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Inhibition of HIV-1 replication by ribozymes that show poor activity *in vitro*

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ABSTRACT

Self-cleaving RNAs (ribozymes) can be engineered to cleave target RNAs of choice in a sequence-specific manner (1). Consequently, they could be used to inhibit virus replication or to analyse host gene function *in vivo*. However, ribozymes that are catalytic *in vitro* are generally disappointing when analysed in cells unless expressed at high levels relative to their target RNAs (2, 3). Here we provide evidence that this can be overcome by optimizing ribozyme structure using cellular rather then cell-free assays. We show that ribozymes of relatively long flanking complementary regions (FCRs), while poor catalysts *in vitro*, can produce profound inhibition of HIV replication in cells. By examining a series of ribozymes in which the FCRs vary from 9 to 564 nucleotides, we establish that the optimum length for activity in the cell is ≥ 33 nucleotides.

INTRODUCTION

Antisense RNAs (AR) and ribozymes offer a means of inhibiting the expression of chosen viral or cellular genes in a sequence-specific manner. The ability of ribozymes to inactivate multiple copies of target RNA should allow one to overcome problems of stoichiometry inherent in the AR approach and thereby give a greater inhibition of gene expression. However, when compared directly, evidence suggests that ribozymes are less effective in cells than their antisense counterparts (4, 5) and depend for activity upon a many-fold excess of ribozyme over substrate RNA (2–4, 6, 7). Since studies had established that the catalytic efficiency of ribozymes *in vitro* is greatest if their FCRs are between 6 and 8nt (8, 9) the ribozymes tested had short flanking complementary regions (FCRs). However, previous work on antisense RNAs against HIV suggested that productive interaction between complementary RNAs in cells required substantially longer duplexes than would be predicted from *in vitro* hybridization (10–12). It seemed reasonable that similar considerations might apply to ribozymes designed to cleave target RNAs within the cell.

In this paper, we report experiments on a family of ribozymes directed against HIV. They share a catalytic motif, derived from the (+) strand of sTobRV (13), and a target site, within the first coding exon of *tat*, but differ in the length of their FCR. We show that, while the optimum FCR length for *in vitro* activity is 9nt, the optimum FCR length for action against HIV in the cell is ≥ 33nt.

MATERIALS AND METHODS

Cell lines and virus stocks

Omega E ecotropic helper cells (14) and PA317 cells (15) were grown in DME supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Uxbridge, UK). Jurkat clone E6-1 cells (16) and C8166 cells (17) were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS. HIV-1_{IIIB} (18) was obtained from the NIH AIDS Research and Reference Program and was propagated by acute infection in C8166 cells, filtered through 0.2μm filters and treated with DNase I (0.1mg/ml, 30min 37°C) to remove detectable proviral DNA. Virus infectivity was determined by $TCID_{50}$ using the polymerase chain reaction (PCR) of HIV LTR DNA to detect the end-point as described (19). P24 ELISA was as described (20).

Construction of HIV-directed ribozymes (See Figure 1)

The antisense RNAs have been described previously (10). The gene for ribozyme $496_{45:75}$ was derived by insertion of a 22nt hammerhead catalytic motif into AR5 in place of the G corresponding to C_{5334} of the HIV-1_{HXB2R} genome (21) using oligonucleotide-mediated mutagenesis (22). This was subsequently sub-cloned into pSPT19 using EcoRI and SacI. Ribozyme $496_{45:564}$ was generated by the addition of a SacI/KpnI fragment of AR2 to the 3′ end of ribozyme $496_{45:75}$. Deletions in the FCRs of ribozyme $496_{45:75}$ were created by PCR using a nested set of primers that contained EcoRI and BamHI sites to facilitate sub-cloning into pSPT19. The gene for the control RNA, $G_5U\text{-}496_{45:70}$, was derived by the replacement of the G_5 for U (numbering according to (23)) using oligonucleotide-mediated mutagenesis of $496_{45:70}$. This mutation renders the ribozyme motif inactive (8) but has no effect on the predicted secondary structure (using the programs of references (24, 25)). Sense $496_{45:70}$ is complementary to ribozyme $496_{45:70}$ and consequently is neither an antisense RNA nor a ribozyme.

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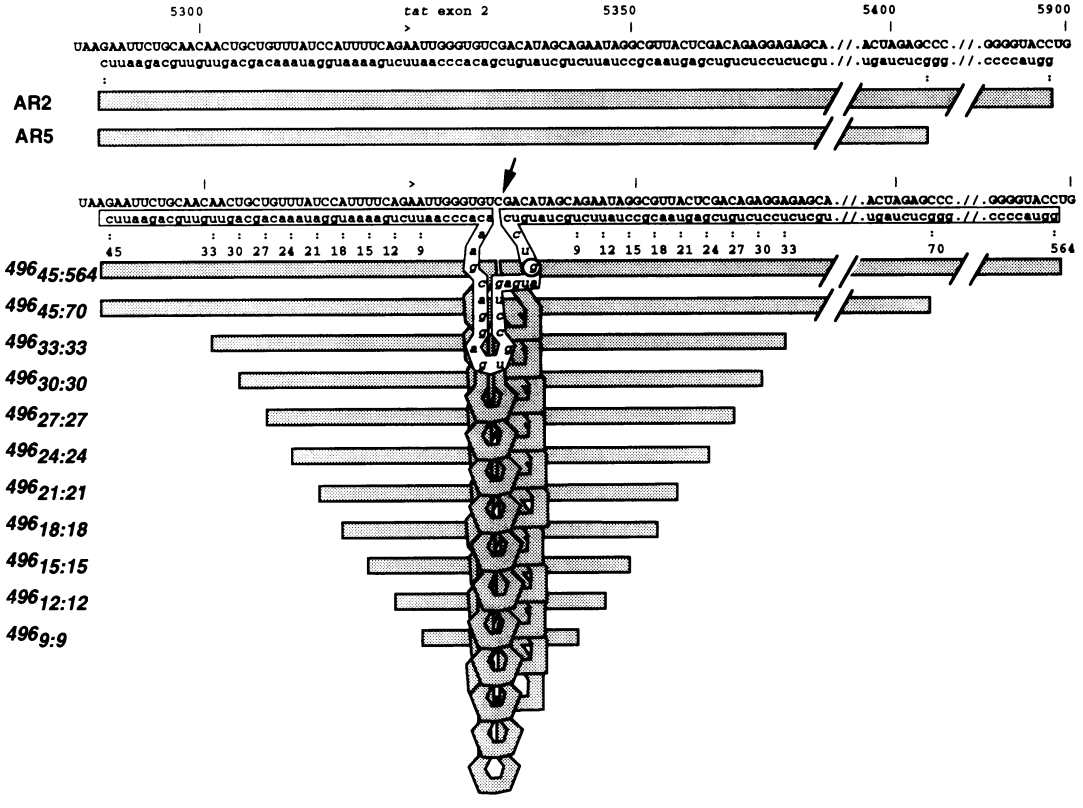

Figure 1. Structure of HIV-directed RNAs. The target region of the primary transcript of HIV-1_{IIIB}, clone HXB2R (21), is shown in upper case and the nucleotide positions and start of the first coding exon of *tat* are indicated (numbering according to GenBank/EMBL accession numbers K03455 M38432). The antisense and ribozyme sequences (upper and lower panel, respectively) are shown in lower case in the 3′−5′ direction and the structure of each construct is indicated with stippled boxes. The site of ribozyme-mediated cleavage is indicated with an arrow. The 3′ and 5′ terminal nucleotides of each construct are indicated with (:) and, in the case of the ribozymes, the distance of this nucleotide from the position of cleavage is indicated. The guanosine mutated to a uracil in the non-catalytic derivative, G_5U-$496_{45:70}$ is circled.

In vitro ribozyme assays

EcoRI-linearized template (30μg/ml), was transcribed *in vitro* using T7 polymerase (Promega, Madison, WI 53711-5399; 2800U/ml) in 100μl of 40mM Tris−HCl pH7.5 containing NaCl (10mM), $MgCl_2$ (6mM), DTT (5mM), spermidine (2mM), ATP, GTP and UTP (0.5mM each), CTP (12μM; 100μCi ^{32}P CTP) and RNasin (200U/ml) for 2 hours at 37°C. The DNA template was removed by digestion with RQ1 DNase (Promega, 1U/μg template, 37°C, 15 minutes) and unincorporated nucleotides removed by exclusion chromatography through aspun column (500*g*, 5min) containing sephadex G-50 (Pharmacia). Ribozyme reactions were carried out in 50mM Tris−HCl, pH 8.0, 20mM $MgCl_2$ at a concentration of 1μM (ribozyme) and 5μM (substrate) at 50°C for 1 hour unless otherwise stated. Under these conditions, substrate RNA was saturating and the rate of substrate cleavage was linearly dependent on ribozyme concentration (data not shown). Reactions were terminated by transferring samples into an equal volume of 95% (v/v) formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 1mM EDTA. Products were heated at 95°C, 1min and then analysed by electrophoresis through 6% polyacrylamide containing 8M urea and 25% formamide, and quantitated by laser densitometry (LKB Ultroscan XL) after exposure to pre-flashed film.

Construction of ribozyme-expressing cell lines

Genes encoding each RNA were sub-cloned between the EcoRI and BamHI sites of pBabe Hygro (14). Recombinant retrovirus was produced by the transient transfection of Omega E cells followed by the transduction of PA317 cells as described (14). Jurkat cells (10^7 cells) were transduced with 10^5 c.f.u. amphotropic retrovirus in the presence of polybrene (8μg/ml) in 4ml for 2h, grown in medium for 2 days and then transferred to medium containing 500μg/ml hygromycin B. A culture of at least 10^7 resistant cells re-grew within two weeks under these conditions. The levels of ribozyme RNA expressed in each cell line was determined by quantitative S1 nuclease analysis using a probe overlapping the 5′ end of the transcript and a Molecular Dynamics PhosphorImager to quantitate the data. All lines were found to express similar levels of RNA: the difference between the highest and lowest was less than a factor of 3, with nosystematic correlation between amount and length of RNA (data not shown).

Primer extension analysis

Ribozyme products were resolved by denaturing PAGE, eluted using RNaid (BIO 101) and mixed with 2.5 fmol of primer (sequence: $^{5'}$GTCTAGGATCTACTG$^{3'}$; labelled at the 5′ end by polynucleotide kinase using (γ^{32}P)ATP (26) in hybridization buffer (10mM PIPES pH 6.8, 0.4M NaCl) in a final volume of 10μl. The mixture was then heated to 80°C for 3min, allowed to anneal at 50°C for 6h and then reverse-transcribed using AMV reverse transcriptase (Promega, 100U/ml) in 100μl 50mM Tris−HCl, pH 8.2, containing $MgCl_2$ (6mM), DTT (10mM), actinomycin D (25μg/ml) and dATP, dCTP, dTTP and dGTP

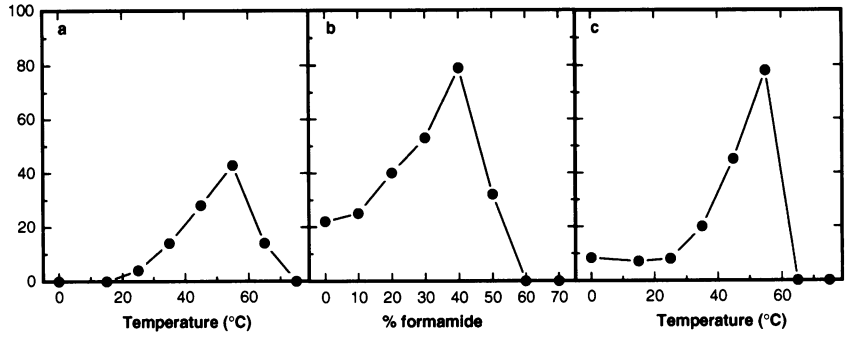

Figure 2. *In vitro* cleavage of surrogate HIV mRNA by ribozyme $496_{45:70}$. **a.** A profile of extent of cleavage by ribozyme $496_{45:70}$ *in vitro* at different temperatures. Substrate and ribozyme RNAs were transcribed *in vitro* using T7 RNA polymerase and ^{32}P CTP, mixed in the molar ratio 5:1 substrate:ribozyme and incubated in 50mM Tris−HCl, pH8, 20mM $MgCl_2$ for 60min. Ribozyme, substrate and cleaved products were analysed by denaturing polyacrylamide gel electrophoresis and quantitated by laser densitometry of autoradiographs. **b.** Effect of formamide concentration on extent of reaction after 60min at 50°C. **c.** Temperature profile of cleavage after 60min in the presence of 40% formamide.

(0.5mM each) for 1 h at 43°C. The cDNA was recovered by ethanol precipitation, washed with 95% ethanol, desiccated and dissolved in 5μl of formamide dyes. In parallel, the sequence of the target strand was determined using a ^{35}S sequencing kit (Amersham) and the primer used in the extension analysis. Products were analysed by denaturing PAGE, as before.

RESULTS

The activity of ribozyme 496 *in vitro*

The structure of ribozyme $496_{45:70}$ and other constructs used in this study is indicated in Figure 1. When *in vitro*-transcribed ribozyme $496_{45:70}$ was incubated with surrogate HIV mRNA, the target RNA was cleaved at the positionpredicted, and the point mutant, G_5U-$496_{45:70}$, produced no cleavage under similar conditions ((27, 28) and data not shown). The amount of cleavage by ribozyme $496_{45:70}$ at equilibrium was determined at a variety of temperatures and the temperature optimum was found to be approximately 55°C (Figure 2a). This is similar to the 50°C optimum previously reported (29). The activity of ribozyme $496_{45:70}$ was assayed *in vitro* in the presence of varying concentrations of formamide (Figure 2b). It was found that increasing concentrations of denaturant to 40% (v/v) increased ribozyme activity and this is reminiscent of work on the Tetrahymena class I intron ribozyme and the Hepatitis δ ribozyme (30−33). The inclusion of 40% formamide in the reaction did not alter the temperature optimum but abolished cleavage at 65°C and allowed significant cleavage to occur at 15°C and below (Figure 2c). Ribozyme $496_{45:70}$, therefore, has the *in vitro* properties that would be predicted from its structure, cleaving its substrate accurately but with a stoichiometry of no more than 2 in the absence of denaturing agents.

Ribozyme-mediated inhibition of HIV-1 replication in cell culture

Using the MLV-based vector, pBabe Hygro (14), Jurkat cell lines were made that expressed the RNAs, AR2 and AR5 (10), their ribozyme derivatives, $496_{45:70}$ and $496_{45:564}$, and the control RNAs, G_5U-$496_{45:70}$ and sense $496_{45:70}$. Each cell line was highly polyclonal, expressed similar levels of RNA and had indistinguishable growth rates over a period of 8 days (results not shown). When challenged with HIV-1_{IIIB}, cells expressing AR5, AR2, sense strand $496_{45:70}$ and G_5U-$496_{45:70}$ supported normal levels of viral replication (see Figure 3). In contrast, theribozyme derivative of AR5, $496_{45:70}$, inhibited HIV replication by up to 98% on day 7 post-infection. The absence of inhibition by the control RNAs is strong evidence that inhibition by ribozyme $496_{45:70}$ is the result of ribozyme-mediated cleavage rather than antisense effects. Ribozyme $496_{45:564}$ produced up to 56% inhibition, although this effect was transitory.

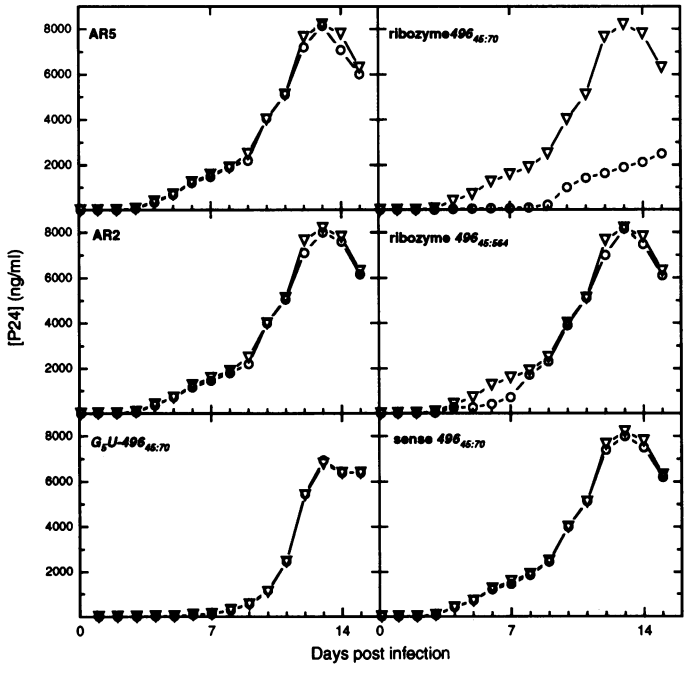

Figure 3. Inhibition of HIV replication in cells by ribozymes with long FCRs. Jurkat cells were transduced with the retroviral vector, pBabe Hygro (14), into which had been cloned genes encoding the RNAs described (Figure 1). The cells were challenged with HIV-1_{IIIB} as described (10) and samples were harvested at daily intervals for assay by P24 ELISA (20) as a measure of HIV replication. (circles), the P24 values from the test RNA-expressing Jurkat cultures; (triangles), the P24 values from the corresponding control Jurkat cultures.

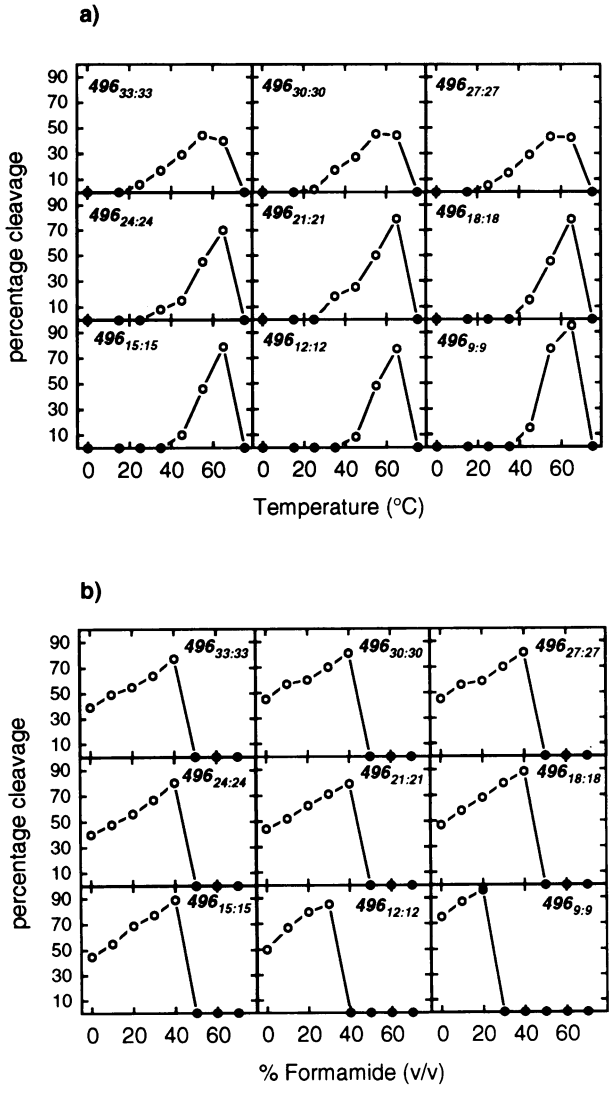

Figure 4. Cleavage of surrogate HIV RNA *in vitro* by ribozymes of differing FCR length. **a.** Profile of extent of cleavage after 60 min at varying temperatures. **b.** Extent of cleavage at 50°C after 60min in the presence of varying concentrations of formamide.

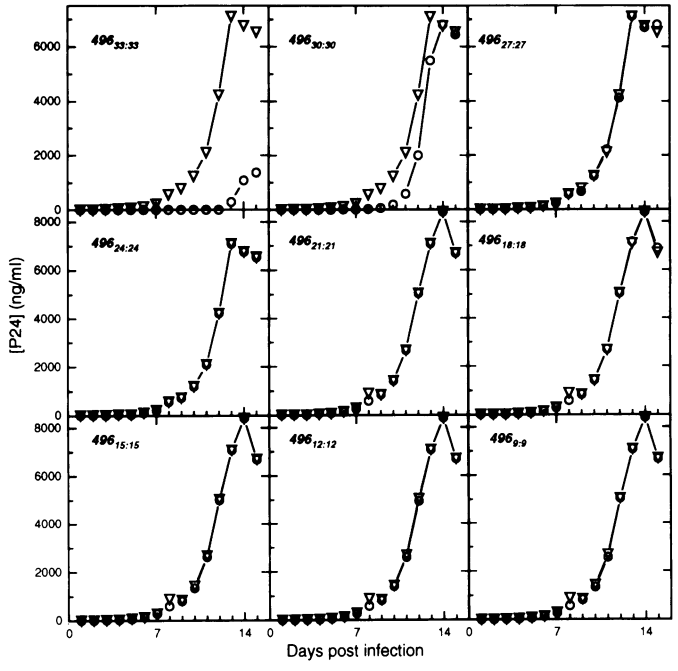

Figure 5. Inhibition of HIV replication in cells by ribozymes of differing FCR length. Ribozyme-expressing polyclonal Jurkat cell lines were derived by retroviral transduction, challenged with HIV-1_{IIIB} and sampled for P24 assay, as before.

Activity of FCR length variants *in vitro*

The inhibition observed in the growth of HIV by a ribozyme with poor *in vitro* properties and the failure of previously reported ribozymes to work effectively in the cellular environment, while working efficiently *in vitro*, suggested that the optimal flanking sequence length for effective ribozyme action might be different in the two contexts. To test this, a nested set of nine derivatives of ribozyme $496_{45:70}$ was constructed in which the FCRs were progressively and symmetrically reduced in length from 33nt to 9nt (Figure 1). These were analysed first under cell-free conditions, as before, and showed a temperature optimum of between 55°C and 65°C. The ability to cleave at temperatures below 45°C declined progressively as the FCR length was reduced (see Figure 4) and this may be the the result of the anomalous retardation of annealing kinetics observed with short 'tracer', long 'driver' reactions (34). However, under most conditions, the efficiency of reaction increased with decreasing length of FCR and this is consistent with previously reported work (8, 9).

Cleavage at 50°C was enhanced progressively by increasing concentrations of formamide: up to 40% (v/v) for ribozymes with FCR ≥ 15nt; up to 30% for that with FCR = 12nt and up to 20% for that with FCR = 9nt. The temperature-dependence of cleavage was determined at the optimal formamide concentration of each ribozyme (see Figure 3d). All the profiles are very similar, with an enhancement of cleavage by formamide at lower temperatures, a temperature optimum of 55°C and no cleavage at 65°C.

Relationship between FCR length and inhibition of HIV replication by ribozymes

Stable Jurkat cell lines expressing each ribozyme were derived as previously described and challenged with HIV1_{IIIB} as before. The results (see Figure 5) show that none of the ribozymes having FCRs < 30nt exhibited measurable inhibitory activity against HIV in cell culture. However, ribozyme $496_{30:30}$ produced over 10^3-fold inhibition of replication during days 1 to 5 after infection, although inhibition was lost in the following week. Ribozyme $496_{33:33}$ produced $> 10^3$-fold inhibition between days 1 and 12 post infection. The degree of inhibition was not the result of differential levels of ribozyme RNA in the cells, as the non-inhibitory RNAs were expressed at between 0.99× and 2.85× the level of the inhibitory RNA, $496_{33:33}$ (data not shown).

DISCUSSION

The results described here reveal an apparently converse relationship between the *in vitro* activity and the cellular potency of a family of ribozymes differing in the length of their flanking complementary regions (FCRs). The shortest FCRs were associated with the greatest activity in cell-free assays, as expected. However, only ribozymes with FCRs 30nt gave significant inhibition of HIV-1 replication ($\geq 10^3$-fold) in cultured cells. It is possible that these observations will turn out to be peculiar to the particular target site or assay system we have used. For example, the information content of the target mRNA or its secondary structure may not only determine its

susceptibility to ribozyme-mediated but also affect the optimal size of ribozyme. Only further systematic studies of the sort described here, aimed at alternative target sites, will resolve this question. Further, our chosen target system does not allow one to measure the effective ratio of ribozyme to substrate RNA in the cell since the proportion of cells expressing HIV mRNAs and the level of expression in each cell varies during the course of the experiment.

On the other hand, it is possible that the 'long FCR rule' will prove to be generally applicable. Interestingly, a ribozyme reported to be less effective against HIV than its parental antisense RNA had FCRs of 20 and 18: below the threshold we propose (5). We believe the apparent discrepancy between intracellular and cell-free activity may be due to the radically different environment within the cell and the consequent effect on the kinetic parameters of ribozyme action (27). It is likely that most interactions between complementary RNAs occur in the nucleus (35) although ribozymes have proved effective against the cytoplasmic RNA virus, LCMV (36). The nucleoplasm contains abundant ssRNA-binding hnRNP proteins (37) and enzymes that modify or unwind long RNA duplexes (38–40). These proteins would be expected to have a substantial effect upon the interaction between complementary nucleic acids, such as ribozymes andsubstrate RNAs. For example, the ssRNA-binding proteins would tend to increase the rate of annealing by stabilizing the hybridization-competent single-stranded forms. Indeed hnRNP protein A1, has been shown to increase the kinetics of nucleic acid annealing by a factor of $>10^3$ compared to those found in protein-free buffers (41). On the other hand, by lowering the free energy of ssRNA, these same proteins might also increase the rate of dissociation of ribozyme from substrate and from cleaved products. Consequently, rather than the two-component interaction between ribozyme and substrate RNAs that occurs *in vitro*, a competition between RNA–RNA interactions and RNA–protein interactions occurs in the nucleoplasm. In certain respects, this situation is better mimicked *in vitro* by the presence of denaturant such as formamide rather than the usual Tris/Mg^{2+} conditions.

In the light of these considerations, we interpret our experimental findings as follows. In the presence of hnRNP proteins, ribozymes with sub-optimal FCRs (<30nt) are unable to bind their target for sufficient time to allow cleavage to occur. This is qualitatively equivalent to the observation that ribozymes with FCRs <15nt are inactive in the presence of 40% formamide. Ribozymes with supra-optimal FCRs (>>70nt) fail to dissociate from their cleaved products. preventing them from initiating multiple rounds of substrate cleavage. Ribozymes with optimal FCRs (~33nt) bind their substrates sufficiently strongly to allow cleavage but are sufficiently easily dissociated by hnRNP proteins to enable multiple rounds of cleavage to occur.

We believe that current *in vitro* assays of ribozyme activity are not helpful inpredicting activity in the cell. By relying on cellular assays for optimization, we have obtained levels of inhibition that have not previously been reported in comparable systems. This approach may yield profoundly inhibitory ribozymes targeted to other viral or cellular genes.

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