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Construction and deployment of triple ribozymes targeted to multicatalytic proteinase subunits C3 and C9

Research Article

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Abbreviations: **AOs**, antisense oligonucleotides; **Rz** , ribozymes; **TRz**, triple ribozyme; **ITRz**, internal targeted ribozyme

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Summary

We have been developing triple ribozyme constructs for modulating gene expression, which consist of 2 cis-acting ribozymes flanking an internal trans-acting ribozyme, which is targeted to a selected cellular RNA. The 2 cis-acting ribozymes function autocatalytically, resulting in liberation of the internal ribozyme with minimal nonspecific flanking sequences. Here, we test 2 triple ribozyme constructs targeted to the multicatalytic proteinase subunits C3 and C9. The liberated internal ribozyme is 5-20 times more active in vitro than the same ribozyme contained with a double-G mutant (which cannot undergo autocatalytic processing) or contained within nonspecific flanking vector sequences. These triple ribozymes were placed within inducible expression vectors, which were used to produce stably transfected hepatocyte cell lines. Both of the constructs distributed between nucleus and cytoplasm, produced substantial growth inhibition in the cell lines, and their effectiveness was due to their catalytic activity and not to antisense effects, as demonstrated with catalytically inactive mutants. Thus, the triple ribozyme approach appears to represent a substantial improvement over conventional ribozymes.

I. Introduction

Use of antisense oligonucleotides (AOs) has afforded the opportunity to delineate functions of specific genes (Helene and Toulme, 1990; Izant and Weintraub, 1985; Zamecnik and Stephenson, 1978), and it has proven possible to differentially modulate endogenous vs. exogenous mRNAs using AOs (Benedict and Clawson, 1996). However, in spite of widespread applicability, use of AOs suffers from a number of limitations. For example, AOs are inherently unstable in cells, necessitating modifications to phosphodiester linkages (Hoke *et al*., 1991; Iverson *et al*., 1992). AOs are also often toxic at concentrations necessary to observe the desired effects, and in addition can trigger myriad non-specific effects (Krieg *et al.*, 1995; Stein and Cheng, 1993; Storey *et al*., 1991; Wagner, 1994). Finally, abundant mRNAs, or RNAs with significant secondary structure, are unlikely to be modulated efficiently.

"Hammerhead" ribozymes provide an alternative approach to downregulating specific gene products (Christoffersen and Marr, 1995; Haseloff and Gerlach, 1988; Ohkawa *et al.*, 1995; Parker *et al.*, 1992; Perreault *et al.*, 1990). Ribozymes (Rz) can be conceptualized as 2 functional elements, a conserved stem-loop structure forming the catalytic core, and

flanking sequences which are reverse complementary to sequences surrounding the target site in an RNA transcript (Goodchild and Kohli, 1991; Haseloff and Gerlach, 1988). Rz-mediated cleavage occurs 3' to a targeted nucleotide triplet NUX (N can be any nucleotide, whereas X can be A, C, or U) (Haseloff and Gerlach, 1988; Kawasaki *et al.*, 1996; Koizumi *et al*., 1989; Ruffner *et al*., 1990): When the third nucleotide is G, cleavage cannot occur. Flanking sequences confer specificity, and extend on both sides of the target site selected. Relatively short flanking sequences (6-9 nt each) allow sufficient specificity for the cleavage reaction, while allowing ready dissociation from the target, which is typically rate-limiting for the catalytic cycle (Goodchild and Kohli, 1991; Parker, et al., 1992). However, in spite of the requirement for relatively short specifier/flanking sequences for efficient catalytic rates in vitro, such constructs are often relatively ineffective in vivo. For example, Rz targeted to HIV-1 RNAs were optimally active in vivo with flanking sequences ≥ 33 nt (Crisell *et al*., 1993), even though these Rz showed relatively poor catalytic activity in vitro. Parameters likely to contribute to this disparity between in vitro vs. in vivo activity are target site accessibility, cellular compartmentation, as well as potential deleterious effects of non-specific flanking sequences present within traditional Rz transcripts.

With this in mind, we have been developing triple ribozyme (TRz) constructs which offer distinct advantages; these constructs consist of an internal targeted ribozyme (ITRz), which is flanked by two cis-acting ribozymes. The 2 cis-acting ribozymes are targeted to sites within the primary TRz construct, so that following transcription, they function autocatalytically liberating the ITRz. Our initial design is similar to one previously described by Taira and colleagues (Ohkawa *et al*., 1992; Ohkawa *et al*., 1993; Taira *et al.*, 1991), and other self-trimming Rz have also been designed (Altschuler *et al*., 1992; Dzianott and Bujarski, 1989; Ruiz *et al.*, 1997). We have previously characterized a TRz targeted to the retinoblastoma gene product (Rb) mRNA, and showed that it was efficiently liberated and functioned effectively in vivo (Benedict *et al*., 1998). Similar results were obtained with a TRz targeted to repetitive B2 transcripts, where we further showed that this approach results in a distribution of liberated ITRz between nucleus and cytoplasm (Crone *et al*., 1998). Here we have designed and produced TRz constructs targeted to the C3 and C9 subunits of the multicatalytic proteinase (MCP), and present results documenting their effectiveness both in vitro and in vivo. Their effectiveness in vivo is clearly due to their catalytic activity, since catalytically inactive mutant TRz do not produce analogous results. The TRz constructs were superior to the same ITRzs contained within TRz constructs which could not undergo self-liberation, or than the same ITRzs flanked by nonspecific vector sequences.

II. Results

We prepared a double ribozyme cassette, consisting of 2 cis-acting ribozymes flanking a cloning site. These ribozymes were targeted to GUCs in the primary transcript, and were designed to function autocatalytically. We then chose target sites in MCP subunits C3 and C9 mRNAs, and constructed ITRz specific for the chosen target sites. These specific ITRz were then inserted into the parent double ribozyme construct to create the targeted TRz constructs (C3TRz and C9TRz). Catalytically inactive mutants (C3mTRz and C9mTRz) were created by changing 2 essential nucleotides. In addition, double-G mutants were created (designated C3GGm and C9GGm), in which the autocatalytic cleavage sites were changed from GUCs to GUGs, so that autocatalytic cleavage could not occur. Finally, “single” Rz were created (designated C3SRz and C9SRz), where the cis-acting Rz were replaced with nonspecific flanking vector sequences. All constructs were then placed into a vector allowing bidirectional transcription, as well as into suitable expression vectors. All constructs were sequenced to confirm identity.

To document self-liberation, we employed pCRII constructs. For the C3TRz, "sense" transcripts were produced with Sp6 polymerase, and after transcription reactions the RNA was analyzed by PAGE (**Figure 1A**). About 80% of TRz transcripts underwent complete or partial processing during the transcription incubation. The expected 5', 3', and ITRz bands were observed, and additional bands were also observed for partially-processed 5' + ITRz and 3' + ITRz products (**Figure 1A**, as indicated). Enzyme "pausing" (premature termination) at the 5' autocatalytic site was generally low with Sp6 polymerase, although in some experiments it occurred in nearly 20% of transcripts (similar to that generally observed with T7 polymerase, see below).

In further experiments, unprocessed or partially-processed transcripts were eluted from gels and incubated for various periods at 37°C. Approximately 75% of the full length transcripts underwent processing (complete or partial) during this subsequent incubation (**Figure 1B**). Autocatalytic cleavage occurred somewhat more efficiently at the 3' site compared with the 5' site under these conditions. With the partially processed transcripts, 85% of the 5' + ITRz transcripts underwent autocatalytic processing, while 55% of the 3' + ITRz transcripts were processed. Since no enzyme "pausing" complicates these experiments, the expected stoichiometry was observed for the various products. Quantitative analyses (for 3 separate preparations) indicate that >80% of C3TRz transcripts undergo complete processing, with consequent liberation of the ITRz, in the initial transcription reaction and the subsequent incubation period.

For the C9TRz, transcription from the Sp6 promoter produced the "sense" TRz, which underwent self-cleavage (**Figure 2A**). Cleavage proceeded efficiently at both autocatalytic sites for 50% of the transcripts, with production of 5'-, 3'-, and liberated ITRz (as designated). A smaller proportion (roughly 20%) of transcripts showed self-cleavage at only one of the internal 5' or 3' sites (**Figure 2**). In addition, quantitative analyses indicated that the 5' Rz was overrepresented by 20% due to enzyme pausing between the 5' Rz and ITRz.

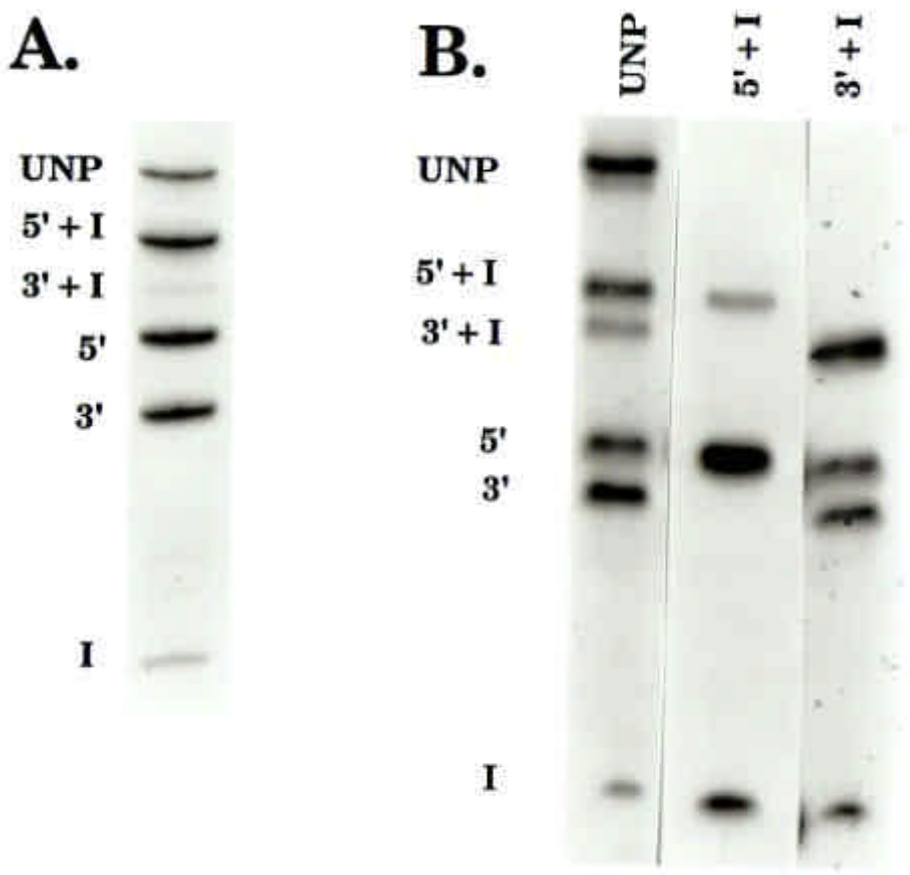


Figure 1. In vitro Characterization of the C3TRz. **Panel A**. Self-liberation. ^{32}P-labeled C3TRz was transcribed in the sense and antisense directions using Sp6 and T7 RNA polymerases (respectively), and the products were examined by PAGE and autoradiography. Transcription with Sp6 produced the active TRz, with consequent liberation of the ITRz (66 nt), the 5' cis-acting Rz (151 nt), and the 3' cis-acting Rz (146 nt), the latter 2 of which remain associated with some vector sequences. Two less intense bands observed represent incompletely processed transcripts, where either the 5' or 3' autocatalytic cleavage did not occur (these are designated 5' + I and 3' + I, respectively). **Panel B**. Reincubation of unprocessed/partially processed transcripts. The residual unprocessed TRz, or the partially processed 5' + I and 3' + I transcripts were excised from gels, and incubated for an additional 30 min at 37 C, and then analyzed by PAGE and autoradigraphy. Lane 1 shows results with transcripts which were unprocessed during the initial transcription reaction, while lanes 2 and 3 depict results from incubation of the 5' + I and 3' + I transcripts (respectively). The band in lane 3 corresponding to the 5' transcript arose from contamination of the excised 3' + I band with 5' + I transcript during the gel-purification procedure (it was present in much greater quantity in this experiment). No self-liberation was observed when transcription was performed in the antisense (T7) direction (data not shown), or when incubations were performed in the presence of excess EDTA.

When unprocessed (full-length) transcripts were eluted from the gel and incubated for 30 min at 37°C, a significant proportion (44%) of the transcripts underwent efficient self-cleavage, although a somewhat higher proportion (50%) of transcripts with only one self-cleavage was observed (**Figure 2B**). In addition, elution and subsequent incubation (30 min) of the partially processed 5' + ITRz and 3' + ITRz transcripts showed that efficient processing occurred (**Figure 2B**, lanes 2 and 3). "Zero-time" controls indicated that no processing of transcripts occurred after RNA extraction. These results (and extended time-course experiments) indicate that the ITRz is effectively liberated from >80% of the C9TRz transcripts. The balance may be contained in complexes (Groebe and Uhlenbeck, 1988), although quantitative liberation might also eventually occur.

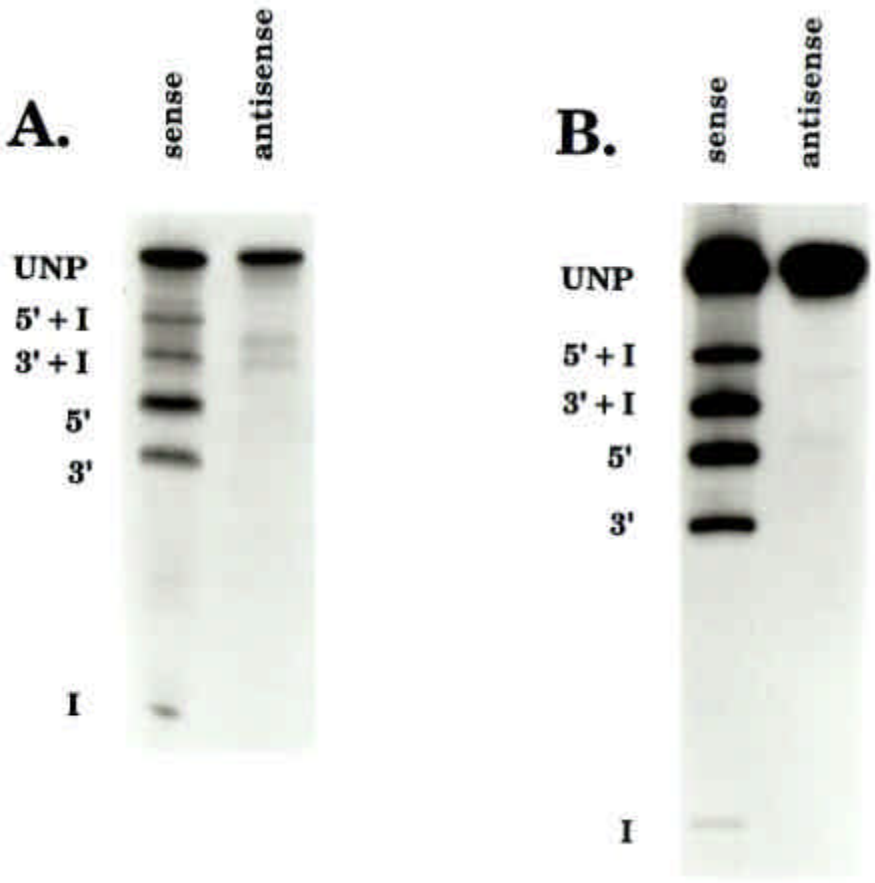


Figure 2. In vitro Characterization of the C9TRz. **Panel A**. Self-liberation. Sense transcripts were obtained with Sp6 polymerase, and antisense transcripts were generated with T7 polymerase. Quantitative analysis of radioactivity in the respective bands (designated as in **Figure 2**) showed that two-thirds of the initial TRz transcripts underwent processing during the transcription reaction, and two-thirds of these were completely processed. No processing of antisense transcripts was observed, either during the initial transcription reaction or the subsequent incubation (Panel B). **Panel B**. Reincubation of unprocessed transcripts. Full length TRz transcripts which were not processed during the initial transcription reaction were excised from gels and further incubated for 30 min at 37 C. 40% underwent processing, and one-half of these were correctly liberated. In the reincubation reaction (where no enzyme pausing occurs) the 3' + I product was elevated, indicating that autocatalytic processing at the 3' site may be somewhat less efficient than the 5' processing for this particular construct. One other round of excision and incubation (not shown) showed that subsequent processing of full-length TRz transcripts contributes another 10% of liberated ITRz. Similar analyses of the 5' + I and 3' + I transcripts suggest that about one-half of these transcripts subsequently undergo complete processing. Taken together, these figures indicate that a similar proportion of C9TRz undergo complete processing (with consequent liberation of the ITRz) as compared with the C3TRz, although the time required for this processing was somewhat longer.

In further experiments, we also examined effects of extending the initial transcription reaction and/or subsequent incubations. When the initial transcription reactions were extended from 1 h to 2 h, 140% more ITRz was liberated. When Mg^{++} was increased to 50 mM for the second h following the 1 h transcription reaction (effectively terminating transcription), we observed a 50% increase in liberation of the ITRz, and when this incubation was extended to 2 h, we observed a 110% increase. No processing occurred after addition of excess EDTA. These results thus confirm efficient, time-dependent liberation of the ITRz from our constructs in vitro.

We then performed a series of experiments to document cleavage of the targeted RNAs in vitro. ^{32}P-labeled target RNAs were synthesized in vitro. Unlabeled C9TRz and C3TRz were also synthesized. For comparative purposes, the C9-targeted ITRz was directly transcribed in vitro, as were

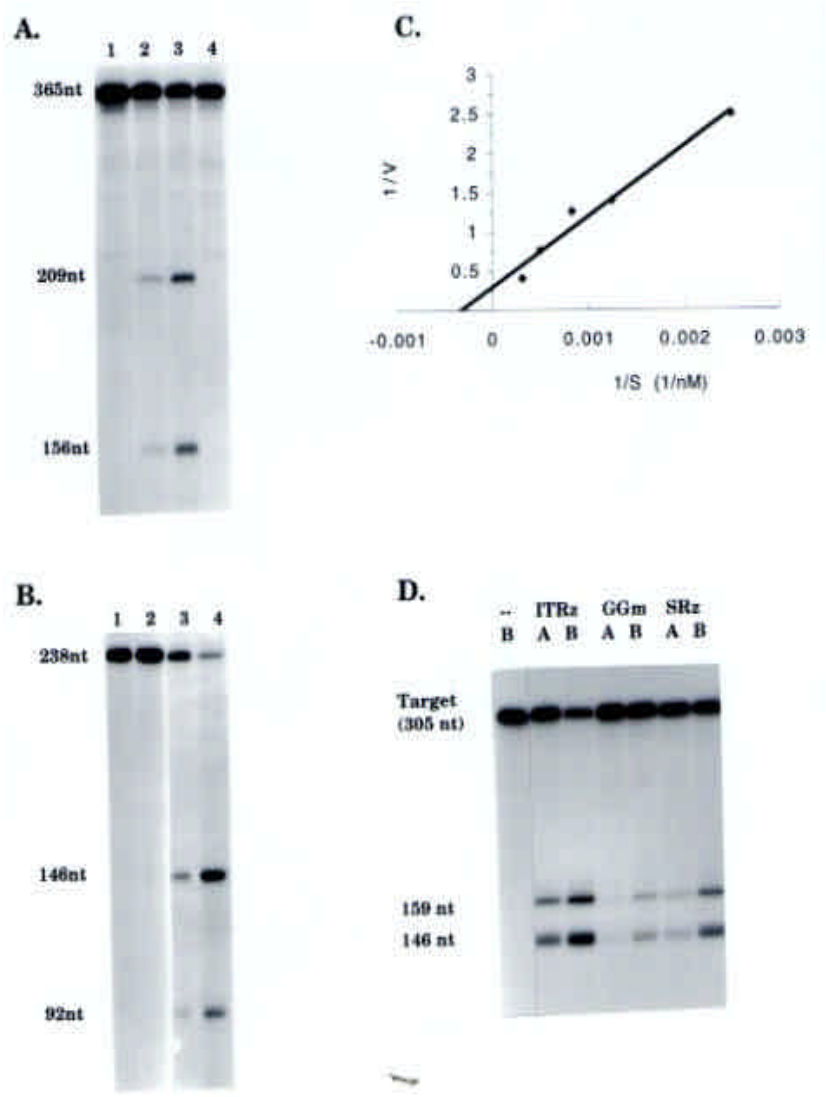


Figure 3. Target cutting in vitro with C3TRz and C9TRz. **Panel A**. Target cleavage by the C9TRz. A ^{32}P-labeled 365 nt partial C9 target was transcribed, gel-purified, and incubated with the C9TRz. Following 0.5 h (lane 2) or 2 h (lane 3) incubations, RNA was examined by PAGE and autoradiography as described. Cleavage produced 156 and 209 nt fragments, with cleavage of 40% of transcripts at 2 h. No cleavage occurred in the absence of Rz (lane 4), or in the presence of excess EDTA. **Panel B**. Target Cleavage by the C3TRz. A ^{32}P-labeled partial C3-target RNA was transcribed, gel-purified, and incubated with C3TRz at 37 C at a 1:1 molar ratio. Following incubation, RNA was examined by PAGE and autoradiography. Here, the target C3 RNA transcript was 238 nt, with cleavage products of 146 and 92 nt. Under the conditions used, no cleavage occurred in the absence of Rz or in the presence of excess EDTA (lane 2). 33% and 80% of transcripts were cleaved after 0.5 and 2 h incubations (lanes 3 & 4, respectively), while 100% cleavage was observed after 6 h (data not shown). **Panel C**. Lineweaver-Burke plot of C9TRz activity in vitro. ^{32}P-labeled C9 target and unlabeled C9TRz were prepared. Extensive time-course incubations were conducted at 37 C with multiple substrate concentrations. The extent of cleavage was ascertained by PAGE and phosphorimager analyses, and linear regression values were obtained. The calculated k_m (under these arbitrary conditions) is about 0.2 µM, and the indicated V_{max} is 10 cleavages/Rz-h. **Panel D**. Comparison of in vitro Catalytic Activities of C9 Rz Constructs. C9TRz preparations, the liberated ITRz, and the C9GGm and C9SRz constructs were purified and incubated with a ^{32}P-labeled 305 nt partial C9 target at 37 C for 0.5 h (A) or 2 h (B). Cleavage produced 159 nt and 146 nt fragments. Following incubations, cleavage was assessed by PAGE and autoradiography.

the C9SRz and C9GGm Rz constructs. Unlabeled Rz and ^{32}P-labeled target RNAs were then incubated for various periods, and the reaction products were examined by PAGE (**Figures 3A** and **3B**). More extensive kinetic analyses with C9TRz preparations and target RNA at various concentrations indicated an apparent k_m of about 0.2 µM, with an apparent V_{max} of about 10 cleavages per h per ribozyme under these conditions (**Figure 3C**). We then compared the activity of the C9 ITRz with the C9GGm and C9SRz constructs. The ITRz was 5 times more active than C9SRz (which is the same ITRz flanked by nonspecific vector sequences) and 20 times more active than C9GGm (which cannot undergo autocatalytic processing (**Figure 3D**). Comparable results were obtained with the analogous C3 constructs.

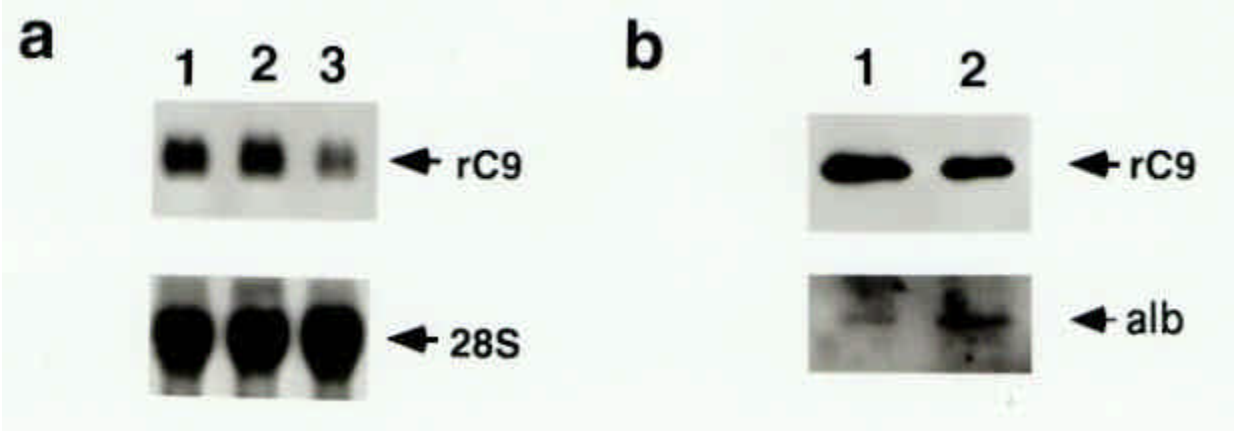


Figure 4. Reduction of C9 mRNA and protein in stably transfected clones expressing the C9TRz. CWSV1 cells were transfected with the C9TRz in the LacSwitch vector, and stably transfected clones were obtained by antibiotic selection. Four of 5 clones showed markedly reduced growth rates, whereas one clone was not expressing the C9TRz and therefore served as an additional control. Of the 4 clones showing growth inhibition, clone 3 was the fastest growing (with a 60% reduction in growth rate), and was used for subsequent analyses; the other 3 clones grew so slowly they were not easily amendable to further analyses. **Panel A**. Northern blot analysis of cellular RNA from control CWSV1 cells (lane 1), from an antibiotic-selected clone not expressing the TRz (lane 2), and from clone 3 (lane 3). The reduction of C9 mRNA in clone 3 was >65%, when corrected for slight differences in loading, as determined with 28 S rRNA probe (lower panel). **Panel B**. Immunoblot analysis of C9 protein. Cellular proteins were prepared from an antibiotic-selected clone not expressing the TRz (left), and from clone 3 (right), separated by SDS-PAGE, and probed with an antibody directed against C9. The membrane was then stripped and reprobed with antibodies to rat albumin (lower panel). Normalization to equivalent loading demonstrated that the actual reduction in C9 protein was > 80%.

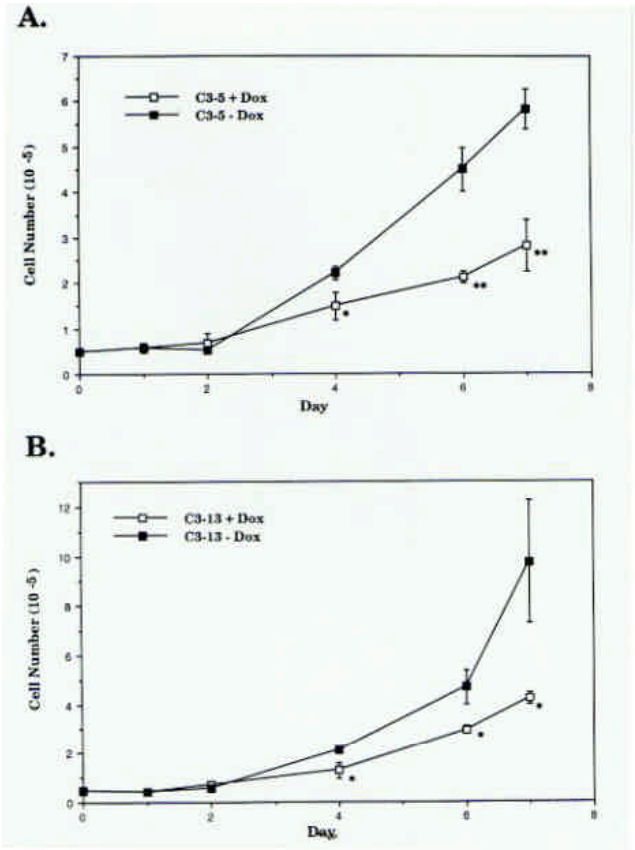
We next tested the TRz constructs in cell culture, first using the LacSwitch vector system. Transient transfection analyses were performed with CWSV1 cells, and cytoplasmic RNA was isolated 24 h later and examined by Northern blot analysis (equivalent loading was verified with GAPDH). We observed marked reductions in targeted RNAs, which were not observed with control cells transfected with the parent double ribozyme, and these reductions were contingent upon induction of TRz expression. With both the C9TRz and C3TRz, we observed 50% reductions in target RNAs in CWSV1 cells (data not shown). Since the transfection efficiency of this procedure is approximately 50%, this suggests very efficient cleavage of the targeted mRNAs within cells in these transient transfections.

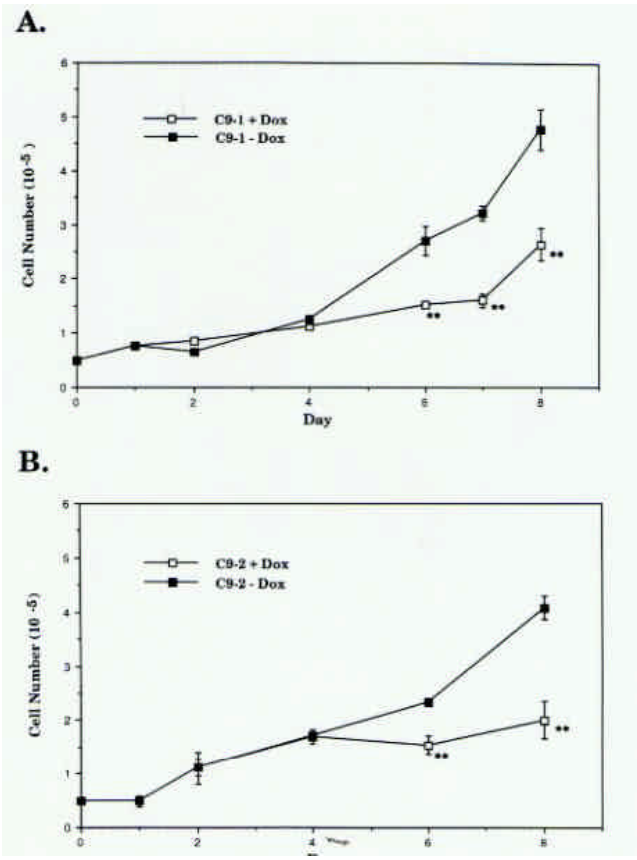
We next developed stably transfected CWSV1 clones expressing the C9TRz or C3TRz with the LacSwitch system. With the C9-targeted TRz, 4 clones expressing the TRz were obtained. All 4 clones showed significant growth inhibition; 3 of these clones grew so slowly that they were not easily amendable to further analyses, whereas one clone (#3) showed a 60% reduction in growth rate, and was further analyzed. Expression of the C9-targeted TRz in clone #3 produced a 65% reduction in C9 mRNA (**Figure 4A**), and this reduction was paralleled by a similar reduction in rC9 protein (**Figure 4B**).

Stably transfected CWSV1 clones were also obtained with C9- and C3-targeted TRz with the pTet-On system, and their growth was compared in the presence or absence of doxycycline (allowing each clone to serve as its own control). Induction of expression of the C3-targeted TRz resulted in a marked reduction in growth of stably transfected clones (**Figure 5A** and **5B**), consistent with previous results following downregulation of C3 mRNA with AOs (Benedict and Clawson, 1996). We consistently observed a 2-3 day delay before growth inhibition was manifested. A similar reduction was observed following induction of expression of the C9-targeted TRz in stably transfected clones (**Figure 6A** and **6B**), although with all of these clones the lag period was consistently 4 days. Northern blot analysis was used to confirm reduction of target RNAs (**Figures 7A** and **7B**).

In contrast to the doxycycline-dependent growth inhibition observed with the C3TRz and C9TRz constructs, expression of the catalytically inactive mutant TRz (C3mTRz and C9mTRz) in a number of stably transfected clones did not produce any significant effects on cell growth (**Figure 8** shows growth studies with 2 stably transfected cell lines expressing the catalytically inactive C9mTRz). A reduction in cell growth was observed with cells expressing the C3mTRz: Cell growth inhibition was about 30% with induction of expression of C3mTRz (but was not statistically significant), compared with the 60% reduction produced by expression of C3TRz (**Figure 5B**, and data not shown).

Figure 5 (⇒). Effects of expression of the C3TRz in vivo. CWSV1 cells were transfected with the pTet-On construct, and stably transfected clones were obtained by antibiotic selection. One of these stably transfected clones which showed good expression levels was then transfected with the C3TRz (and a hygromycin resistance construct) and stably transfected clones were obtained by hygromycin/neomycin selection. **Panels A** and **B**. Results of growth studies with 2 individual clones ± doxycycline, with each clone thus serving as its own control. In each case, growth of the clones was significantly inhibited (* $p < 0.05$; ** $p < 0.01$) when expression of the C3TRz was induced by addition of doxycycline.





⇐ **Figure 6**. Effects of Expression of the C9TRz in vivo. Individual clones were obtained as described in Figure 5 and growth studies were conducted. **Panels A** and **B** show results with 2 individual clones ± doxycycline. In both cases, growth of the clones was significantly inhibited when expression of the C9-targeted TRz was induced by addition of doxycycline.

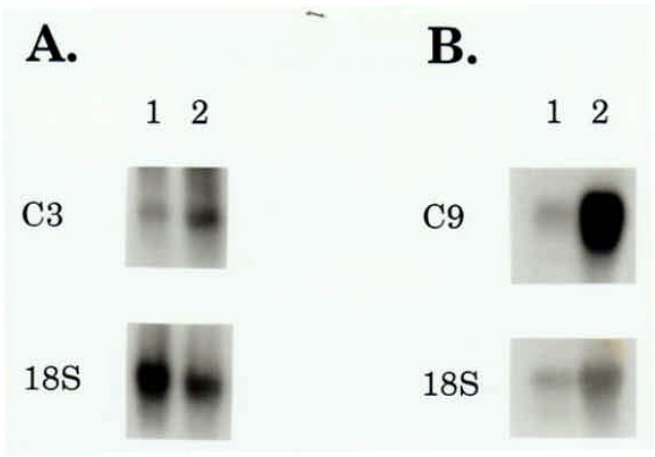
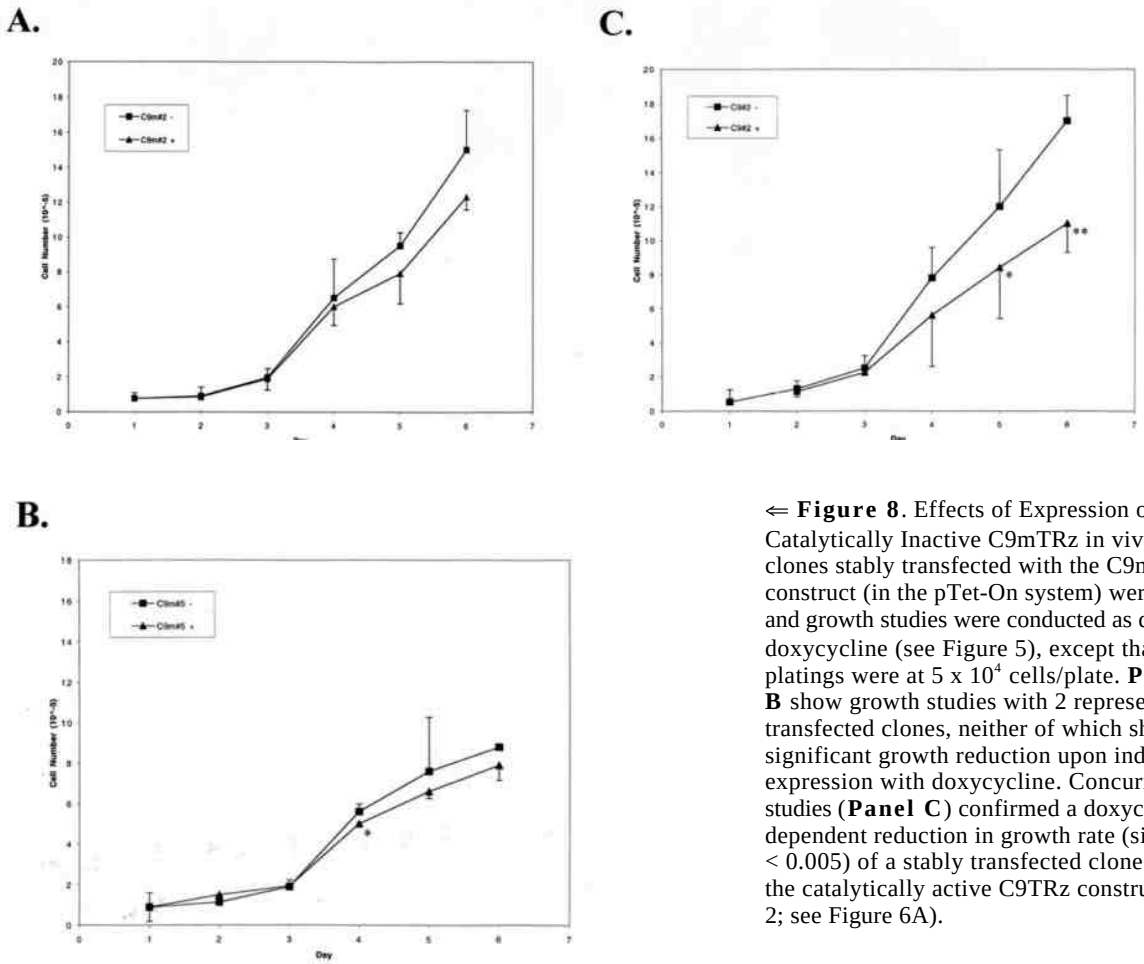


Figure 7 (⇒). Effects of Expression of C3TRz and C9TRz on target mRNAs in vivo. **Panel A**. Northern blot analysis of C3 mRNA. Cytoplasmic RNA was isolated (from the clone shown in Figure 5A) after 4 days of TRz expression, separated by PAGE, and examined by Northern blot analysis with ^{32}P-labeled C3 probe. A major reduction in C3 target RNA (upper panel) was observed in clones expressing the C3TRz (lane 1) compared to the same clone not expressing it (lane 2). The membrane was then stripped and equivalent loading was verified by reprobing with 18S rRNA (lower panel). C3TRz expression was then examined using an ^{32}P-labeled oligonucleotide antisense to the ITRz (data not shown). **Panel B**. Northern blot analysis of cytoplasmic RNA (from the clone shown in Figure 6A) isolated 4 days after induction of C9TRz expression. A major reduction in C9 target mRNA was observed when TRz expression was induced (lane 1) compared with the same clone not expressing the TRz (lane 2). The membrane was then stripped and reprobed with 18S rRNA for documentation of loading (lower panel), and with an ^{32}P-labeled oligonucleotide antisense to the ITRz (data not shown).



⇐ **Figure 8**. Effects of Expression of Catalytically Inactive C9mTRz in vivo. CWSV1 clones stably transfected with the C9mTRz construct (in the pTet-On system) were obtained, and growth studies were conducted as described ± doxycycline (see Figure 5), except that initial platings were at 5 x 10^4 cells/plate. **Panels A** and **B** show growth studies with 2 representative stably transfected clones, neither of which showed significant growth reduction upon induction of expression with doxycycline. Concurrent growth studies (**Panel C**) confirmed a doxycycline-dependent reduction in growth rate (significant at $p < 0.005$) of a stably transfected clone expressing the catalytically active C9TRz construct (clone C9-2; see Figure 6A).

In preliminary further experiments (using the pIND vector system which is inducible with the ecdysone analog ponasterone A), the constructs unable to undergo self-liberation (C3GGm and C9GGm), or the ITRz flanked by nonspecific vector sequences (C3SRz and C9SRz) were also not effective in reducing growth rate (data not shown).

Cytoplasmic and nuclear RNA was isolated from two of the stably transfected clones expressing C3TRz and C9TRz, and TRz expression and self-liberation was examined by RT/PCR using "inner" and "outer" primer pairs (the inner primer pair amplifies both processed and unprocessed TRz transcripts, whereas the outer primer pair amplifies only unprocessed transcripts). RT/PCR analyses confirmed doxycycline-dependent expression of the TRz constructs in cytoplasmic RNA, and showed that essentially all detectable transcripts were processed in vivo (**Figure 9**). Interestingly, doxycycline-induction of TRz expression was not observed in nuclear RNA **(Figure 9C**), even though inductions of 5- to 10-fold were characteristically observed in cytoplasmic RNA from the same clones (**Figure 9A**). Given that nuclear RNA represents approximately 10% of total cellular RNA, our results indicate that about 90-95% of the ITRz is found in the cytoplasm. These results parallel those previously reported for Rb- and B2-targeted TRz constructs (Benedict *et al.*, 1998; Crone *et al.*, 1998).

III. Discussion

This communication documents the general effectiveness of targeted TRz constructs, in which the ITRz (targeted to a cellular RNA) is flanked by 2 cis-acting ribozymes. The two cis-acting ribozymes are targeted to nucleotide sequences within the primary transcript, so that cleavage results in autocatalytic liberation of the ITRz . This design creates an uncapped 5' end for the relatively short liberated ITRz, and is based upon a design previously described by Taira and colleagues.

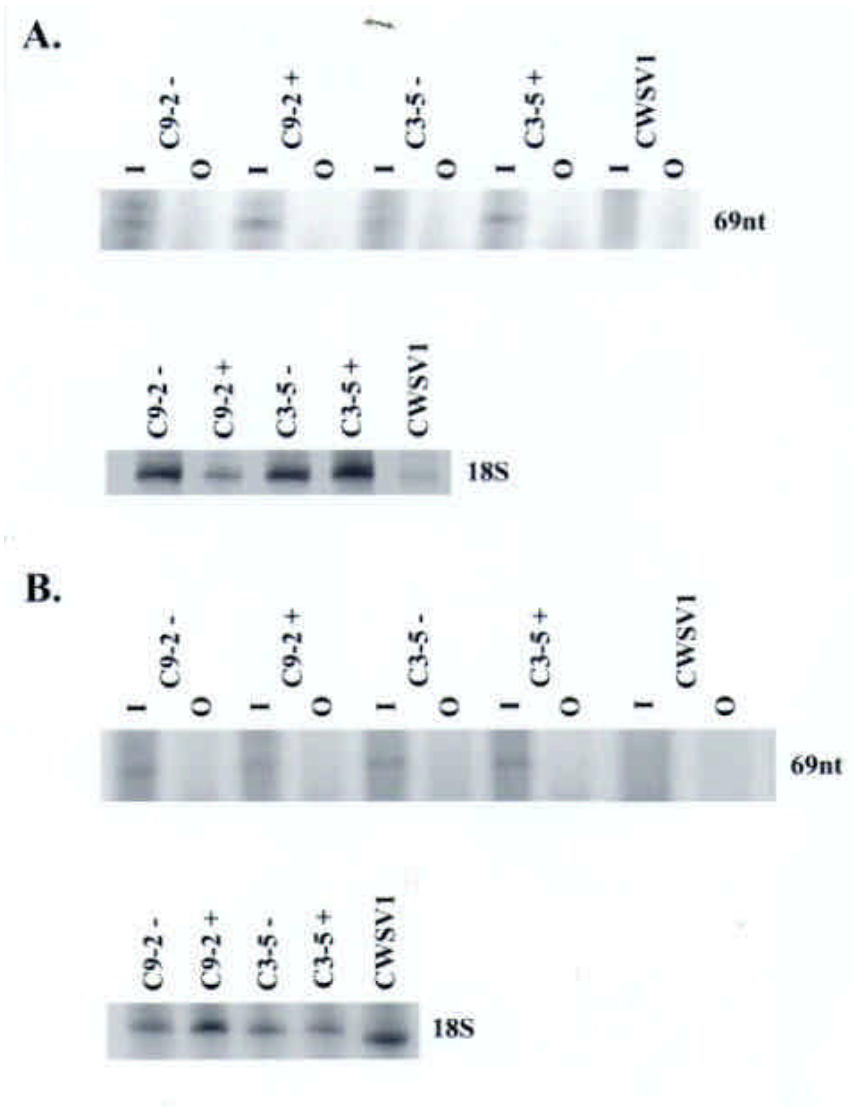


Figure 9. RT/PCR Detection and Processing of ITRz in vivo. Stably transfected clones expressing C9TRz (clone #2) or C3TRz (clone #5) were grown under inducing (+) or non-inducing (-) conditions, and cytoplasmic and nuclear RNA was prepared. 1 µg of RNA was used in RT/PCR analyses with the inner (I) or outer (O) primer pairs as described. Following reactions, products were separated on 8% polyacrylamide gels, and the amplified products were visualized by autoradiography. **Panel A** shows TRz expression with cytoplasmic RNA, and **B** shows results with nuclear RNA. Concurrent amplification of 18S rRNA was performed for standardization. Experiments using double-G mutant RNA transcripts confirmed that products were obtained with both primer pairs, with the inner primer pair being 1.8X as efficient. With the cellular RNAs, products were observed only with the inner primer pair, indicating that essentially all (> 90%) of the ITRz was liberated in vivo.

Thus far, we have obtained reasonably effective cleavage of targeted RNAs by choosing target sites based on RNA structural modeling using the mFold program, although optimal definition of target sites requires additional analyses, for example use of ribozyme expression libraries (Lieber and Strauss, 1995). In three extensive comparisons thus far, we have obtained 10-10^3-fold greater activity with library-selected TRz vs. TRz designed based on mFold modeling.

The liberated ITRz appears to effectively distribute between nuclear (about 10%) and cytoplasmic (about 90%) compartments (Benedict, et al., 1998; Crone, et al., 1998; Figure 9). This presumably reflects a competition between autocatalytic processing vs. nucleocytoplasmic transport of capped transcripts (see Benedict, et al., 1998; Crone, et al., 1998), and may alleviate potential problems resulting from physical separation of ribozyme and target within cells (Cotten and Birnstiel, 1989; Sullenger and Cech, 1993). Perhaps even more importantly, the liberated ITRz contains minimal non-specific flanking sequences, which may otherwise significantly hamper Rz catalytic activity in vivo.

These reagents appear to provide a number of important advantages over AOs. First, given their catalytic activity, each ITRz could potentially cleave a considerable number of target transcripts in vivo during its life-time. This advantage is apparent in experiments where the catalytically inactive mutant TRzs did not significantly affect cell growth whereas the catalytically active TRz produced marked (and highly significant) growth inhibition. The lag period before effects of the TRz activity seen presumably reflects a build-up in ITRz concentration. Second, the liberated ITRz is not compromised by significant non-specific flanking sequences, which should be expected to hamper antisense transcripts. This may well underlie the obvious discrepancy between in vitro catalytic activities of Rz vs. their in vivo effectiveness (Crisell, et al., 1993). Additionally, use of TRz constructs in suitable expression systems provides continuous production, and is not likely to be complicated by problems with non-specific toxicity or long-term stability, and verification of their efficiency is straightforward, with destruction of targeted RNAs and reduction in the corresponding proteins (where appropriate).

Finally, the constructs described here represent "first generation" constructs. A number of improvements have now been introduced, including: A) Use of 2 contiguous trans-acting ITRz, whose activities do not adversely affect each other; B) Redesign of the cis-acting Rz flanking sequences, which markedly improves the autocatalytic self-liberation activity of the TRz constructs; C) Addition of a short hairpin loop and/or protein-binding domains, to the 3' end of the double ITRz insert, to improve stability of the insert after liberation; and D) Development of a streamlined library selection procedure, which identifies target sites which yield TRz with 10-10^3 X greater catalytic activities. A number of these constructs are currently being tested in vivo, with the goal of developing suitable therapeutic reagents for clinical trials.

IV. Materials and Methods

A. Cell lines

An SV40-immortalized rat hepatocyte **cell line** (Benedict *et al*., 1995; Woodworth and Isom, 1987), designated CWSV1, was used in these studies. The CWSV1 cells were maintained in chemically defined medium (Woodworth and Isom, 1987).

B. Vectors

Vectors used in these experiments included:

(**i**) pCRII (Invitrogen). This vector contains Sp6/T7 RNA polymerase promoters for bidirectional transcription, and was used to produce RNA transcripts for in vitro studies.

(**ii**) LacSwitch system (pOPRSVICAT, from Stratagene), which was used for transfection analyses. This system contains a neomycin-resistance gene for antibiotic selection. When used in conjunction with the repressor vector, this system is inducible with IPTG.

(**iii**) A tetracycline/doxycycline inducible system, which consists of the pBI-L and pTet-On vectors (Clontech). pTet-On contains the tetracycline transactivator, which is active in the presence of doxycycline, and stably transfected clones expressing pTet-On were obtained by neomycin selection. The TRz constructs were cloned into the multiple cloning site in pBI-L. Clones stably transfected with pTet-On were then transfected with the TRz constructs in pBI-L (along with the pTKHyg vector to provide hygromycin resistance), and stably transfected clones were obtained by hygromycin selection.

(**iv**) The ecdysone-inducible expression system (InVitrogen). This system consists of the pIND expression vector and a vector (pVgRXR) expressing a heterodimeric ecdysone receptor. The heterodimer of the ecdysone receptor (VgEcR, modified to contain the VP16 transactivation domain) and the retinoid X receptor binds a modified ecdysone response element in pIND in the presence of the ecdysone analogs muristerone A or ponasterone A, thereby activating transcription. Stably transfected clones expressing the various ribozyme constructs were developed by antibiotic (zeocin and G418) selection.

C. Ribozyme synthesis

The parent double ribozyme (designated pLSClip) was initially synthesized in the LacSwitch vector as previously described (Benedict, et al., 1998). Internal targeted ribozymes (ITRz) were then designed for MCP subunits C3 (EMBL locus TATC3AA, accession number J02897) and C9 (EMBL locus RNPTSC9, accession number X53304). Target sites were selected after analysis of predicted secondary structures generated using the mFold program, which is based upon previous analyses (Jaeger *et al.*, 1989), and database searches were performed to check for collateral target redundancy. For C3, GUU_{22} was selected, and for C9 GUC_{101} was selected. For the C3-targeted ITRz, the 5' and 3' flanking sequences were UCGAAGCUGU and CCGCGUUGA respectively, and for the C9-targeted ITRz, they were UCUUCGA and CAUGGCU, respectively. These ITRz were then synthesized using reverse complementary oligodeoxynucleotides, which were designed to

include pre-cut *Bgl*II sites after annealing. Catalytically inactive ITRz were also created (Haseloff and Gerlach, 1988), where the catalytically essential G and A nucleotides were replaced with A and G, respectively. After annealing, product was ligated into *Bgl*II digested pLSClip at a 1:5 molar ratio. The identities of the final targeted TRz were verified by sequencing.

D. In vitro expression of ribozymes

For in vitro expression, the targeted TRz were removed from pLSClip using *Not*I and inserted into the *Not*I site of the pCRII vector (Invitrogen). Forward and reverse M13 primers were used in PCR protocols to amplify the region of pCRII containing the TRz (and T7 and Sp6 promoters), which was then used as template in vitro , using the Riboprobe Transcription System (Promega) with 50 µCi ^{32}P-CTP. Based on sequence analysis, T7 or Sp6 RNA polymerase was used to produce transcripts in the sense direction, and the other polymerase was used to produce antisense transcripts. Reactions were incubated at 37°C for 1 or 2 h; they were then digested with DNase, extracted with phenol/chloroform, and RNA was precipitated with ethanol and resuspended in 10 µl H_2O. RNA was incubated at 80°C for 5 minutes in an equal volume of loading buffer (80% formamide, 100 mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF), and analyzed in a 6% urea/polyacrylamide gels, followed by blotting and autoradiography. In some experiments, unprocessed (full-length) or partially processed transcripts were purified by PAGE. The bands were excised, homogenized in buffer (20 mM Tris-HCl, pH 7.6, 250 mM NaCl) and then incubated for 2 h at 4°C and for 5 min at 65°C. Following centrifugation at 2,000 x g for 5 min, the supernate was removed and RNA was precipitated with ethanol. RNA was resuspended and incubated for various periods at 37°C, and then analyzed as described. Zero-time incubations of unprocessed or partially-processed transcripts showed that no processing occurred during the handling procedures.

E. Target cleavage reactions

For target cleavage reactions, ^{32}P-labeled target RNAs were prepared as described above, using cloned partial target transcripts contained in pCRII. The C3 target used was 238 nt, yielding products of 146 and 92 nt after Rz cleavage, while the C9 target was 365 nt, giving 156 and 209 nt products after Rz cleavage. Following transcription, DNA template was removed by incubation with RNase-free DNase for 15 min at 37°C, loading buffer was added, and the RNA was separated by PAGE on 6% polyacrylamide gels. The ^{32}P-labeled target RNA was then gel-purified as above.

F. Preparation of triple ribozymes (TRz)

Unlabeled TRz were transcribed in vitro as above. Following transcription, the liberated ITRz were gel-purified, precipitated with ethanol, and resuspended in 50 µl H_2O. Labeled target RNA was incubated at 37°C for various times with (or without) Rz in buffer (50 mM Tris-HCl, pH 7.6, 25 mM KCl, 20 mM $MgCl_2$). In some cases, residual unprocessed and partially-processed TRz transcripts were also gel-purified and analyzed. The molar ratio of target:ribozyme generally used for standard analyses was 1:1, with final concentrations of about 50 nM. Reactions were terminated with an equal volume of stop buffer and the products were examined using an 6% urea/polyacrylamide gel followed by autoradiography.

G. Transfection experiments

For transfection experiments, CWSV1 cells were grown to mid-logarithmic phase and transfected with the various vectors, using either Lipofectin reagent (Gibco) as described (Benedict and Clawson, 1996; Benedict, et al., 1995) or electroporation. For electroporation, cells were resuspended in RPMI + 10% bovine calf serum. Each 0.4-cm gap cuvette contained 0.5 ml (5 x 10^6 cells) and 10 ug vector DNA. We used a BioRad Gene Pulser II, with capacitance 950 µF at 250 V/cm (t=20-25 msec). Transfection analyses using the green fluorescent protein construct (pEGFP-N1, from Clontech) indicated an efficiency of approximately 50% at 24 h. For development of stable transfectants, geneticin (at 0.5 mg/ml, from Sigma) was added after 48 h and selection was continued for approximately 4 weeks. Individual geneticin-resistant colonies were harvested using pipettes. As controls, clones stably transfected with the parent double ribozyme (designated Clip) or the catalytically inactive mutants were also produced. With the pTet-On expression system, cells stably transfected with pTet-On were obtained by geneticin selection. These cells were then transfected with the TRz constructs in pBI-L (along with pTKHyg), and stably transfected clones were obtained by hygromycin selection.

H. Growth studies

For growth studies, cells were generally plated at a density of 5 x 10^4 cells (or in some instances at 10^5 cells) and grown on 60 x 15mm tissue culture dishes. Plates were trypsinized and cells were counted in triplicate using a hemocytometer throughout a 7-8 day period.

I. Northern blotting

For Northern blot analyses, RNA was isolated from cells by the guanidinium thiocyanate method (Ausubel *et al*., 1996). RNA was fractionated by agarose-formaldehyde gel electrophoresis using a 1.2% gel and then transferred to MagnaGraph nylon transfer membrane (MSI) by capillary transfer. Membranes were subsequently irradiated using a Stratalinker UV-Crosslinker (Stratagene) at 24 x 10^4 mjoules. Hybridization was at 42°C overnight in 5 ml formamide hybridization buffer (Ausubel, et al., 1996), with 5 x 10^6 cpm/ml ^{32}P-DNA probe, which was labeled using the Prime-a Gene System (Promega), the cloned target inserts described above, and 50 µCi ^{32}P-dCTP. Following overnight hybridization, blots were washed extensively, with final rinses at 60°C in 0.1X SSC with 0.1%SDS prior to autoradiography. Before reprobing, blots were stripped with two rinses for 2 h at 70°C in 60% formamide, 50 mM Tris-HCl (pH 8.0), with 1% SDS, and stripping was verified by autoradiography.

J. TRz expression levels and RT/PCR amplifications

An RT/PCR protocol was used for assessing TRz expression levels and the extent of self-liberation in vivo. These utilized "inner" and "outer" primer pairs as previously described (Benedict, et al., 1998; Crone, et al., 1998). Briefly, the inner primer pair utilized sites located internal to the autocatalytic cleavage sites (that is, an upstream region just 3' to the 5' autocatalytic cleavage site, and a downstream sequence reverse

complementary to the region just 5' to the 3' autocatalytic cleavage site). This inner primer pair amplifies both unprocessed and processed (liberated ITRz) Rz transcripts. The outer primer pair utilizes sequences just external to the autocatalytic cleavage sites (i.e., sequence just 5' to the 5' autocatalytic cleavage site, and reverse complementary to the region just 3' to the 3' autocatalytic cleavage. The double-G mutants (which cannot undergo autocatalytic processing) were used to establish relative efficiencies of the primer pairs; the inner primer pair was 1.8X more efficient. The inner primer pair amplified a 69 nt product, whereas the outer primer pair amplified a 100 nt product. In some experiments, the 3' inner primer was used with the 5' outer primer for amplification of unprocessed TRz transcripts; results were analogous to those with the outer primer pair, producing an 86 nt product.

One of the primers was end-labeled with ^{32}P using T7 polynucleotide kinase, and 1.5×10^5 cpm was used in RT/PCR amplifications with 0.5 μg RNA. Following reactions (generally 25 cycles), products were separated by PAGE in 6% gels, and radioactivity in the bands was quantitated. To establish relative amounts of RNA used in the reactions, a 323 nt portion of 18S rRNA (nt 527-849) was amplified concurrently (15 cycles) and quantitated as above.

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References

Altschuler, M., Tritz, R., and Hampel, A. (**1992**) A method for generating transcripts with defined 5' and 3' termini by autolytic processing. **Gene** 122, 85-90.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (**1996**) **"Current Protocols in Molecular Biology."** John Wiley & Sons, New York.

Benedict, C., and Clawson, G. (**1996**) Nuclear multicatalytic proteinase subunit RRC3 is important for growth regulation in hepatocytes. **Biochemistry** 35, 11612-11621.

Benedict, C., Pan, W., Loy, S., and Clawson, G. (**1998**) Triple ribozyme-mediated down-regulation of the retinoblastoma gene. **Carcinogenesis** 19, 1223-1230.

Benedict, C., Ren, L., and Clawson, G. (**1995**) Nuclear multicatalytic proteinase _ subunit RRC3: differential size, tyrosine phosphorylation, and susceptibility to antisense oligonucleotide treatment. **Biochemistry** 34, 9587-9598.

Christoffersen, R., and Marr, J. (**1995**) Ribozymes as human therapeutic agents. **J. Med. Chem**. 38, 2023-2037.

Cotten, M., and Birnstiel, M. (**1989**) Ribozyme mediated destruction of RNA *in vivo*. **EMBO J.** 8, 3861-3866.

Crisell, P., Thompson, S., and James, W. (**1993**) Inhibition of HIV-1 replication by ribozymes that show poor activity *in vitro*. **Nucl. Acids Res**. 21, 5251-5255.

Crone, T., Schalles, S., Benedict, C., Pan, W., Ren, L., Loy, S., Isom, H., and Clawson, G. (**1998**) Growth Inhibition by a triple ribozyme targeted to repetitive B2 transcripts. *Submitted.*

Dzianott, A., and Bujarski, J. (**1989**) Derivation of an infectious viral RNA by autolytic cleavage of *in vitro* transcribed viral cDNAs. **Proc. Natl. Acad. Sci**.(USA) 86, 4823-4827.

Goodchild, J., and Kohli, V. (**1991**) Ribozymes that cleave an RNA sequence from human immunodeficiency virus: The effect of flanking sequence on rate. **Arch. Biochem. Biophys**. 284, 386-391.

Groebe, D., and Uhlenbeck, O. (**1988**) Characterization of RNA hairpin loop stability. **Nucleic Acids Res.** 16, 11725-11735.

Haseloff, J., and Gerlach, W. (**1988**). Simple RNA enzymes with new and highly specific endoribonuclease activities. **Nature** 334, 585-591.

Helene, C., and Toulme, J. (**1990**) Specific regulation of gene expression by antisense, sense, and antigene nucleic acids. **Biochim. Biophys. Acta** 1049, 99-125.

Hoke, G., Draper, K., Freier, S., Gonzalez, C., Driver, V., Zounes, M., and Ecker, D. (**1991**) Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization, and antiviral efficacy versus herpes simplex virus infection. **Nucleic Acids Res.** 19, 5743-5748.

Iverson, P., Zhu, S., Meyer, A., and Zon, G. (**1992**) Cellular uptake and subcellular distribution of phosphorothioate oligonucleotides into cultured cells. **Antisense Res. Devel**. 2, 211-222.

Izant, J., and Weintraub, H. (**1985**) Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA. **Science** 229, 345-352.

Jaeger, J., Turner, D., and Zuker, M. (**1989**) Improved predictions of secondary structures for RNA. **Proc. Natl. Acad. Sci. USA** 86, 7706-7710.

Kawasaki, H., Ohkawa, J., Tanishige, N., Yoshinari, K., Murata, T., Yokoyama, K., and Taira, K. (**1996**) Selection of the best target site for ribozyme-mediated cleavage within a fusion gene for Adenovirus E1A-associated 300 kDa protein (p300) and luciferase. **Nucleic Acids Res.** 24, 3010-3016.

Koizumi, M., Hayase, Y., Iwai, S., Kamiya, H., Inoue, H., and Ohtsuka, E. (**1989**) Design of RNA enzymes distinguishing a single base mutation in RNA. **Nucleic Acids Res**. 17, 7059-7071.

Krieg, A., Yi, A., Matson, S., Waldschmidt, T., Bishop, G., Tesdale, R., Koretzy, G., and Klinman, D. (**1995**) CpG motifs in bacterial DNA trigger direct B-cell activation. **Nature** 374, 546-549.

Lieber, A., and Strauss, M. (**1995**) Selection of efficient cleavage sites in target RNAs by using a ribozyme expression library. **Mol. Cell. Biol**. 15, 540-551.

Ohkawa, J., Koguma, T., Kohda, T., and Taira, K. (**1995**) Ribozymes: From mechanistic studies to applications in vivo. **J. Biochem**. 118, 251-258.

Ohkawa, J., Yuyama, N., and Taira, K. (**1992**) Activities of HIV-RNA targeted ribozymes transcribed from a 'shot-gun' type ribozyme-trimming plasmid. **Nucl. Acids Symp. Ser**. 27, 15-16.

Ohkawa, J., Yuyama, N., Takebe, Y., Nisikawa, S., Homann, M., Sczakiel, G., and Taira, K. (**1993**) Multiple site-specific cleavage of HIV RNA by transcribed ribozymes from shotgun-type trimming plasmid. **Nucleic Acids Symp. Ser**. 29, 121-122.

Parker, R., Muhlrad, D., Deshler, J., Taylor, N., and Rossi, J. (**1992**). *In* **"Gene Regulation: Biology of Antisense RNA and DNA"** (R. Erickson and J. Izant, eds.), pp. 55-70. Raven Press, New York.

Perreault, J., Wu, T., Cousineau, S., Ogilvie, K., and Cedergren, R. (**1990**) Mixed deoxyribo- and ribo-oligonucleotides with catalytic activity. **Nature** 344, 565-568.

Ruffner, D., Stormo, G., and Uhlenbeck, O. (**1990**) Sequence requirements of the hammerhead RNA self-cleavage reaction. **Biochemistry** 29, 10695-10702.

Ruiz, J., Wu, C., Ito, Y., and Wu, G. (**1997**) Design and preparation of a multimeric self-cleaving hammerhead ribozyme. **BioTechique**s 22, 338-345.

Stein, C., and Cheng, Y. (**1993**) Antisense oligonucleotides as therapeutic agents - is the bullet really magic? **Science** 261, 1004-1012.

Storey, A., Oates, D., Banks, L., Crawford, L., and Crook, T. (**1991**) Anti-sense phosphorothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16. **Nucleic Acids Res.** 19, 4109-4114.

Sullenger, B., and Cech, T. (**1993**) Tethering ribozymes to a retroviral packaging signal for destruction of viral RNA. **Science 262,** 1566-1569.

Taira, K., Nakagawa, K., Nishikawa, S., and Furukawa, K. (**1991**) Construction of a novel RNA-transcript-trimming plasmid which can be used both in vitro in place of run-off and (G)-free transcriptions and in vivo as multi-sequences transcription vectors. **Nucleic Acids Res.** 19, 5125-5130.

Wagner, R. (**1994**) Gene inhibition using antisense oligodeoxynucleotides. **Nature** 372, 333-335.

Woodworth, C., and Isom, H. (**1987**) Regulation of albumin gene expression in a series of rat hepatocyte cell lines immortalized by simian virus 40 and maintained in chemically defined medium. **Mol. Cell. Biol**. 7, 3740-3748.

Zamecnik, P., and Stephenson, M. (**1978**) Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. **Proc. Natl. Acad. Sci**. **USA** 75, 280-284.