Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10802–10806, November 1992
Medical Sciences

Inhibition of human immunodeficiency virus type 1 expression by a hairpin ribozyme

(catalytic RNA/intracellular immunization/antiviral therapy)

JOSHUA O. OJWANG[†], ARNOLD HAMPEL[‡], DAVID J. LOONEY[†§], FLOSSIE WONG-STAAL[†¶], AND JAY RAPPAPORT[†‖]

[†]Department of Medicine, Clinical Sciences Building, University of California, San Diego, La Jolla, CA 92093-0665; [§]San Diego Veterans Administration Medical Center, San Diego, CA 92161; and [‡]Department of Biological Sciences, Northern Illinois University, Montgomery Hall 319, Dekalb, IL 60115

Communicated by Helen M. Ranney, August 5, 1992 (received for review May 20, 1992)

ABSTRACT **Ribozymes are RNAs that possess the dual properties of RNA sequence-specific recognition, analogous to conventional antisense molecules, and RNA substrate destruction via site-specific cleavage. The cleavage reaction is catalytic in that more than one substrate molecule is processed per ribozyme molecule. We have designed a hairpin ribozyme that cleaves human immunodeficiency virus type 1 (HIV-1) RNA in the leader sequence (at nucleotides +111/112 relative to the transcription initiation site). The ribozyme was tested *in vitro* and gave efficient and specific cleavage of RNA containing the leader sequence. To test the antiviral efficacy of this ribozyme, we have cotransfected into HeLa cells HIV-1 proviral DNA and a plasmid expressing the ribozyme from the human β-actin promoter. HIV-1 expression was inhibited as measured by p24 antigen levels and reduced Tat activity. The antiviral effect of the ribozyme appears to be specific and results from directed RNA cleavage; activity requires both a target sequence and a functional RNA catalytic center. These results suggest that this HIV-1-directed hairpin ribozyme may be useful as a therapeutic agent.**

Since the identification of human immunodeficiency virus (HIV) as the causative agent for acquired immune deficiency syndrome (AIDS), there has been a concerted effort to develop strategies which inhibit virus replication. In theory, inhibition of HIV-1 replication should be achievable by interfering with various key steps in the viral life cycle, including virus entry, reverse transcription, transcription, transactivation, translation, packaging, or release of virus particles (1). The diverse approaches that have been taken include antisense and ribozyme technologies. Both approaches target the same step in the HIV-1 life cycle, the utilization of viral mRNA (reviewed in ref. 2). Conventional antisense RNAs and DNAs have been shown to impair gene expression (3, 4) and may have utility as antiviral and anticancer agents. The stoichiometric nature of antisense inhibition imposes limits on this approach. Ribozymes are RNA molecules that possess RNA catalytic activity. A catalytic strand cleaves a specific site in target RNAs; the number of cleaved RNAs is greater than what might be predicted based on stoichiometry (5–7). These experiments demonstrate the feasibility of using ribozymes to confer intracellular resistance (8–12) to productive HIV infection.

The ribozyme used in this study is of the "hairpin" type (13). This ribozyme is derived from the minimum catalytic center of the negative strand of tobacco ringspot virus (14). The original native minimum-sequence hairpin ribozyme had a 50-nucleotide (nt) catalytic RNA cleaving a 14-nt substrate RNA in a trans reaction. The catalytic RNA/substrate RNA complex forms a hairpin two-dimensional structure having four helical domains and five loop structures. Two helices form between the substrate and the ribozyme, which allow specificity of binding. Located between these two helices in the substrate is a N*GUC sequence where GUC is a required sequence and cleavage occurs at *. Certain sequences are cleaved with high efficiency *in vitro*, with k_{cat} and K_m as high as 2.1/min and 30 nM, respectively, for the native sequence at pH 7.5, 37°C, low salt, and 12 mM Mg^{2+} (13). Since cleavage occurs efficiently *in vitro* under near-physiological conditions, the hairpin ribozyme may also be functionally favorable *in vivo* (15).

The ribozyme used in this study was designed to cleave a target site at position +111/112 relative to the transcription initiation site, within the 5′ untranslated leader sequence present in all HIV-1_{HXB2} mRNA species. This target is conserved in most of the known HIV-1 isolates. *In vitro* analysis of this ribozyme revealed a highly efficient cleavage activity. We further tested the effectiveness of the hairpin ribozyme in a transient-expression assay in HeLa cells and observed a reduction in p24 protein as well as Tat activity. These data support the potential application of ribozymes as therapeutic agents directed against HIV.

MATERIALS AND METHODS

Enzymes and Chemicals. Restriction enzymes were from Bethesda Research Laboratories and Boehringer Mannheim, DNA Modification enzymes from Pharmacia and Boehringer Mannheim, the DNA sequencing kit from Pharmacia, T7 RNA polymerase from United States Biochemical, and [α-^{32}P]CTP from ICN. Bovine calf serum, antibiotic solution (containing penicillin and streptomycin), L-glutamine, sodium pyruvate, phosphate-buffered saline (PBS), and Dulbecco's modified Eagle's medium (DMEM) were from GIBCO. The HIV-1 p24 antigen-detection kit was from Coulter.

Plasmid Constructions. Unless stated otherwise, recombinant DNA techniques were essentially as described in ref. 16. DNA sequences were confirmed by published procedures (17, 18). Nucleotide sequence from the HXB2 clone (19) was used to design the HIV-1-specific ribozyme.

The hairpin ribozyme directed against the 5′ leader sequence of HIV-1 was constructed as follows. Double-stranded oligodeoxyribonucleotides containing wild-type (HR+:HR−) and disabled (dHR+:dHR−) ribozyme sequences were chemically synthesized. These oligonucleotides were flanked by

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HIV, human immunodeficiency virus; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; nt, nucleotide(s).

¶To whom reprint requests should be addressed.
‖Present address: Department of Medicine, Division of Renal Diseases and Hypertension, George Washington University and Laboratory of Oral Medicine, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892.

Xho I and *Bam*HI sites at the 5′ end and by an *Mlu* I site at the 3′ end. The oligonucleotides were cloned in the *Xho* I/*Mlu* I sites of pHC to generate plasmids pHR and pdHR. Plasmid pHC contained a hairpin autocatalytic cassette along with *Xho* I and *Mlu* I sites in pTZ18R (United States Biochemical). The autocatalytic hairpin cassette contained the native hairpin and substrate RNA sequences (13) with the 3′ terminus of the substrate covalently linked to the 5′ terminus of the ribozyme with a CCUCC sequence. Plasmids pHR and pdHR therefore contained the HIV-1 ribozyme upstream of the hairpin autocatalytic cassette.

RH+ = 5′-ctcgaggatccACACAACAAGAAGGCAACCAGAGAAACA-
CACGTTGTGGTATATTACCTGGTAcgcgt-3′

RH− = 5′-acgcgTACCAGGTAATATACCACAACGTGTGTTTCTCTG-
GTTGCCTTCTTGTTGTGTggatcctcgag-3′

dHR+ = 5′-ctcgaggatccACACAACAAGAAGGCAACCAGAG[CGT]CA-
CACGTTGTGGTATATTACCTGGTAcgcgt-3′

dHR− = 5′-acgcgTACCAGGTAATATACCACAACGTGTG[ACG]CTC-
TGGTTGCCTTCTTGTTGTGTggatcctcgag-3′.

Lowercase letters correspond to restriction sites, and underlining corresponds to HIV-1 sequences flanking the catalytic domain of the ribozyme, and the mutated bases to generate the disabled ribozyme are bracketed. The correct clones were identified by filter hybridization (16) and confirmed by DNA sequencing. For tissue culture studies, the DNA fragment (*Xho* I–*Hin*dIII) containing the ribozyme from pHR or pdHR was subcloned downstream of the human β-actin promoter at the *Sal* I and *Hin*dIII sites of pHβApr-1 (20). The resultant vectors were called pβ-HR (wild type) and pβ-dHR (disabled).

***In Vitro* Transcription and Reaction Properties.** All ribozymes and substrate were transcribed by T7 RNA polymerase from partially double-stranded synthetic DNA templates (double-stranded at the promoter site for transcription initiation) (21). Transcription was carried out (14) with [α-^{32}P]CTP and transcripts were purified by electrophoresis in 15% acrylamide/7 M urea gels. The *in vitro* cleavage reactions were carried out by incubating the ribozyme and substrate RNA at 37°C in 12 mM $MgCl_2$/2 mM spermidine/40 mM Tris, pH 7.5. After addition of 7 M urea/bromophenol blue/xylene cyanol, products were resolved by electrophoresis in a 15% acrylamide/7 M urea gel. Components were identified by autoradiography, and following excision from the gel, radioactivity was quantitated by Cerenkov counting.

Comparative Binding Assay. Unlabeled active or disabled ribozyme (0.05 μM) was mixed with [α-^{32}P]CTP-labeled substrate (0.10 μM) to give an excess of substrate in 2 mM spermidine/40 mM Tris, pH 7.5. The mixture was heated at 90°C for 2 min, incubated sequentially on ice for 2 min, at 37°C for 10 min, and on ice for 2 hr. Finally, the resultant material was run in a 15% acrylamide gel in 40 mM Tris·HOAc, pH 7.5/12 mM MgOAc at 4°C (nondenaturing conditions which will not disrupt complexes between the ribozyme and substrate).

Cells and Transfections. HeLa cells were propagated in DMEM containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were plated at ≈70% confluency in a 12-well plate (10^5 cells per well) 1 day prior to transfection. Before transfection, the medium was replaced. Calcium phosphate-precipitated (22) plasmid DNA (20 μg) was added to the cells. DNA concentration in each reaction was normalized by adding pUC19 DNA. After 24 hr the medium was removed and the cells were rinsed three times with PBS. The cultures were then maintained in 2 ml of medium. After 48 hr the cells were harvested. Chloramphenicol acetyltransferase (CAT) activity of cell lysates and p24 in culture medium were determined.

Dot Blot Analysis. Total RNA from untransfected or HIV-1 ribozyme-transfected cells was isolated by a rapid procedure (16). Briefly, the cells were washed twice with ice-cold PBS without Ca^{2+} and Mg^{2+} and then lysed with 10 mM EDTA, pH 8.0/0.5% SDS. Then 0.1 M NaOAc, pH 5.2/10 mM EDTA was added to the lysed cells. Total RNA was recovered from the cell lysate by a single extraction with water-saturated phenol followed by multiple ethanol precipitations. To remove the template DNA, the isolated RNA pellet was resuspended in 200 μl of 10 mM Tris, pH 8/1 mM EDTA and treated with RNase-free pancreatic DNase I (2 μg/ml) in 10 mM $MgCl_2$/0.1 mM dithiothreitol/10 mM RNase inhibitor for 60 min at 37°C. The reaction was stopped by adding 10 mM EDTA and 0.2% SDS, and the RNA was extracted by phenol/chloroform treatment and precipitated by ethanol. The recovered total RNA was redissolved in diethyl pyrocarbonate-treated water and 20 μg was immobilized onto a GeneScreen*Plus* membrane (DuPont) by gentle suction with a blotting manifold (Bethesda Research Laboratories). The membrane was then probed with a 5′-end-radiolabeled synthetic oligodeoxyribonucleotide complementary to the ribozyme RNA (50 nt).

p24 Antigen Determination. The Coulter HIV-1 p24 ELISA kit was used according to the manufacturer's instructions to quantitate viral core antigen. Absorbance was read at 450 nm with an ELISA plate reader. Viral protein concentration in the culture supernatant was determined from the absorbance by using a standard curve. The values were then expressed as percent activity. 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.

CAT Assay. Lysates were prepared by freeze–thawing of cells 48 hr after transfection and were assayed for CAT enzyme activity. For quantitation, the unacetylated and acetylated forms of [^{14}C]chloramphenicol were cut out of the thin-layer chromatogram and radioactivity was analyzed in a scintillation counter. The values were expressed as percentage of control value. Transfection experiments were performed three or more times, and the data are presented as the mean ± SD.

RESULTS

Specific Cleavage of HIV-1 RNA by Hairpin Ribozyme *in Vitro*. The hairpin ribozyme was engineered to cleave a site in the 5′ long terminal repeat (LTR) of the HXB2 clone of HIV-1 (Fig. 1*A*). The target sequence is UGCCC*GUCUGUUGUGU, with cleavage occurring at *. The hairpin ribozyme was engineered so that it could base pair to the two sequences flanking the C*GUC to form helices 1 and 2 (Fig. 1*B*). Helix 2 is fixed in length at four bases by the functional requirements of the hairpin ribozyme; however, the length of helix 1 could be varied. The length of helix 1 that provided optimal catalytic activity was experimentally determined to be 8 nt (A.H., unpublished observation).

In vitro this ribozyme cleaved the target substrate with high efficiency as determined by its kinetic parameters (Fig. 2). Under very mild reaction conditions (37°C in 12 mM $MgCl_2$/40 mM Tris/2 mM spermidine at pH 7.5), K_m was 100 nM and k_{cat} was 1.6/min. This gives an enzyme efficiency of $k_{cat}/K_m = 0.016\ nM^{-1}\cdot min^{-1}$ as compared to 0.07 for original native hairpin ribozyme (14). The catalytic efficiency, relative to the original tobacco ringspot ribozyme, is therefore 23%. A disabled ribozyme was prepared with the same sequence as in Fig. 1*B* except that nt 22–24, AAA (Fig. 1*C*), were changed to CGU (Fig. 1*D*). No catalytic activity was detected for this ribozyme *in vitro*, showing that it was disabled (data not shown).

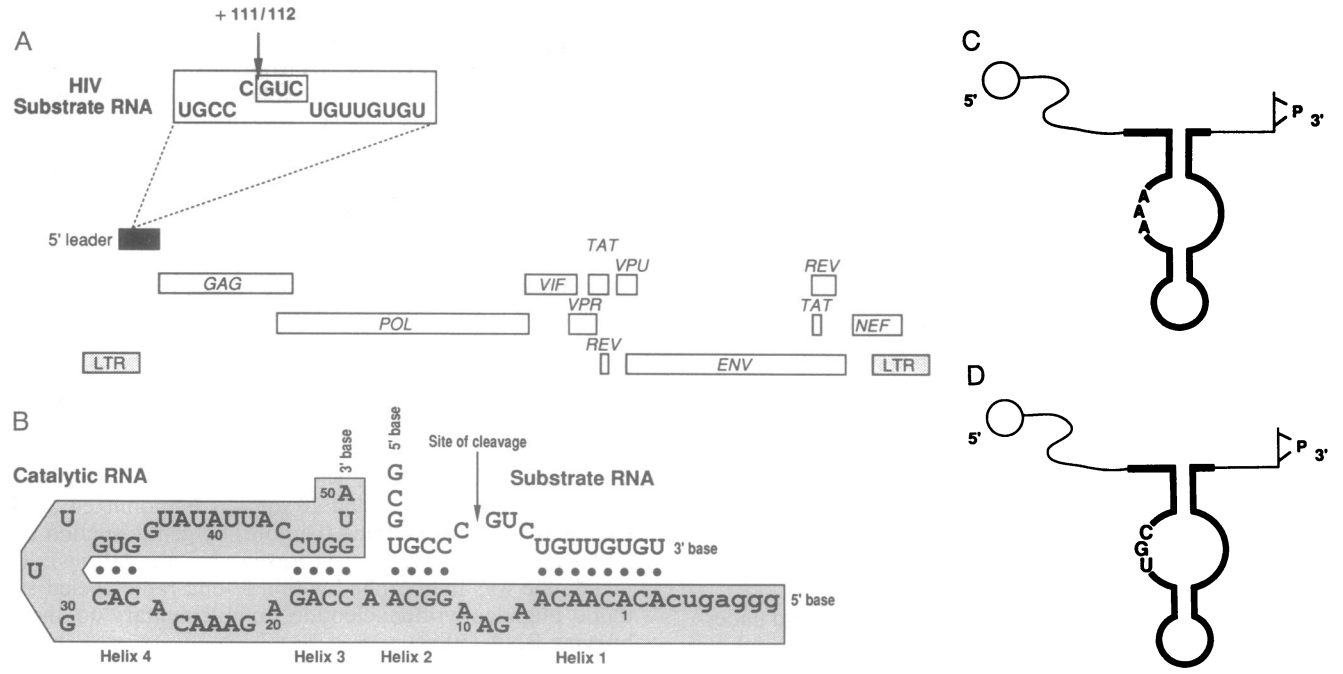

FIG. 1. (*A*) Sequence and location of the target site in the HIV-1 genome. Cleavage occurs at nucleotides +111/112 (arrow) relative to the transcription initiation site (HIV-1_{HXB2}). The 16-base target site is found in the leader of all known HXB2 mRNA species. (*B*) The hairpin ribozyme used to cleave the HIV-1 substrate RNA. Substrate RNA has the target sequence shown plus additional gcg sequence at its 5′ end. Hairpin catalytic RNA is designed as shown. It also has additional vector sequence shown (lowercase letters). (*C*) Wild-type (from plasmid pβ-HR) ribozyme. (*D*) Disabled ribozyme (from pβ-dHR). The 5′ cap site and 3′ terminus with 2′,3′-(cyclic)phosphate are depicted. See text for construction.

Effects of Ribozyme on HIV-1 Expression in a Transient-Transfection Assay. The practical application of ribozymes as therapeutic agents *in vivo* will depend on their ability to function in a complex cellular environment. This requires stable expression of the ribozyme in the cell, specificity for the target RNA, accessibility of mRNA targets for cleavage, and lack of cytotoxicity of the endogenously expressed ribozyme. To address these issues, the sequences containing the HIV-directed ribozyme and its disabled counterpart were cloned into a mammalian expression vector (23) under the control of the human β-actin promoter (20). The resultant plasmid DNAs were used to transfect HeLa cells. Expression of the wild-type and disabled ribozymes was confirmed by dot blot analysis of total cellular RNA (Fig. 3). The wild-type and disabled ribozyme mRNAs were detected at equal levels from transfected HeLa cells but not from cells transfected with pHβAPr-1 vector or from nontransfected HeLa cells.

To demonstrate that the ribozyme inhibited gene expression of (HIV-1), the plasmid pHXB2gpt (24) was employed in a transient assay. In this assay, pHXB2gpt provides the target mRNA which is translated into viral proteins, including Tat. Tat protein in turn transactivates the CAT-linked LTR promoter (pC15CAT) (25). If the ribozyme is functional, then it is expected to cleave the HIV-1 mRNA, resulting in reduced *tat* and *gag* expression and, hence, reduced transactivation. Tat activity is reflected by the amount of CAT enzyme activity (Fig. 4 *A* and *B*), whereas the amount of *gag* product is reflected by the amount of p24 protein detected by ELISA (Fig. 4*C*). The results are shown as a relative percentage of the control value. The expression of HIV-1 ribozyme in this transient assay inhibited HIV-1 expression and virus production by 70–80%. The ratio given above was the optimum. Further increase in ribozyme concentration had no additional effect.

Ribozyme Activities in Intact Cells Involve Target Cleavage. Ribozyme action involves hybridization with target RNA (Fig.

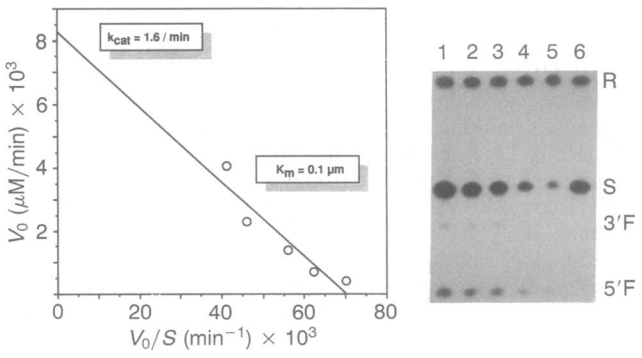

FIG. 2. *In vitro* reaction kinetics. The reaction shown was carried out at 37°C for 5 min with the ribozyme at 5 nM and the substrate at 0.1 μM (lane 1), 0.05 μM (lane 2), 0.025 μM (lane 3), 0.012 μM (lane 4), 0.006 μM (lane 5), or 0.025 μM (lane 6; this is control lane at zero time). From the time course, 35% of the substrate was uncleavable. This was subtracted for these calculations. These results give K_m = 100 nM and k_{cat} = 1.6/min, which are outstanding catalytic RNA parameters. Additional experiments showed that this reaction under these conditions was linear with time (data not shown). R, ribozyme; S, substrate; 3′F, 3′ cleaved fragment; 5′F, 5′ cleaved fragment.

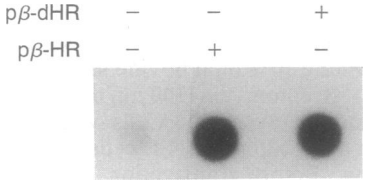

FIG. 3. Expression of wild-type and disabled ribozymes in HeLa cells. Cells were transfected with pβ-HR or pβ-dHR by a calcium phosphate method. Forty-eight hours later, total RNA was rapidly isolated (15) from both transfected and mock-transfected cells. Total RNA (20 μg per slot) was hybridized with 5′-end-labeled ribozyme-specific probe (10^7 cpm/μg).

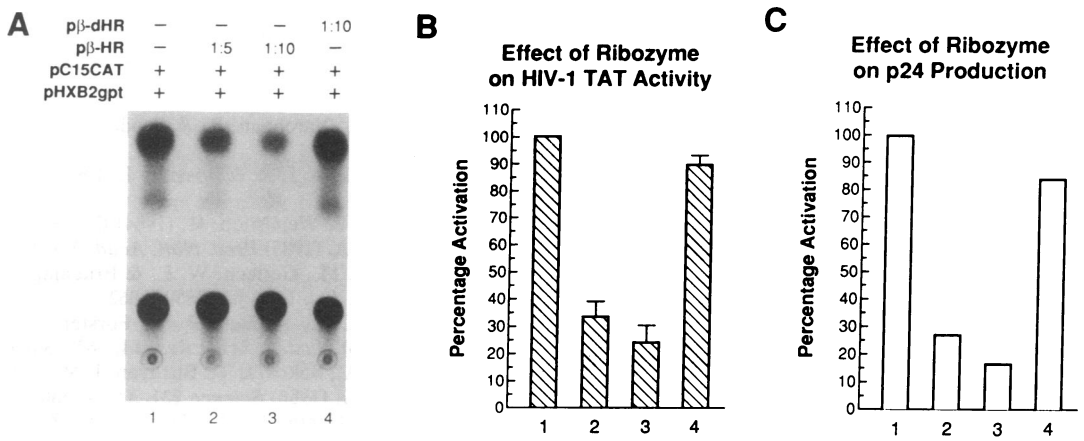

FIG. 4. Effect of ribozyme on HIV-1 expression in transiently transfected HeLa cells. This effector plasmid (pHXB2gpt, which provides the ribozyme target) and a reporter plasmid (pC15CAT) were cotransfected into HeLa cells either with HIV-1 5′ leader sequence-specific ribozyme containing plasmid (pβ-HR) or with disabled ribozyme construct (pβ-dHR) in a molar ratio of 1:5 or 1:10 (effector plasmid/ribozyme plasmid). The control consisted of HeLa cells transfected with pHXB2gpt, pC15CAT, and pHβApr-1 vector. The DNA concentration was normalized to 20 μg with pUC19. (*A*) After 48 hr the cells were harvested (see text) and subjected to CAT assay. (*B*) Radioactivity in slices of the thin-layer chromatogram corresponding to unacetylated and acetylated chloramphenicol was measured by scintillation counter, and results are expressed relative to the control. (*C*) Culture supernatant was subjected to p24 ELISA. The experiments were repeated three times or more and values are represented as relative percentage. Numbers below bars in *B* and *C* correspond to numbers below lanes in *A*.

5*A*) and subsequent cleavage. To distinguish between an antisense and a ribozyme effect in the cotransfection assay, a disabled ribozyme (pβ-dHR) was constructed and tested. The binding of the disabled ribozyme to substrate was not affected by the mutation (Fig. 5*B*). However, this mutation was found to inactivate the catalytic activity of the ribozyme *in vitro* (A.H., unpublished data). Since these changes are outside the substrate binding site, antisense activity, if present, should be unaffected. The differential inhibitory activity of pβ-HR or pβ-dHR was used to determine the catalytic contribution of the ribozyme. The disabled ribozyme yielded only ≈10% inhibition, compared with 70–80% inhibition by the wild type. These results suggest that the inhibition of HIV-1 replication and expression is attributable mainly to the catalytic property of the ribozyme and not its antisense features.

Ribozyme Specificity. To confirm the specificity of the ribozyme effect, pTAT, a plasmid lacking the target sequence, was used instead of the target plasmid (pHXB2gpt) to supply Tat protein for transactivation. The experiment was performed as described above. This approach eliminated the ribozyme target in the cells but still provided Tat for transactivation from a source devoid of the ribozyme target. In the absence of the ribozyme target, no inhibition of CAT expression was observed (Fig. 6). These results indicate that the HIV-1 ribozyme inhibits gene expression only in the context of its appropriate target sequence.

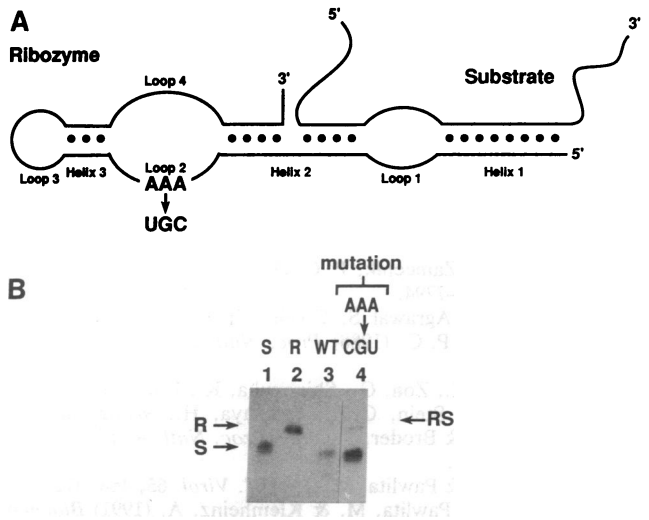

FIG. 5. Comparative binding of disabled and wild-type ribozymes. (*A*) Ribozyme/substrate complex. (*B*) Lanes: 1, substrate with no added ribozyme; 2, [α-^{32}P]CTP-labeled active ribozyme used as mobility reference; 3, unlabeled HIV-1-specific active ribozyme and [α-^{32}P]CTP-labeled substrate (9% bound); 4, disabled ribozyme and [α-^{32}P]CTP-labeled substrate (18% bound). S, substrate; R, ribozyme; RS, ribozyme/substrate complex.

DISCUSSION

Antisense RNA and DNA can inhibit HIV expression in mammalian cells (26–28). When antisense RNAs were expressed intracellularly from hybrid genes, a transient inhibition of HIV expression was observed (29, 30). Ribozymes

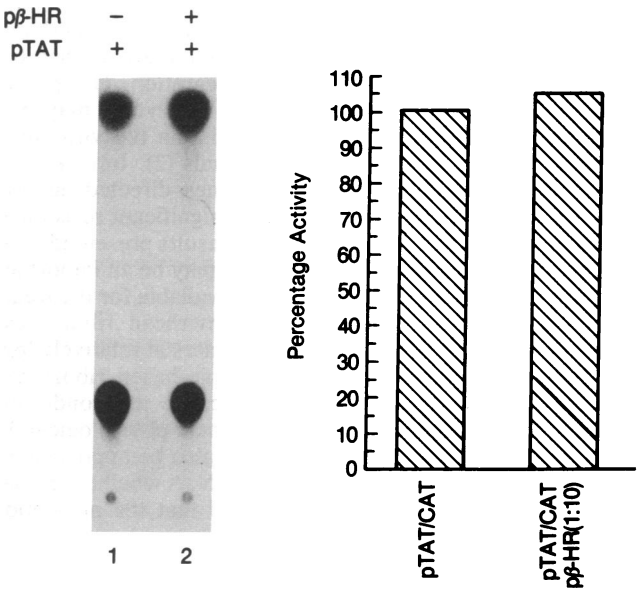

FIG. 6. Ribozyme specificity. HeLa cells were transfected with pTAT plus pC15CAT (lane 1) or pTAT plus pC15CAT plus pβ-HR (lane 2). After 48 hr the cells were harvested and subjected to CAT assay. Percent conversion was quantified by scintillation counting as in Fig. 4*B*. Results are expressed as percentage of control activity.

have all the properties of an antisense RNA with the additional property of catalytic cleavage (31, 32). In this study, we have used an HIV-1 specific hairpin ribozyme under the control of the human β-actin promoter and analyzed the effect on HIV gene expression. A target within the 5′ leader sequence was chosen because of its high degree of conservation among most known isolates and because of its presence in both early and late viral gene products. By cleaving at this site, the ribozyme renders the RNA capless and presumably exposed to degradation. In addition, uncapped mRNAs are poorly translated. We have demonstrated the activity of this hairpin ribozyme in inhibiting HIV gene expression in a transient transfection system. The exact mechanism by which ribozymes function intracellularly, however, has been difficult to address. For example, ribozymes may perform catalytic cleavage or they may act as conventional antisense molecules. Many investigators have employed an antisense strand to the ribozyme as a control. This strategy has little utility because these RNAs cannot function as antisense to the target or perform catalytic cleavage. To separate antisense from cleavage effects, we created a 3-base mutation in the ribozyme between helix 3 and helix 4 within the catalytic center. The disabled ribozyme presumably can still bind to the substrate, but catalytic activity should have been abolished. Any activity observed with the disabled ribozyme should, therefore, have been from its antisense property. Since the disabled ribozyme retained little inhibitory activity relative to the wild type, the observed effect was primarily due to the ribozyme catalytic activity.

In vivo activity of a ribozyme is impossible to predict, despite evidence of function *in vitro*. Cellular proteins and formation of RNA–protein complexes may either inhibit or facilitate target cleavage. The level of inhibition observed in HeLa cells in this case was slightly higher than the maximal percent cleavage observed *in vitro* (65%). The inhibition was maximal when the ribozyme expression plasmid was present at a 10-fold molar excess. The lack of complete inhibition in both cases may reflect target accessibility in a portion of the RNA molecules. A slightly higher level of inhibition in the cells (70–80%) may be due to an increased accessibility of target RNA or, alternatively, may result from an additional antisense effect.

In addition to inhibition of endogenous HIV expression, catalytic RNA may have the potential to cleave and perhaps eliminate HIV-specific RNA prior to integration, thereby inhibiting the viral life cycle prior to integration. This possibility remains to be tested. Although ribozymes may not eliminate HIV infection, they may maintain the virus in a dormant state in HIV-infected individuals (2). Intracellular expression of "hammerhead" ribozymes directed against HIV-1 RNA has been shown to confer significant resistance to productive HIV-1 infection (8–10). Results presented here suggest that the hairpin ribozyme, too, may be an important addition to the therapeutic modalities available for the treatment of AIDS. As opposed to hammerhead ribozymes, hairpin ribozymes cleave at maximum rates at relatively low Mg^{2+} concentrations. Therefore, hairpin-based ribozymes may be particularly advantageous because the conditions required for their optimum function are near physiological. In addition to HIV, this approach should also be applicable to eliminate a wide variety of deleterious RNAs whether cellular (i.e., oncogene) (33) or viral, provided that the nucleotide sequences are known.

We thank Paul Klotman and Michael Lan for helpful discussions and for critical reading of the manuscript, Patricia Badel for help with tissue culture work, and Jeff Aspinall and Bob Hoenle for vector constructions. This work was supported in part by U.S. Army Grant DAMD17-90-C-0094 (to F.W.-S.), National Institutes of Health Grant 1R-29AI31823-01 and California Universitywide AIDS Task Force Grant R90SD045 (to J.R.), and National Institutes of Health and Biotechnology Research and Development Corporation grants (to A.H.). J.O.O. is supported by World Laboratory Fellowship MCD-2/17/R.PE and California Universitywide AIDS Task Force Grant R91SD056.

1. Mitsuya, H., Yarchoan, R. & Broder, S. (1990) *Science* **249**, 1533–1544.
2. Sarver, N., Zaia, J. A. & Rossi, J. J. (1992) *AIDS Res. Rev.*, in press.
3. Izant, J. G. & Weintraub, H. (1984) *Cell* **36**, 1007–1015.
4. Melton, D. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 144–148.
5. Buzayan, J. M., Gerlach, W. L. & Bruening, G. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8859–8862.
6. Hutchins, C. J., Rathjen, P. D., Forster, A. C. & Symons, R. H. (1986) *Nucleic Acids Res.* **14**, 3627–3640.
7. Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R. & Bruening, G. (1986) *Science* **231**, 1577–1580.
8. Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Landne, P. A., Stephens, D. A. & Rossi, J. J. (1990) *Science* **247**, 1222–1225.
9. Weerasinghe, M., Liem, S. E., Asad, S., Read, S. E. & Joshi, S. (1991) *J. Virol.* **65**, 5531–5534.
10. Dropulic, B., Lin, N. H., Martin, M. A. & Jean, K.-T. (1992) *J. Virol.* **66**, 1432–1441.
11. Baltimore, D. (1988) *Nature (London)* **335**, 395–396.
12. Trono, D., Feinberg, M. B. & Baltimore, D. (1989) *Cell* **59**, 113–120.
13. Hampel, A., Tritz, R., Hicks, M. & Cruz, P. (1990) *Nucleic Acids Res.* **18**, 299–304.
14. Hampel, A. & Tritz, R. (1989) *Biochemistry* **28**, 4929–4933.
15. Keese, P. & Symons, R. H. (1987) in *Viroids and Viroid-Like Pathogens*, ed. Selmancik, J. S. (CRC, Boca Raton, FL), pp. 1–47.
16. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
17. Messing, J., Crea, R. & Seeburg, P. H. (1981) *Nucleic Acids Res.* **9**, 309–321.
18. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
19. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Joseph, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C. & Wong-Staal, F. (1985) *Nature (London)* **313**, 277–284.
20. Leavitt, J., Gunning, P., Porreca, P., Ng, S.-Y., Lin, C.-S. & Kedes, L. (1984) *Mol. Cell. Biol.* **4**, 1961–1969.
21. Milligan, J., Groebe, D., Witherall, G. & Uhlenbeck, O. (1987) *Nucleic Acids Res.* **15**, 8783–8798.
22. Graham, F. C. & Van der Eb, A. J. (1973) *Virology* **52**, 456–457.
23. Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y. & Kedes, L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4831–4835.
24. Ratner, L., Fisher, A., Jagodzinski, L. L., Mitsuya, H., Liou, R. S., Gallo, R. C. & Wong-Staal, F. (1987) *AIDS Res. Hum. Retroviruses* **3**, 57–69.
25. Arya, S. K., Guo, C., Josephs, S. F. & Wong-Staal, F. (1985) *Science* **229**, 69–73.
26. Agrawal, S., Ikeuchi, T., Sun, D., Sarin, P. S., Konopa, A., Maizel, J. & Zamecnik, P. C. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7790–7794.
27. Goodchild, J., Agrawal, S., Civeira, M. P., Sain, P. S., Sun, D. & Zamecnik, P. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5507–5511.
28. Matsukura, M., Zon, G., Shinozuka, K., Robert-Guroff, M., Shimada, T., Stein, C. A., Mutsuya, H., Wong-Staal, F., Cohen, J. S. & Broder, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4244–4248.
29. Sczakiel, G. & Pawlita, M. (1991) *J. Virol.* **65**, 468–472.
30. Sczakiel, G., Pawlita, M. & Kleinheinz, A. (1991) *Biochem. Biophys. Res. Commun.* **169**, 643–651.
31. Haseloff, J. & Gerlach, W. L. (1988) *Nature (London)* **334**, 585–591.
32. Cameron, F. H. & Jennings, P. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9139–9143.
33. Chrisey, L., Rossi, J. & Sarver, N. (1991) *Antisense Res. Dev.* **1**, 57–63.