Functional Characterization of a U5 Ribozyme: Intracellular Suppression of Human Immunodeficiency Virus Type 1 Expression

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We have designed a ribozyme that cleaves human immunodeficiency virus type 1 (HIV-1) RNA in U5 (at nucleotide +115). This ribozyme was tested in vitro and was found to give efficient and specific digestion of RNA containing the HIV-1 U5 sequence. When the U5 ribozyme was placed into the HIV-1 genome, virus replication was suppressed in tissue culture. Introduction of this ribozyme into cells by using an amphotropic retrovirus vector significantly reduced expression of U5-containing RNA in cells chronically infected with HIV-1. Naive T cells were cocultivated with packaging cells that produce defective amphotropic retroviruses containing the U5 ribozyme. These lymphocytes were found to be partially protected from HIV-1 infection.

Hammerhead ribozymes were first described for the avocado sunblotch viroid and the satellite RNAs of the tobacco ringspot virus. These small RNA genomes use *cis* self-cleaving ribozymes in rolling-circle replication (4, 12, 13, 17, 26; reviewed in reference 32). Ribozyme activity in vitro requires a secondary structure that contains three stems and a catalytic center of 13 nucleotides (34). The substrate for the hammerhead ribozyme needs only a 3-nucleotide GUC sequence (16).

Demonstrating the ability of the hammerhead ribozyme to cleave RNAs in vivo has been difficult. There are many examples which suggest that ribozymes can effectively cleave selected RNAs intracellularly (5, 8, 10). However, in most cases the efficiency of cleavage has been much less than quantitative. In early reports it was shown that HeLa CD4⁺ cells transfected with a β -actin promoter-driven ribozyme (targeted to *gag* mRNA) partially protected cells from human immunodeficiency virus type 1 (HIV-1) infection (6, 29). The present study explored the effects of a ribozyme directed against a U5 sequence when the ribozyme was delivered into T lymphocytes.

We used two approaches. First, we created an infectious HIV-1 genome that contained the U5 ribozyme integrated into the *nef* gene. This genome was found to be greatly reduced in infectivity compared with the wild type. Second, we generated defective amphotropic viruses that contain the U5 ribozyme sequence. We found that these defective virus vectors which delivered ribozymes into T cells could partially protect these cells from HIV-1.

MATERIALS AND METHODS

Plasmid constructions. A synthetic oligonucleotide containing the U5 ribozyme sequence 5'-TCACACAACACG TATGAGGCCGGAAGGCCGAAACGGGCACACACTA CTT-3' was inserted into the *Hind*III and *Bam*HI sites of the pGEM 4Z vector (Promega) for in vitro transcription. The same sequence was placed into the *Xho*I site of the *nef* gene either in the pNL4-3 (1) or in the LAI (35) genome in sense and antisense orientations. A defective amphotropic retrovirus containing the U5 ribozyme was constructed by ligat-

ing the ribozyme sequence into the *Bam*HI and *Hind*III sites of the murine leukemia virus (MuLV) pBabeNeo vector (23).

In vitro transcription and hybridization reaction. pGEM-U5 ribozyme and a pHIV-CATSV (24) derivative (containing the U5 substrate sequence positioned downstream of a T7 promoter) were linearized with appropriate enzymes (either *Eco*RI or *Nco*I). The linear templates were transcribed in vitro by using 0.5 μ g of DNA, 20 U of RNasin, 50 μ Ci of [α -³²P]UTP, 2.5 mM ATP, 2.5 mM GTP, 2.5 mM CTP, 100 mM dithiothreitol, and 20 U of T7 RNA polymerase in 1 $\times$ transcription buffer (40 mM Tris-HCl [pH 7.5], 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl) (Promega). The reaction mixture was incubated for 60 min at 37°C, and the specific activity was determined by precipitation with 5% trichloroacetic acid. DNA was removed by digestion with 1 U of RQ1 RNase-free DNase (Promega) for 15 min at 37°C. EDTA was added to 3 mM to sequester free magnesium. After transcription, equal volumes of ribozyme and substrate were mixed, heated to 65°C for 5 min, and cooled stepwise manually to 37°C in a water bath (30 s each at 55, 42, and 37°C). The reaction mixture was then adjusted to 20 mM MgCl₂ to initiate cleavage. After 1 or 5 min the reaction was stopped by the addition of formamide followed by boiling for 3 min, and the product was analyzed on an 8% denaturing polyacrylamide gel.

Transfections. HeLa CD4⁺ cells (7) maintained in complete medium (Dulbecco modified Eagle medium containing 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 2 mM glutamine) were transfected with infectious HIV-1 provirus genomes containing the U5 ribozyme by using calcium phosphate (15, 36). GP+envAM12 packaging cell lines (21, 22) were grown in complete medium plus 7.5 μ g of hypoxanthine per ml, 125 μ g of xanthine per ml, 12.5 μ g of mycophenolic acid per ml, and 100 μ g of hygromycin B per ml (Sigma). To generate amphotropic virus-producing clones, cells were transfected with the pBabeNeo vector (23) containing the ribozyme placed within the sequence from the *Bam*HI to *Eco*RI restriction sites and then subjected to selection in 200 μ g of G418 (GIBCO) per ml for 2 weeks. Single-cell-derived clones were assayed for the presence of virus by using a MuLV reverse transcriptase assay. In this assay Mn²⁺ was used as the divalent cation (28).

Virus infections. H9 cells (1 $\times$ 10⁵), chronically infected

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with HIV-1, were cocultivated with cells producing either amphotropic ribozyme-containing retrovirus (ARR) or amphotropic retrovirus without ribozyme (AR). HIV-1 production (an indication of ribozyme efficiency) was monitored by periodic assays of reverse transcriptase in the supernatant media (37). MT4 cells (1×10^5) were cocultivated for 3 days with confluent monolayers (2×10^6 cells) of packaging cell lines producing either ARR or AR, prior to infection with HIV-1. These MT4 cells were then harvested and infected with 1,500 ^{32}P cpm (reverse transcriptase activity) of HIV-1 per μl , added as a 100- μl inoculum to cells for 2 h. The resulting reverse transcriptase activities are values obtained after subtracting for that produced by naive packaging cell lines.

In vivo cleavage determination. Cleaved and uncleaved RNAs in cells infected with HIV-1 containing ribozyme were quantitated by RNA polymerase chain reaction (PCR) (Invitrogen). Briefly, RNA was isolated from cells with hot phenol (20). Three ^{32}P -end-labeled primers were made: primer a (5'-GCTCTCTGGCTAACTAGGG-3' from +34 to +52), primer b (5'-TTTGAGCACTCAAGGCAAGC-3' from +97 to +78, and primer c (5'-AAAGGGTCTGAGG GATC-3' from +154 to +138) (map positions are from pNL4-3, with +1 being the start of R [1]) (see Fig. 5). Primer b, when incubated with RNA and reverse transcriptase, synthesizes a 97-nucleotide cDNA from both cleaved and uncleaved mRNA, whereas primer c synthesizes a 154-nucleotide cDNA only from uncleaved mRNA (see Fig. 5A). The cDNA fragment(s) were then amplified upon addition of primer a with *Taq* polymerase in the chain reaction (Perkin-Elmer). The amplified fragments are either a 121-nucleotide (ac) fragment from uncleaved RNA or a 64-nucleotide (ab) fragment from cleaved RNA (Fig. 5A). The ratio of the ac to the ab fragment indicates the relative amounts of cleaved and uncleaved RNA.

RESULTS

Specific cleavage of HIV-1 RNA by ribozymes in vitro. Targets for hammerhead ribozymes contain the GUCN consensus sequence. Eleven such GUCU targets are present in the HIV-1 genomic RNA. We designed a ribozyme to one such consensus present in the U5 long terminal repeat (LTR). This U5 ribozyme contains a catalytic domain (16) flanked by 10 and 17 nucleotides that are complementary to sequences 5' and 3' to GUCU, respectively (Fig. 1A). It was inserted into a plasmid downstream of the T7 promoter for in vitro transcription (Fig. 1A). We also constructed a plasmid that directs the in vitro synthesis of the U5 substrate RNA by using T7 RNA polymerase (24). When [α - ^{32}P]UTP-labeled ribozyme and substrate transcripts were mixed, the substrate RNA was specifically cleaved (see P1 and P2 product fragments in Fig. 1B); at a ratio of 1:1 and at 37°C, maximal cleavage occurred within 5 min. Quantitation of the reaction products (P1 and P2) indicated that 73% of the starting U5 LTR target RNA was cleaved. The reaction failed to reach completion (100% cleavage), most probably because the kinetics of hybridization of ribozyme to substrate become rate limiting at the late stages of digestion.

The length of the substrate or ribozyme RNA does not significantly affect cleavage. To explore whether the length of the ribozyme affected its ability to cleave, the U5 LTR ribozyme or substrate plasmids were digested with restriction enzymes and then transcribed in vitro as short or long ribozymes or substrate RNAs (Fig. 2A). The in vitro transcripts were then mixed in different combinations, and the

relative cleavage efficiencies were determined by quantitating P2 production. P2 rather than P1 was assayed because the lengths of the P1 fragments and their differential incorporation of [^{32}P]UTP (i.e., longer RNA has more [^{32}P]UTP per molecule) made them difficult to quantitate in 8% polyacrylamide gels (Fig. 2B). We found that neither the amount of P2 nor the kinetics of its production varied significantly in incubations of the different ribozyme-substrate combinations. Under these assay conditions the efficiency of cleavage was not significantly dependent on the length of either the ribozyme or the substrate (Fig. 2B).

Efficient ribozyme cleavage within the HIV-1 genomic RNA. We next examined ribozyme function in vivo (in tissue culture). To optimally constrain the ribozyme and its substrate to the same subcellular locale, we placed the ribozyme and its target onto the same RNA molecule by inserting a small (52-nucleotide) self-cleaving RNA (*cis* ribozyme) into the *nef* gene (Fig. 3A). In human T-cell lines, the *nef* gene is dispensable for virus replication (3, 19). This short RNA contains both the catalytic and the substrate components for cleavage. Following transcription of proviral DNA into RNA in transfected cells, a specific self-induced breakage of the RNA genome would be expected (Fig. 3A and B).

Six independent plasmid clones of infectious HIV-1 proviral DNA containing *cis* ribozyme in either the sense [91-7(s), 91-9(s), and 91-14(s) (Fig. 3C)] or the antisense [91-4(as), 91-8(as), and 91-10(as) (Fig. 3C); antisense-oriented ribozymes do not self-cleave] orientation were separately introduced into monolayers of HeLa CD4⁺ cells. The virions produced from these parallel transfections were assayed following cocultivation with MT4 cells by monitoring reverse transcriptase activity in the supernatant medium. Virus production was 100-fold lower in cells transfected with the 91-7(s), 91-9(s), and 91-14(s) constructs ("sense" proviruses) than in cells transfected with the 91-4(as), 91-8(as), and 91-10(as) constructs ("antisense" proviruses) (Fig. 3C). This inhibition of virus production persisted during a 10-day observation period, at which time the experiment was terminated (data not shown). We therefore concluded that no cellular or viral repair mechanism exists permitting HIV-1 to survive a cleavage of its RNA genome.

We next placed the U5 ribozyme into the *Xho*I site of the *nef* gene of pNL4-3 (1) or LAI (35) proviral DNAs (Fig. 4A); Fig. 4B illustrates the possible mechanisms of cleavage by such a U5 ribozyme. For example, intergenomic cleavage can occur between two opposing viral genomes (upper left). The ribozyme can also cleave its own RNA through intragenomic folding such that the ribozyme sequence meets the substrate sequence on the same RNA molecule (upper right). Alternatively, subgenomic (spliced) ribozyme transcripts can cleave either genomic or subgenomic RNAs (lower diagram).

Independent plasmid isolates of infectious clones containing the U5 ribozyme were separately transfected in HeLa CD4⁺ monolayers were cocultivated with 1×10^5 MT4 cells and the supernatants were monitored for production of reverse transcriptase activity. We found that the antisense ribozyme-containing virus of pNL4-3 and its LAI counterpart both had replication kinetics comparable to those of wild-type virus (Fig. 4C and D). This contrasted with results from the sense infectious genomes, which exhibited marked inhibition in progeny virus production (Fig. 4C and D); peak virus production lagged by 1 week (Fig. 4C and D). This was evident by monitoring either supernatant reverse transcriptase (Fig. 4C and D) or syncytium formation (Fig. 4E).

Ribozymes have two inherent activities. First, they must

FIG. 1. In vitro cleavage of HIV-1 substrate RNAs by a U5 ribozyme. (A) Schematic representations of substrate (upper) and ribozyme (lower). (B) Kinetics of cleavage of the substrate (S) by cognate U5 ribozyme (R). P1 and P2 are the resulting product fragments after cleavage. Lanes: 1, ribozyme alone; 2, substrate alone; 3, mixture of substrate and ribozyme at time 0; 4, mixture of substrate and ribozyme after 1 min; 5, mixture of ribozyme and substrate after 5 min. The substrate is 380 nucleotides (nt) in length; the ribozyme is 90 nucleotides; and the cleaved products P1 and P2 are 265 and 115 nucleotides, respectively. MW, molecular size.

FIG. 2. Effect of length on cleavage by ribozyme. (A) Schematic diagram of substrates (upper panel) and ribozymes (lower panel) transcribed to various lengths. Abbreviations: SS, short substrate; SL, long substrate; RS, short ribozyme; RL, long ribozyme. (B) Cleavage of short (SS) and long (SL) U5 LTR substrates by using short (RS) and long (RL) ribozymes. The amount of the constant-sized P2 product was used to quantitate the efficiencies of cleavage. Lanes 1, 2, and 3 contain the short substrate incubated with the short ribozyme with samples assayed at 0, 1, and 5 min, respectively; lanes 4, 5, and 6 contain the long substrate incubated with the short ribozyme assayed at 0, 1, and 5 min, respectively; lanes 7, 8, and 9 contain the short substrate incubated with the long ribozyme at 0, 1, and 5 min, respectively; and lanes 10, 11, and 12 contain the long substrate incubated with the long ribozyme at 0, 1, and 5 min, respectively. P1 is the 3' fragment produced from the cleavage of the short substrate; P1' is the 3' fragment produced from the cleavage of the long substrate; P2 is the conserved 5' fragment produced from the cleavage of either substrate (SS or SL). The slight difference in input for the long substrate between lanes 1 and 4 appears to be a sampling error. This difference does not appear on other gels.

be able to hybridize via base pairing to their substrate RNAs. Thus ribozymes have the potential of producing an antisense RNA effect. In addition, once hybridized, ribozymes can cleave the substrate RNA. This second activity distinguishes ribozymes from short antisense oligoribonucleotides. To verify that our observed reduction in virus production reflected the cleavage function of the ribozyme, we performed RNA PCR to verify RNA strand scission. RNAs were isolated from MT4 cells 4 days after cocultivation with HeLa CD4⁺ cells that had been transfected with either a sense or an antisense U5 ribozyme virus. Primer pairs were chosen to discriminate between uncleaved and cleaved RNAs (Fig. 5A). For example, whereas the ab primer pair would not distinguish between cleaved and uncleaved RNA (Fig. 5A), the ac primer pair would amplify only uncleaved RNA (Fig. 5A). The ratio ab/ac is thus a measure of cleavage.

We quantitated the amounts of amplified ac (121-nucleotide) and ab (64-nucleotide) fragments by denaturing polyacrylamide gel electrophoresis and determined the ratio of ab to ac. As shown in Fig. 5B and C, the ratio of uncleaved fragments (ac) to total fragments (ab) was approximately 1

for the antisense virus. However, in cells infected with the sense virus (Fig. 5B, lane 1), reduced ratios of uncleaved to total RNA were measured (i.e., a ratio of ac to ab of 0.3 [Fig. 5C]), suggesting that the substrate RNAs were being correctly cleaved intracellularly.

Suppression of HIV-1 expression by an amphotropic retrovirus vector containing the U5 ribozyme. The U5 ribozyme was inserted into a MuLV retrovirus vector (23) such that both genomic and subgenomic (spliced) RNAs would contain the ribozyme sequence (Fig. 6A). To make high-titer viral supernatants, Gp+envAM12 packaging cells (21, 22) were transfected with an amphotropic MuLV vector containing a U5 ribozyme. Because the vector contains a drug resistance marker (neomycin), clonal cell lines could be selected and analyzed by RNA PCR for the expression of the U5 ribozyme. In two of the clonal lines (Fig. 6B, arrow in lanes 2 and 4), a ribozyme-specific signal was detected. To rule out a possible artifact due to DNA contamination, we amplified the same samples without the reverse transcription step (Fig. 6B, lanes 3 and 5); no bands were detected. Three of the eight clonal lines characterized in this manner pro-

FIG. 3. Inhibition of virus expression by a *cis* self-cleaving ribozyme placed into the HIV-1 genome. (A) Schematic depiction of the ribozyme sequence and the site of insertion into the pNL4-3 genome. The ribozyme sequence was inserted in the sense ($\rightarrow$) or antisense ($\leftarrow$) orientation at the *Xho*I site. (B) Secondary structure of the *cis*-cleaving ribozyme. The site of cleavage is indicated by the arrow. (C) Reverse transcriptase assay of virus production for the sense or antisense virus. Three independent isolates of infectious genomes with sense (s) or antisense (as) ribozymes were compared. HeLa CD4⁺ cells transfected with sense or antisense virus were cocultivated with MT4 cells, and virus production was monitored by reverse transcriptase assays of the supernatant medium. Reverse transcriptase reaction mixtures were spotted onto DEAE-paper, and autoradiography was carried out (37). The intensity of the dots reflects the amount of supernatant virus. Three parallel sets of virus assays are shown. Antisense viruses are 91-4(as), 91-8(as), and 91-10(as). Sense viruses are 91-7(s), 91-9(s), and 91-14(s). Mock is a mock transfection of HeLa CD4⁺ cells with calcium phosphate alone followed by cocultivation with MT4 cells.

FIG. 4. Inhibition of virus expression by a *trans* U5 LTR ribozyme placed into the pNL4.3 or LAI infectious genome. (A) Diagram depicting the position of the U5 substrate site (wavy box) and the position of the inserted ribozyme (checked box). The ribozyme was inserted at the *Xho*I site in the sense (→) or antisense (←) orientation. The *Xho*I site is destroyed in the process of insertion. (B) Schematic representations of the possible interactions between ribozyme and U5 substrate RNA. Intergenic cleavage occurs when the ribozyme from one RNA cleaves the U5 target on another RNA (upper left); intragenomic cleavage occurs when the ribozyme cleaves the target on the same RNA strand by bending (upper right); subgenomic RNAs containing ribozyme can also cleave genomic or subgenomic RNA (lower panels). (C) Kinetics of virus production comparing LAI wild-type virus, LAI sense virus, and LAI antisense virus. Infectious genomes were transfected into HeLa CD4⁺ cells and then cocultivated with MT4 cells. Virus production was assayed by measuring the reverse transcriptase activity. (D) Counterpart results with the pNL4.3 infectious genome. (E) Syncytium formation by MT4 cells after cocultivation with HeLa CD4⁺ cells transfected with either wild-type (upper), antisense (center), or sense (lower) ribozyme containing HIV-1. Cocultivation was done 48 h after transfection. Photographs were taken on day 4 after cocultivation.

FIG. 5. In vivo cleavage of HIV-1 RNA. (A) Diagrammatic depiction of the PCR assay for cleaved and uncleaved RNAs. Uncleaved RNA can be amplified by both the ab and the ac primer pairs, whereas cleaved RNA cannot be amplified by the ac primer pair. (B) Autoradiogram showing PCR-amplified fragments from infected-cell RNA comparing amounts of uncleaved (ac) and total (ab) RNA. The sense virus as compared with the antisense virus showed a decrease in the amount of ac fragment compared with ab. (C) Quantitation of the ac and ab fragments by using a phosphorimager (Molecular Dynamics). Relative areas for pNL4-3 sense virus are 0.3 for ac and 1 for ab; those for pNL4-3 antisense virus are 1 for ac and 0.8 for ab.

FIG. 6. Amphotropic retrovirus vector suppresses HIV infection in T cells. (A) Schematic representation of the ribozyme inserted into the MuLV amphotropic retroviral vector (pBabeNeo [23]). (B) RNA PCR results verifying the production of retroviruses carrying the U5 ribozyme sequence. RNAs in lanes 2 and 4 (arrow) contain ribozyme sequence as compared with RNA from parental GP+envAM12 control cells (lane 1). RNAs from clonal cells were also assayed by PCR without first adding reverse transcriptase (lanes 3 and 5). (C) Kinetics of virus production from H9 cells chronically infected with HIV-1 after cocultivation with ARR-producing (solid squares [U5]) or AR-producing (open square [control]) cells. (D) Kinetics of virus production from MT4 cells that were cocultured with ARR-producing cells (solid triangles) for 3 days prior to challenge with 1,500 cpm of HIV-1 per μ l in a 100- μ l suspension. Control cocultures with GP+envAM12 cells producing AR were also infected in this way (open triangles).

duced high titers of virus, as confirmed by RNA PCR and reverse transcriptase production, and were used in infectivity assays.

We first examined the effect of the U5 ribozyme on

chronic virus production. H9 cells, chronically infected with HIV-1, were cocultivated with packaging cells producing the ARR, and supernatant reverse transcriptase was monitored on a daily basis (Fig. 6C). For comparison, H9 cells were

cocultivated with cells producing the AR (Fig. 6C). Over the course of 6 days, virus production from the ARR-cocultivated H9 cells was moderately suppressed.

In a separate experiment, MT4 cells were preinfected with wild-type virus, AR, or ARR and then exposed to HIV-1 (1,500 cpm of reverse transcriptase activity per μ l); virus production was monitored over the next 7 days (Fig. 6D). During this period, supernatant reverse transcriptase activities from the ARR samples were consistently lower than that generated from the AR samples. These results suggest that ARR-producing cells can partially protect MT4 cells from exogenous HIV infection.

DISCUSSION

Antisense RNA has been shown to affect HIV expression in mammalian cells (2, 14). In other reports, when antisense RNAs were produced intracellularly from hybrid genes, a transient inhibition of HIV expression was seen (30, 31). However, the stoichiometric requirements for this inhibition may limit pragmatic use. Ribozymes have all of the attributes of an antisense RNA that functions catalytically (16). Essentially, ribozymes are antisense RNAs with an added domain that cleaves substrate RNA (5). When appropriately introduced into cells, ribozymes theoretically can efficiently and specifically destroy (viral) RNAs, thus inhibiting (virus) expression. Because ribozymes function as enzymes, each molecule remains unconsumed during a given reaction and, in principle, can be used repeatedly.

We have synthesized a ribozyme targeted to the U5 region of HIV-1. The rationale for this design is that all viral RNAs are susceptible targets for this ribozyme, since they contain the U5 sequence. Because U5 is the RNA leader that links the coding sequence of the mRNA to its cap structure, cleavage in U5 renders the RNA "capless." These capless RNAs cannot be efficiently translated by ribosomes into proteins.

To characterize our U5 ribozyme for potential use inside cells, we examined its activities in vitro and in vivo (tissue culture). Our in vitro assays demonstrated that HIV-1 genomic RNA was readily cleaved at the U5 site (Fig. 1B). At a ratio of 1:1, the reaction was at least 73% complete within 5 min of incubation. This is considerably better than that previously reported (11), in which a 500- to 1,000-fold molar excess of ribozyme to substrate was used to achieve comparable results. Similarly, others (29) have used extended incubations (14 h) to achieve similar levels of HIV-1 *gag* RNA digestion. Interestingly, despite apparently unfavorable in vitro kinetics, the same investigators reported efficient cleavage and inhibition of virus expression in vivo (29). Our in vitro results suggest that the particular U5 site that we chose could be highly accessible to its cognate ribozyme.

The relative efficiencies of ribozymes that differed in overall length were also examined. RNAs of different lengths were generated by varying sequences that have no direct role in either the hybridization or cleavage reactions. We found that the length of noncomplementary flanking arms did not significantly affect cleavage of the substrate by the U5 ribozyme (Fig. 2B). This result suggests that U5 ribozymes of both genomic and subgenomic lengths may work well.

One cannot extrapolate directly from in vitro results to in vivo (intracellular) settings. For example, Cotten and Birnstiel (10) demonstrated that efficient cleavage by ribozyme of U7 RNA in vitro was not reflected intracellularly. We therefore tested our ribozyme in vivo. We did so by first placing both the ribozyme and substrate onto the same RNA

molecule. In this fashion both substrate and ribozyme are expected to be expressed in the same subcellular locale and in similar stoichiometry. *cis* (Fig. 3A) and *trans* (Fig. 4A) ribozymes were placed into HIV-1 at the *nef* gene. Both types of cleavage reactions were found to inhibit virus production (Fig. 3C and 4C and D). This effect was observed for up to 1 week in tissue culture, after which significant virus production (escape) occurred.

The mechanism of this escape phenomenon is not presently understood. However, we suspect that the error-prone property of the HIV-1 reverse transcriptase (25, 27) results in the emergence of viral genomes containing altered substrate or ribozyme sequences. If this occurs frequently, ribozymes must be targeted to multiple sites in the viral genome. The viral RNA then would have to undergo changes at each of the targeted sites to escape ribozyme cleavage. The probability of multisite changes should be significantly lower than that of changes at a single site.

Ultimately, if ribozymes are to be used in humans, they must be delivered in an efficient and reproducible manner. Many possibilities exist. The particular amphotropic retrovirus vector (Fig. 6A) used in this study produces high-titer defective virus with low helper activity (21–23). We found that cocultivation of chronically infected H9 cells with ARR-producing cells modestly inhibited HIV-1 expression compared with control cocultivations (Fig. 6C). In addition, MT4 cells, which were first cocultivated with cells producing ARR, became partially resistant to subsequent infection by virus (Fig. 6D). Control cocultivations did not confer this phenotype (Fig. 6D). These results suggest that amphotropic virus can enter HIV-1-infected T4 lymphocytes and that upon entry they can carry out ribozyme function. The incompleteness of the effect may be due to inefficiencies either in entry or in ribozyme cleavage. The former is a possible explanation since results from other investigators (9, 18) suggest that at least 50% of cells can be infected by amphotropic retrovirus vectors in culture. Future optimization of retroviral vector technology should improve these numbers.

HIV-1 strains do not all replicate at the same rate. There are slow, low viruses and fast, high viruses (33). In general, primary isolates tend to grow poorly, whereas laboratory-adapted strains tend to grow rapidly and to high titers in tissue culture. In our assays of ribozyme function in tissue culture, we used extremely robust laboratory-adapted molecular clones (pNL4-3, pLAI) in cells (MT4) that are known to be exquisitely sensitive to HIV. Given these parameters, our results probably underestimate the efficiency of ribozymes on slow viruses in less hospitable host cells (e.g., peripheral blood leukocytes or monocytes/macrophages).

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