmid/ribozyme vector weight (μg) ratio was cotransfected. (*B*) Ribozyme inhibition of MN. For HIV-1 pMN, a 0.25:10 effector plasmid/ribozyme vector weight (μg) ratio was cotransfected. After 48 hr, the cells were harvested and the supernatant was subjected to p24 antigen analysis.

4*A*). Slightly less but still significant inhibition was observed for the MN strain (Fig. 4*B*), suggesting that the single-base substitution in target sequence has some effect on ribozyme activity.

DISCUSSION

We have been interested in developing ribozymes as antiviral agents in gene therapy for the treatment of HIV-1 infection. The use of catalytic RNA provides a unique approach to the reduction and perhaps elimination of HIV-specific RNA (incoming HIV genome and *de novo*-synthesized transcripts) and may interfere with more than one step of the infection cycle. We have shown (11) the effect of a hairpin ribozyme driven by the β-actin promoter on HIV expression. In exploring more efficient systems for high and ubiquitous expression of the ribozyme, we have expressed the ribozyme under the control of a pol III promoter (Fig. 1) and determined its effect on HIV-1 replication and expression. The current study shows that the ribozyme in pol III expression cassette can inhibit HIV-1 expression up to 95% (p24) by transient transfection assay (Fig. 2*B*, pJT-HR). The inhibition of *gag* gene-encoded p24 concomitant with a substantial reduction in tat activity suggests that the ribozyme can affect HIV-1 replication. Furthermore, HIV-1 RNAs were degraded with high specificity (Fig. 2*C*).

The use of the 5′ leader sequence of HIV-1 as the target is desirable since it would inhibit expression of both early and late viral gene products due to its presence in all HIV-1 RNA transcripts. By cleaving at this site, the ribozyme renders the RNA capless, so that it is poorly translated and presumably more quickly degraded. Another important consideration in the choice of this 5′ leader sequence of HIV-1 as the target is its conservation among most of the HIV-1 isolates. As an effective therapeutic agent, it is essential for the ribozyme to be able to target a variety of HIV-1 strains. We tested several diverse HIV-1 strains in addition to HXB2, including SF-2, MN, and Eli. SF-2 and Eli contain the same target sequence, but the overall genomic sequences are quite diverse, especially Eli, which is a Zairean virus. Therefore, the accessibility of the same target sequence embedded in divergent genomes will be important to address. Of all the available HIV-1 sequences, only MN contains one nucleotide substitution in the target sequence (20). Although *in vitro* studies suggested that such a conservative $G \rightarrow A$ substitution at that position may be tolerated in a ribozyme–substrate interaction (21), one cannot predict whether such is also the case *in vivo*. In designing experiments to address the various issues of strain diversity, we utilized ribozymes expressed in retroviral vector constructs. Since retroviral vectors are still the preferred vehicles for gene delivery to human hematopoietic cells, including stem cells, these constructs were prepared as a further step toward gene therapy. The results showed that SF-2 and Eli were efficiently inhibited by the ribozymes (70–95%, Figs. 3*A* and 4*A*) expressed from internal promoters in the Moloney murine leukemia virus-based vectors, and MN was inhibited only to a slightly lesser extent (50–70%, Fig. 4*B*). These results are encouraging to our long-term efforts in continuously refining the expression and delivery of hairpin ribozymes as a gene-therapy approach to the treatment of HIV-1 infection. However, to ensure efficacy of the hairpin ribozyme, we have developed the second HIV-1 ribozyme that targets the HIV-1 pol sequence (bp 2490–2504 in the HXB2 clone). Attacking multiple targets should yield greater inhibition as well as safeguard against the generation of escape mutants.

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