J. Biochem. 123, 684-692 (1998)

Comparison of *In Vivo* Activities of 5′-Connected and 3′-Connected *cis*-Acting Ribozymes: Selection of Intracellularly Active Ribozymes Using the Gene for Dihydrofolate Reductase (DHFR) as a Selective Marker in *Escherichia coli*

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Received for publication, October 30, 1997

If ribozymes are to be exploited *in vivo*, it is necessary to select ribozymes that are functional in the intracellular environment. Ribozymes selected in the intracellular environment should retain their function *in vivo* as well as *in vitro*. We have devised a novel system for selection of active ribozymes from pools of active and inactive ribozymes using the gene for dihydrofolate reductase (DHFR) as a selective marker. In our first attempt, a sequence encoding either an active or an inactive ribozyme was connected upstream of the gene for DHFR. Each plasmid was designed such that, when the ribozyme was active, the ribozyme would cleave the target site and, as a result, the rate of production of DHFR would be high enough to endow resistance to trimethoprim (TMP). However, a critical defect may be associated with introduction of a ribozyme upstream of the DHFR gene because, during actual screening for active ribozymes on the 5′ side from a pool of random sequences, there is the danger of selecting sequences that are not related to the activity of ribozymes. Indeed, some upstream linker sequences affected the level of expression of the DHFR protein and, as a result, the resistance of *Escherichia coli* to TMP. Therefore, we newly constructed a 3′-connected ribozyme system, and activities *in vivo* of 5′-connected and 3′-connected ribozymes were compared. We found that the cleavage efficiencies *in vivo* were nearly identical for the two types of ribozyme, 24% for the 5′-side ribozyme and 23% for the 3′-side ribozyme, indicating that polysomes did not seem to inhibit the action of the 3′-connected ribozyme. In both cases, when cells were transformed with a 1 : 1 mixture of active and inactive ribozyme-coding plasmids, it was mainly the cells that harbored the active ribozyme that survived in the presence of TMP.

Key words: DHFR, hammerhead, *in vivo* selection, ribozyme, translation.

Catalytic RNAs, known collectively as ribozymes, were discovered in the early 1980s in the group I intron of *Tetrahymena* by Cech and as the RNA subunit of RNase P by Altman (*1, 2*). Various types of ribozyme have been identified, including group II introns; hammerhead, hairpin, and hepatitis delta virus ribozymes; and ribosomal RNA. Natural ribozymes have RNA-cleavage activity and exhibit high substrate specificity. Therefore, ribozymes (as well as antisense technologies) appear to have potential as tools for suppressing the expression of specific genes (*3-13*). They are expected to be useful in gene therapy for some diseases that are caused by the expression of abnormal mRNA, including diseases caused by infectious agents such as HIV (*4, 7, 9, 13, 14*). The hammerhead ribozyme belongs to the class of molecules known as antisense RNAs (hereafter, the term ribozyme refers exclusively to hammerhead ribozymes unless otherwise noted). However, because of the short extra sequences that form the so-called catalytic loop capturing metal ions, it can act as a metalloenzyme (*15-24*). Since the substrate specificity of ribozyme molecules is high, ribozyme strategies seem likely to have some value as therapeutic agents (*6, 8, 25*).

When the hammerhead ribozyme was engineered such that it could cleave specific RNA sequences "in *trans*" (*26, 27*), it was postulated that this ribozyme might be much more effective than simple antisense molecules in several respects (*26-30*). However, because the activity and stability of ribozymes are highly dependent on the cellular environment (*31*), ribozymes have not yet proven their significant superiority to antisense molecules. There seem to be several reasons for the low activity of ribozymes *in*

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vivo. (i) Various cellular proteins might exist *in vivo* that inhibit their catalytic activity (*32, 33*). (ii) The intracellular concentration of Mg^{2+} ions is much lower than that used *in vitro* in assays of ribozyme activity (*34-36*). (iii) Several cellular RNases contribute to the instability of ribozymes (*7, 37-40*). (iv) Unlike certain proteinaceous enzymes (*41*), it seems unlikely that ribozymes reach their target sites by a sliding mechanism. Many attempts have been made to overcome some of these problems, for example, by chemical modification and substitution of nucleotides to improve the stability and activity of ribozymes (*37-40, 42*). Selection *in vitro* of functional nucleic acids is also of considerable current interest (*43-45*). This method has successfully been used to engineer specific ribozymes (*46-51*). New functional ribozymes with ligase, kinase, amino-acid cleavage, or self-alkylating activities have already been selected by this method (*52-55*). It might also be possible to select very active ribozymes using this method. However, a functional ribozyme selected *in vitro* might not be the best ribozyme in the cellular environment, in which there are potential inhibitory factors, a limited concentration of mandatory Mg^{2+} ions, and so on (*56, 57*).

If ribozymes are to be exploited *in vivo*, it is necessary to select ribozymes that are functional in the intracellular environment. Ribozymes selected in the intracellular environment should retain their function *in vivo* as well as *in vitro*. To this end, we attempted to construct a positive selection system *in vivo* by connecting genes for a toxin and a ribozyme in tandem. When a toxin is expressed, cells harboring the gene for the toxin should be killed. If mRNA for the toxin can be successfully cleaved by the ribozyme that is co-expressed with the mRNA for the toxin, then cells harboring active ribozymes should survive and should form colonies. Consequently, all surviving colonies should harbor information about the sequences of active ribozymes. In our first attempt, we selected the gene for RNase T_1 as the gene for the toxin. However, despite considerable effort, we failed to generate any plasmids that encoded both genes for a toxin and a ribozyme that were connected in tandem. No constructs with a gene for RNase T_1 were rescued from transformed *Escherichia coli* cells. Only frame-shifted constructs, with aborted production of RNase T_1, were rescued. In this first attempt, we were unable to control the extent of the toxicity of RNase T_1.

We next chose a potentially more controllable gene as a selective marker, namely, the gene for dihydrofolate reductase (DHFR) (*58*). DHFR is an essential proteinaceous enzyme in the pathway to thymidylic acid (*59*). Because the synthesis of DNA is required by all proliferating cells, inhibition of this process is one of the most effective ways of controlling cell division. Several drugs, such as methotrexate (MTX) and trimethoprim (TMP), are potent inhibitors of DHFR and, consequently, they inhibit DNA synthesis and the multiplication of cells (*59-63*). We designed our vector such that the level of expression of DHFR would be high when a ribozyme successfully cleaved its target site. Thus, our method involves positive selection and operates as follows. When an inhibitor of DHFR, such as TMP, is present in the culture medium at a certain concentration, DHFR-producing clones, which have already been transfected by a DHFR expression vector, would be expected to survive and grow more rapidly than non-expressing clones. When the level of expression of DHFR exceeds the inhibitory capacity of TMP, *E. coli* cells can proliferate on TMP-containing plates. Furthermore, we can regulate the toxicity of TMP by changing its concentration. Therefore, if we can control the level of expression of the gene for DHFR, which depends on the activity of a ribozyme, we should be able to select ribozymes, that are active in the cellular environment by monitoring resistance to TMP (*58*).

In our previous study, a ribozyme-encoding sequence was placed upstream of the gene for DHFR for the following reasons (*58*). In the case of prokaryotes such as *E. coli*, transcription is coupled with translation so that, if the target RNA had been transcribed prior to transcription of the ribozyme, there would be less of a chance that the ribozyme would cleave the target site. Moreover, polysomes could protect a target site that was located downstream of a strong SD sequence from attack by ribozymes. Therefore, we placed the ribozyme upstream of its target site simply to allow transcription of the ribozyme prior to the transcription of the target site and before its protection by polysomes.

In this study, we newly constructed a 3′-connected ribozyme system and compared its activities with those of the 5′-side ribozyme to examine whether the cleavage efficiency of the 3′-side ribozyme might be affected by the potential protection by polysomes.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—E. coli HB101 (*rec*A13, *sup*E44; Takara Shuzo, Kyoto) was used as the recipient for transformation. Several ribozyme expression vectors were constructed by modifying the DHFR expression vector pTZDHFR20 (*64*).

Synthesis of Oligonucleotides and Construction of Plasmids—The oligodeoxynucleotides were synthesized with a DNA synthesizer (model 392; Applied Biosystems, Foster City, CA) and purified by chromatography on OPC columns (oligonucleotide purification columns; Applied Biosystems). The construction of 3′-connected ribozyme expression vectors was based on the previously prepared 5′-side vectors (*58*). We synthesized and purified six kinds of oligodeoxynucleotide for construction of 3′-connected ribozyme expression vectors [primers for PCR for copying the 5′-connected active ribozyme (forward, 5′-AGA CGT ATC TCG AGC GTC GTT AAA ACT AAT TGA ATT CCT GAT GAG TCC-3′; and reverse, 5′-GCG TAC GTG GAT CCT AAA AAA AAA AGC CCG CTC ATT AGG CGG GCT TTA GTT AGT TAG TCC ATG GTT TCG TCC CTA-3′), primers for PCR for copying the 5′-connected inactive ribozyme (forward, 5′-AGA CGT ATC TCG AGC GTC GTT AAA ACT AAT TGA ATT CCT AAT GAG TCC-3′; and reverse, 5′-GCG TAC GTG GAT CCT AAA AAA AAA AGC CCG CTC ATT AGG CGG GCT TTA GTT AGT TAG TCC ATG GTT TCG TCC CTA-3′), and linkers for the replacement of the 5′-connected ribozyme (forward, 5′-CCG GAG TCA TGG TAG CAA GGT TTC CGC AAA ATT GTT CGT GAC CAT CAC ATA ACC TAG CGG ACA-3′; and reverse, 5′-AGC TTG TCC GCT AGG TTA TGT GAT AAT CAC GAA CAA TTT TGC GGA AAC CTT GCT ACC ATG ACT-3′). A single base change (G^5 to A^5) was introduced within the catalytic core of the active ribozyme (Fig.

1). These changes had been shown previously to destroy cleavage activity (*30, 65*).

The primers for PCR were complementary to the upstream region and downstream region of the ribozyme and were tailed with a recognition sequence for restriction endonuclease *Xho*I in the case of the forward primer and for *Bam*HI in the case of the reverse primer. Linkers instead of a ribozyme were tailed with a recognition sequence for restriction endonuclease *Hin*dIII at the 5′ end and for *Acc*III at the 3′ end. Each oligonucleotide linker was denatured at 95°C in a water-bath, then gradually cooled to room temperature in TE buffer. After annealing, each linker set was ligated to the appropriately digested vector. For the construction of 3′-connected ribozyme expression vectors, the region that contained 5′-connected ribozymes was cut out from 5′-connected ribozyme vectors by restriction enzymes *Hin*dIII and *Acc*III, and a linker was ligated to the digested vector, replacing the ribozyme portion (Fig. 2). DNA fragments containing 5′-connected ribozyme sequences and restriction sites (*Xho*I and *Bam*HI) were amplified by PCR and were cleaved at the restriction sites by *Xho*I and *Bam*HI. These fragments were ligated to the digested ribozyme-free vector *via* the *Xho*I and *Bam*HI restriction sites.

Composition of Culture Media—Plates of LB-modified (LBM) medium, containing polypeptone, yeast extract, NaCl, and 16 mM $MgSO_4$, were used for experiments to check the growth rates of individual clones. For incubation of transformed *E. coli* cells on LBM plates, the medium was supplemented with ampicillin (100 μg/ml) and/or TMP (130–140 μg/ml).

Northern Blotting Analysis—Plasmid vector pMH-3′-RIB, harboring both a 3′ ribozyme and a gene for DHFR, was used to transform *E. coli* HB101. After overnight incubation at 37°C, total RNA was isolated with ISOGEN™ (Nippon gene, Toyama) from 2 ml of a culture of cells in 2× YT medium. Ten micrograms of total RNA per sample were denatured in glyoxal and dimethyl sulfoxide, subjected to electrophoresis in 2.5% NuSieve (3 : 1)™ agarose gel (FMC, Rockland), and transferred to a Hybond-N™ nylon membrane (Amersham, Buckinghamshire). The membrane was probed with a synthetic oligonucleotide (5′-ATT CGC TGA ATA CCG ATT CCC AGT CAT CCG GCT CGT AAT C-3′; complementary to DHFR mRNA) that had been labeled with ^{32}P by use of T4 polynucleotide kinase (Takara Shuzo, Kyoto). Prehybridization and hybridization were performed in the same solution (5× SSPE, 50% formamide, 5× Denhardt's solution, 0.5% SDS, 150 mg/ml calf thymus DNA). Final washing was performed in 0.1× SSPE, 0.1% SDS at 70°C for 30 min.

RESULTS AND DISCUSSION

Design and Construction of the Screening Vector with a 3′-Connected Ribozyme—In our original construct, as mentioned above, the ribozyme sequences were inserted on the 5′ side of the gene for DHFR so that the ribozyme would be transcribed upstream of the target site of the ribozyme. In this screening system in *E. coli*, we attempted to distinguish between two vectors, one containing an active ribozyme and one containing an inactive ribozyme as a result of a single base substitution (Fig. 1A). The active ribozyme sequence was the same as that of the wild-type hammerhead ribozyme, and the inactive ribozyme sequence differed from the active ribozyme by a single G^5 to A^5 mutation within the catalytic core of the ribozyme (Fig. 1A). These mutations completely abolish the activity of the ribozyme (*30, 65*). During actual screening for active ribozymes on the 5′ side from a pool of random sequences, however, there is the danger of selecting sequences that are not related to the activity of ribozymes. Such sequences might include sequences that regulate transcription, for example, promoter sequences and anti-terminators, or sequences that yield tertiary structures that promote reinitiation among others. If such sequences were selected from the random pool, they might affect the level of expression of the DHFR protein and, as a result, the resistance of *E. coli* to TMP. To avoid these possibilities, we must place the ribozyme downstream of the DHFR gene. If the activity of the 3′-side ribozyme were as high as that of the 5′-side ribozyme, there would clearly be an advantage to using the 3′-side ribozyme because accidental selection of the above-mentioned regulatory sequences would be avoided. Our preliminary data indicate that some 3′ ribozymes are more effective than the corresponding 5′ ribozymes in some eukaryotic cells (Ohkawa and Taira, unpublished results). In eukaryotic cells, after mRNA has been transcribed in the nucleoplasm, the mRNA moves to cytoplasm and is translated into protein there. Thus, in eukaryotic cells, unlike in prokaryotic cells, ribozymes might have a better chance of encountering their target site since transcription and translation are not coupled. At any rate, we felt that it was worth examining 3′-side ribozymes in prokaryotic cells also to determine whether we could achieve the same or greater selective power than that

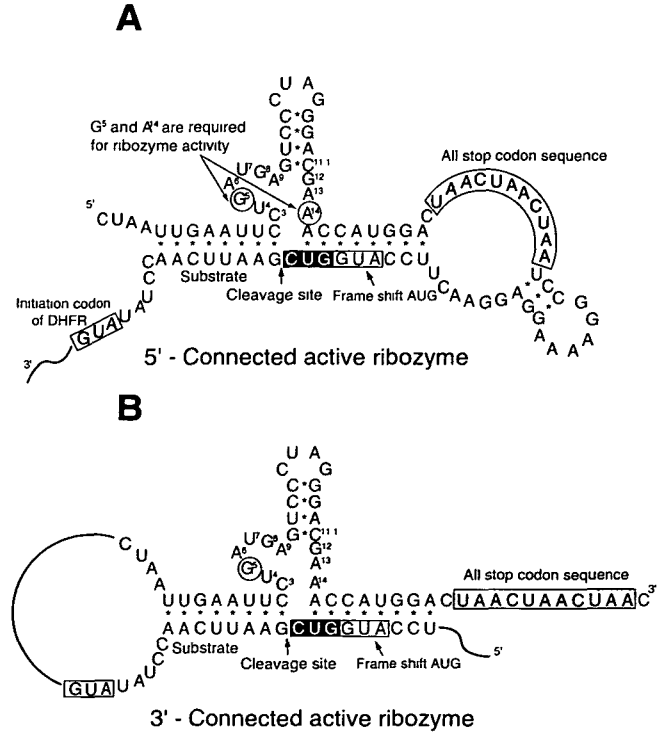

Fig. 1. **Secondary structures of the 5′-connected ribozyme (A) and the 3′-connected ribozyme (B).** A single point mutation (G^5 to A^5 or A^{14} to G^{14}; circled) eliminates the ribozyme activity (*65*).

obtained with 5′-side ribozymes.

We constructed 3′-connected ribozyme expression vectors containing either an active ribozyme or an inactive ribozyme sequence (Fig. 1B). These vectors were based on the 5′-connected ribozyme expression vectors (Fig. 2). The ribozyme region and the "all stop codon" between the promoter and the SD sequences of the 5′-connected ribozyme expression vector were cut out and then inserted between the DHFR gene and the terminator sequence. The "all stop codon" was transferred with the ribozyme region only because of the convenience of manipulation. In order to maintain the same distance between the promoter and the strong SD sequence in the two kinds of construct (5′-connected and 3′-connected ribozyme vectors), we replaced the *Hin*dIII-*Acc*III region by a linker with the same length in nucleotides as the corresponding region containing the ribozyme and the "all stop codon." As a result, the final 3′-connected ribozyme expression vector pMH-3′-RIB (bottom construct in Fig. 2) contained, from the upstream to the downstream region, the promoter, the linker, the strong SD sequence, the pseudo-initiation codon, the ribozyme target site, the original initiation codon for the DHFR gene, the DHFR gene, the ribozyme-coding region, and the terminator. The inactive ribozyme sequence differed from the active one by a single G^5 to A^5 mutation within the catalytic core of the ribozyme, as in the 5′-side ribozyme construct (Fig. 1B). The target site of the 3′-connected ribozyme was exactly the same as that of the 5′-connected ribozyme (Fig. 3).

If the ribozyme were targeted to the gene for DHFR itself, the growth of cells that had been transformed by the vector with the active ribozyme should be slower in the presence of inhibitors of DHFR, such as trimethoprim (TMP) and methotrexate (MTX), because of a lower rate of production of the essential enzyme DHFR. Clones surviving in the presence of TMP or MTX would turn out to have a vector with an inactive ribozyme sequence: in other words, this method corresponds to negative selection. We need a positive selection method to find active ribozyme sequences. Therefore, we took advantage of a frame shift in the AUG codon. We introduced, from the upstream to the downstream direction, an efficient Shine-Dalgarno (SD) sequence, a frame shift initiation codon that was out of frame relative to the gene for DHFR, a target site for the ribozyme and the correct initiation codon for the gene for DHFR. In our vectors, the ribozyme was not targeted to the gene for DHFR itself but to the region between the two AUG codons (Fig. 1), of which one was the original initiation codon of the DHFR gene itself and the second (the pseudo-initiation codon) was located upstream of the original initiation codon to introduce a frame shift (Fig. 3). The second frame-shifted AUG triplet was associated with a strong SD sequence. If the ribozyme failed to cleave the target site, a ribosome would be expected to associate with

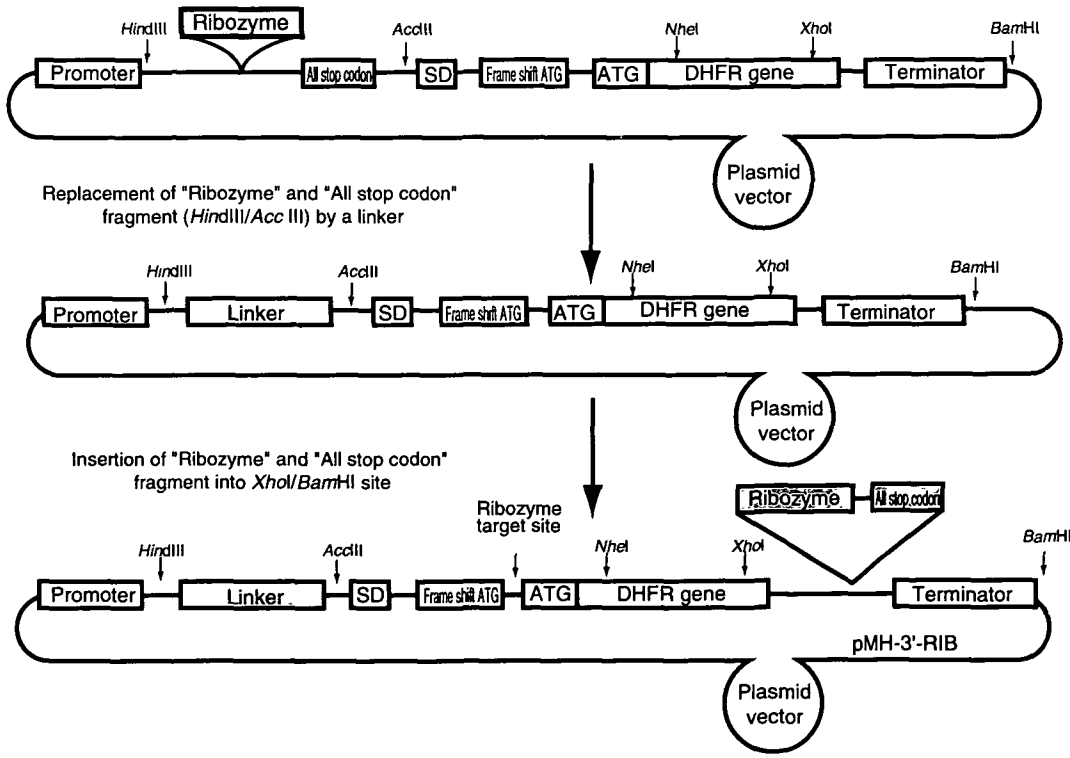

Fig. 2. **Construction of the 3′-connected ribozyme expression vector, pMH-3′-RIB.** The ribozyme region and the "all stop codon" between the promoter and SD sequences in the 5′-connected ribozyme expression vector (top) were cut out and then inserted between the gene for DHFR and the terminator sequence (bottom). In order to maintain the same distance between the promoter and the strong SD sequence in the two kinds of construct (5′-connected and 3′-connected ribozyme vectors), we replaced the *Hin*dIII-*Acc*III region by a linker with the same length in nucleotides as the corresponding region containing the ribozyme and the "all stop codon." The inactive ribozyme sequence differed from the active sequence by a single G^5 to A^5 mutation within the catalytic core of the ribozyme, as in the 5′-side ribozyme construct (Fig. 1). The target site of the 3′-connected ribozyme was the same as that of the 5′-connected ribozyme (see Fig. 3).

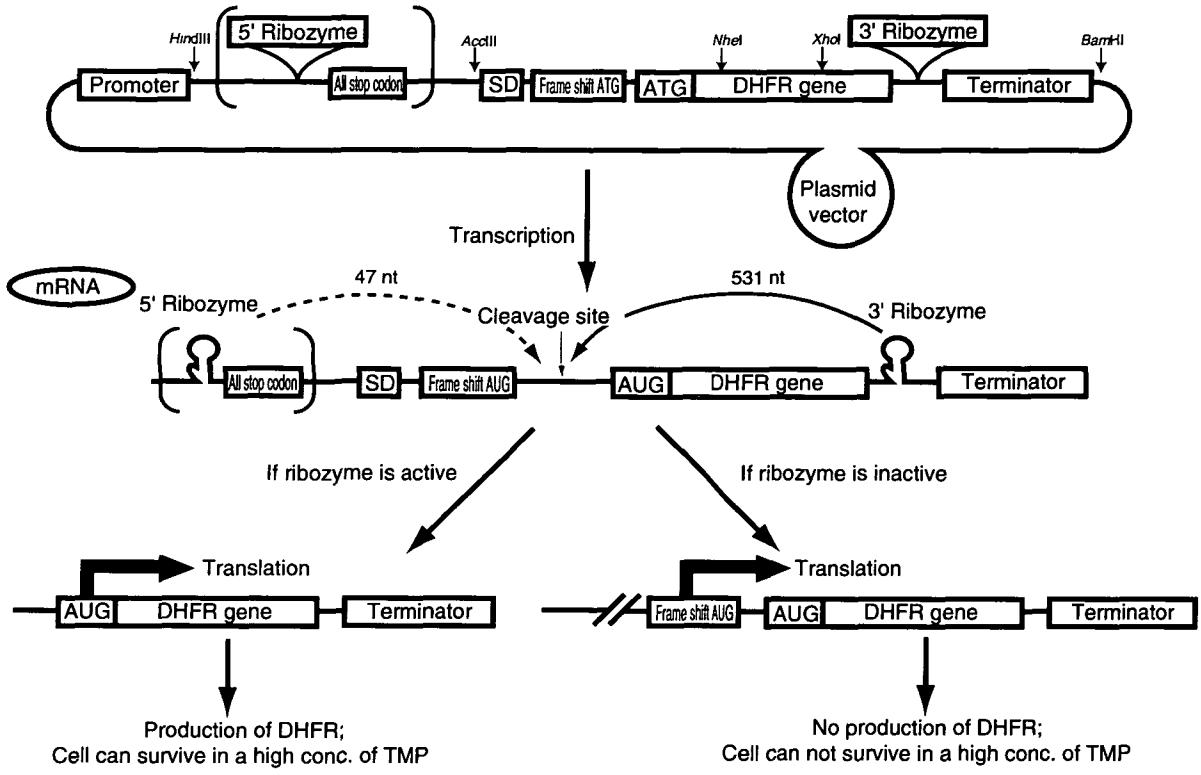

Fig. 3. **The [5′-connected] or 3′-connected ribozyme expression vector.** The plasmid vector has two ATG codons, one of which is a pseudo-initiation codon, located upstream of the authentic ATG codon, which is the initiation codon for the DHFR gene. If, upon transcription, the primary transcript is cleaved by the *cis*-acting ribozyme at the predetermined site between the two AUG codons, the excised mRNA can produce DHFR. Otherwise, the translation of the primary transcript starts at the pseudo-initiation codon, which is associated with a strong Shine-Dargano sequence and is out of frame with respect to the DHFR gene. The distance between the ribozyme and the cleavage site is 47 nucleotides (nt) for the 5′ ribozyme and 531 nt for the 3′ ribozyme.

the strong SD sequence for the frame-shifted AUG and the subsequent translation would not produce DHFR. However, when the ribozyme cleaves the target site, the strong SD sequence and frame-shifted initiation codon are disconnected and a weak SD sequence associated with the correct initiation codon for DHFR within the DHFR mRNA becomes operational, with resultant production of DHFR (Fig. 3).

Furthermore, we introduced a terminator sequence (*66, 67*) downstream of the DHFR gene to facilitate the analysis of transcripts. If the active ribozyme were to attack the target site and cleave the primary transcript, we should be able to detect cleaved transcripts by Northern blotting analysis. In the case of the 5′-connected ribozymes, it was necessary to avoid any readthrough from upstream regions and, therefore, the "all stop codon" sequence (TAACTAACTAA) had been introduced between the ribozyme and the strong SD sequence. In this region, three stop codons should terminate translation in all possible frames (*58*).

Discrimination of Active Ribozymes from Inactive Ribozymes, Connected on the 3′ Side of the DHFR Gene, in the Presence of TMP—The newly constructed 3′-connected ribozyme was then examined by optimizing the level of discrimination between active and inactive constructs as a function of the concentration of TMP, taking advantage of the direct relationship between the level of expression of DHFR and the strength of resistance to TMP (*61*). Figure 4 shows the difference in growth rates between active and inactive ribozyme-expressing colonies at 27°C at 130 μg of TMP per ml of culture medium. *E. coli* cells that had been transformed with the active ribozyme-expressing vector grew more rapidly and made larger colonies than the cells that had been transformed with the inactive ribozyme-expressing vector, as we had observed previously with the 5′-connected ribozyme construct (*58*). For some unknown reason, cells harboring the C^5-inactive ribozyme vector grew more rapidly than cells with the other inactive ribozyme vector. Of the concentrations of TMP tested, we found that the difference in colony size between active ribozyme- and inactive ribozyme-expressing clones was greatest in the range of 125-140 μg per ml of culture medium. This range is higher than the 70 μg of TMP per ml of culture medium used in the assay with the 5′ construct (*58*). The increased resistance to TMP might have originated from an increased level of the transcript (see the next section) and a higher rate of production of DHFR. The level of mRNA might have changed since the sequence of the *Hin*dIII/*Acc*III region strongly influenced the rate of transcription, as confirmed in experiments with different kinds of linker (data not shown). We found that the shorter was the linker, the higher was the level of the transcription. This clearly demonstrates the defect associated with introduction of random sequences upstream of the DHFR gene.

We then carried out a random screening assay for the 3′ ribozyme construct, following the procedure used for the 5′ ribozyme construct (see Fig. 4 in Ref. *58*). *E. coli* cells

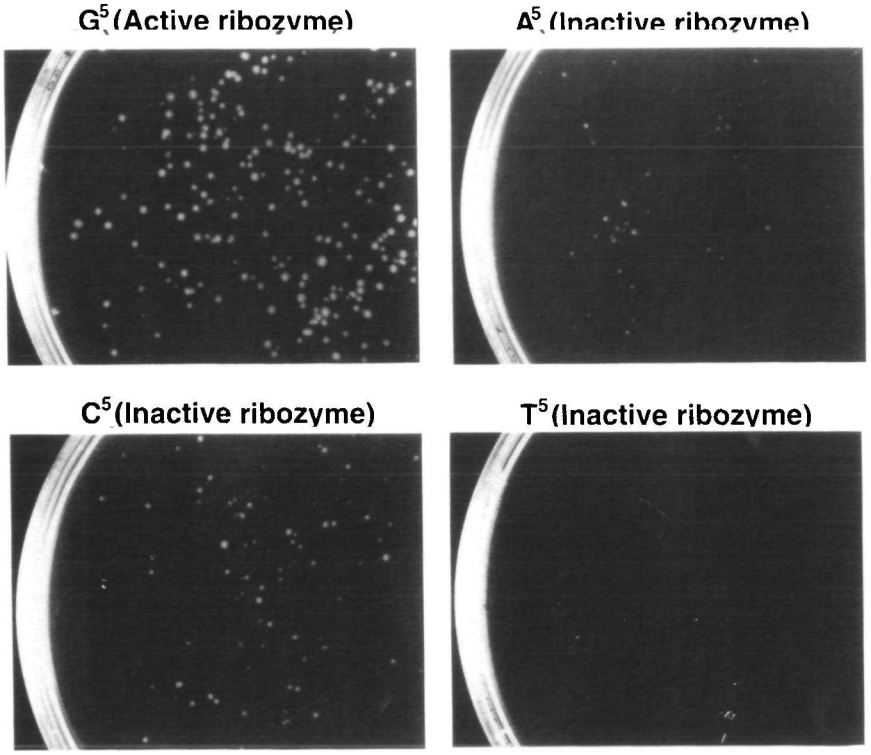

Fig. 4. **Colonies of *E. coli* HB101 cells that had been transformed with the 3′-connected ribozyme expression plasmid.** G^5 (Active ribozyme): Active ribozyme. A^5 (Inactive ribozyme): Inactive ribozyme with G^5 replaced by A. C^5 (Inactive ribozyme): Inactive ribozyme with G^5 replaced by C. T^5 (Inactive ribozyme): Inactive ribozyme with G^5 replaced by U. Competent cells (100 μl) were transformed with 50 ng of plasmids that encoded either an active or an inactive ribozyme, then plated on a plate containing either ampicillin (100 μg/ml) alone or both ampicillin (100 μg/ml) and TMP (130 μg/ml). The same numbers and sizes of colonies were detected on 2 plates containing ampicillin (100 μg/ml) alone, for both an active and inactive ribozyme-coding plasmids (data not shown). By contrast, as shown in this figure, in the presence of 130 μg/ml TMP, colonies expressing the active ribozyme grew more rapidly than colonies that expressed inactive ribozymes.

TABLE I. **Numbers of selected colonies with 5′-connected and 3′-connected active and inactive ribozymes on trimethoprim-containing and/or ampicillin-containing plates.** Plates were incubated at 27°C for 2 to 3 days, and then larger colonies were picked up at random. Trimethoprim plates contained 70 μg of TMP and 100 μg of Amp per ml for 5′-connected ribozyme screening, and 130, 133, or 140 μg of TMP and 100 μg of Amp per ml for 3′-connected ribozyme screening. Ampicillin plates contained 100 μg Amp per ml without TMP. The results for the 5′-connected ribozyme are cited from Ref. *58* for the purpose of comparison.

"G^5 and A^5" mixture	5′-Connected ribozyme		3′-Connected ribozyme			
	Ampicillin	Trimethoprim (70 μg/ml)	Ampicillin	Trimethoprim (130 μg/ml)	Trimethoprim (133 μg/ml)	Trimethoprim (140 μg/ml)
Active ribozyme	29	75	8	9	7	27
Inactive ribozyme	28	1	11	0	2	3

transformed with a mixture of active and inactive ribozyme-coding plasmids in a molar ratio of 1 : 1 were plated on a plate containing both ampicillin (100 μg/ml) and TMP (125–140 μg/ml). Faster growing colonies were picked up at random from the plate and ribozyme sequences were confirmed. The results are shown in Table I, together with the previously obtained results with the 5′ ribozyme construct (*58*). For some unknown reason, the level of background colonies was very sensitive to the concentration of TMP, and the reproducibility was lower with the 3′ ribozyme construct than with the 5′ ribozyme construct. In general, selection was better when freshly prepared TMP was used. Nevertheless, we did achieve limited success even though we could not eliminate the background colonies (Table I).

Detection by Northern Blotting Analysis of a Fragment Cleaved by the 3′-Connected Ribozyme—To confirm that the above-described phenotypic differences were associated with the cleavage activity of the ribozyme, we performed Northern blotting analysis with total RNA from *E. coli* HB101 cells that had been transfected with the ribozyme expression vectors. Northern blotting analysis is the most direct method for identification of cleavage activities of ribozymes *in vivo*. However, since cleaved fragments tend to undergo rapid degradation *in vivo*, Northern blotting analysis has failed in the past to detect some cleaved fragments (*68, 69*). To confirm that the 3′-side active ribozyme cleaved the target site *in vivo* and that the phenotype reflected the ribozyme's cleavage activity, as well as to compare the efficiency of cleavage between the 5′-side and 3′-side ribozymes, we performed Northern blotting analysis for both types of construct under identical conditions (Fig. 5). As mentioned above, the level of the transcript was higher when the 3′-side ribozyme was used than with the 5′-side ribozyme (lanes 3 and 4). The 3′ transcripts (lanes 3 and 4) are larger than the 5′ transcripts (lanes 1 and 2) because they contain the 3′-ribozyme portion in addition to the gene for DHFR (Fig. 3). As indicated in lanes 2 and 4, both 5′- and 3′-connected ribozymes recognized and cleaved specifically the target site. Although the inactive ribozyme lanes (lanes 1 and 3) appear to show a weak signal at the size of the cleaved fragment, this has been proven to be an artifact signal due to the design of the probe since another primer used for

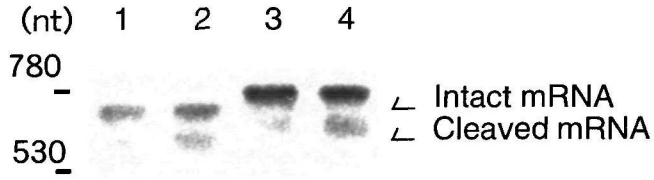

Fig. 5. **Northern blotting analysis (Pictrographic printout) for comparison of the cleavage efficiencies of the 5′-connected and 3′-connected ribozymes.** Ten micrograms of total RNA from *E. coli* cells transformed with the 5′-connected or 3′-connected ribozyme expression vector were subjected to electrophoresis in a 2.5% NuSieve (3:1)™ agarose gel. After transfer to a membrane filter, the RNA was allowed to hybridize with a synthetic oligonucleotide probe (40-mer) that was complementary to part of the gene for DHFR. Lane 1: 5′-connected inactive ribozyme, with A^5 at the catalytic core. Lane 2: 5′-connected active ribozyme with G^5 at the catalytic core. Lane 3: 3′-connected inactive ribozyme, with A^5 at the catalytic core. Lane 4: 3′-connected active ribozyme with G^5 at the catalytic core. Both active ribozyme expression vectors produced the excised short fragment (lanes 2 and 4), but no such fragment was produced by inactive ribozymes (lanes 1 and 3). The extent of cleavage was determined by quantitation of radioactivity in bands of the initial transcript and the cleaved fragment with a Bio-Image Analyzer (BAS 2000, Fuji Film). The RNA marker used was 0.16-1.77 kb RNA ladder (GIBCO BRL). Cleavage efficiencies were the same in lanes 2 and 4.

primer extension analysis identified only a single cleavage product of the expected size (*58*). Most importantly, the cleavage efficiencies were nearly identical for the two types of ribozyme, 24% for the 5′-side ribozyme and 23% for the 3′-side ribozyme. (The extent of cleavage was determined by quantitation of radioactivity in bands of the initial transcript and the cleaved fragment with a Bio-Image Analyzer.) It is noteworthy that polysomes did not seem to inhibit the action of the 3′-connected ribozyme in our specific construct.

That we able to detect the cleaved fragments, while others have failed, is probably because, in our case, the target site of the ribozyme was located upstream of the DHFR gene (Fig. 3), and the DHFR mRNA itself remained intact before and after the ribozyme-mediated cleavage. Protection (by the binding of ribosomes, *etc.*) from digestion by RNases, which must be an intrinsic property of the sequence of DHFR mRNA, allowed the mRNA to remain unchanged after the ribozyme-catalyzed cleavage.

Concluding Remarks—Successful selection *in vitro* of tailored RNA has been reported by others and is of considerable current interest (*12, 46-51*). However, efforts to construct selection systems *in vivo* have met with only limited success (*70*). For use of ribozymes *in vivo*, we need RNAs that function optimally in the intracellular environment. To this end, we attempted to construct a positive selection system *in vivo* based on a controllable gene as a selective marker, namely, the gene for DHFR. Our previous analysis of the 5′-side ribozyme confirmed the possibility of selecting active ribozymes *in vivo* with DHFR as a selective marker. However, we later found that different linker sequences influenced differently the levels of transcription and translation when the 5′ ribozyme construct was used. In order to avoid problems associated with changes in levels of translation that are not related to the function of the ribozyme, we constructed the 3′-connected ribozyme vectors, in this report, and compared the trimming activities of the two types of ribozyme. Compared with the 5′ construct, the background noise in the case of the 3′-connected ribozyme could not be reduced (in fact, the noise appears to be higher with the 3′ construct), even though, for the most part, active ribozymes could also be selected in the presence of TMP (Table I). In its present form, the method for selection of an active mutant ribozyme by the 3′ construct from a completely randomized large pool is inadequate since the significant background noise would easily obscure identification of an active mutant from a large pool of inactive molecules. Nevertheless, we gained useful information from this study regarding the efficiency of cleavage *in vivo* by the 3′ ribozyme under the conditions of potential inhibition by polysomes (*71*).

Northern blotting analysis revealed that the efficiencies of ribozyme-mediated cleavage *in vivo* were identical for 5′- and 3′-connected ribozymes (Fig. 5), despite the fact that, in the case of the 3′ construct, the target site had been transcribed prior to the ribozyme and the possibility existed of polysome-mediated protection against ribozymes. We should also emphasize that the distance between the ribozyme and the cleavage site is 47 nucleotides (nt) for the 5′ ribozyme and 531 nt for the 3′ ribozyme (Fig. 3) and, therefore, the *cis*-acting ribozyme activity for the 3′ ribozyme appeared less favorable than for the 5′ ribozyme. Although the background noise could not be reduced by placing the ribozyme on the 3′ side, it might be advantageous to improve the 3′ ribozyme construct rather than the 5′ construct if selections are to be made with a large pool of completely randomized RNA. In the case of the 3′-connected ribozymes, we can at least minimize effects on levels of transcription and translation. Efforts are underway in our laboratory to reduce the background noise by examining genes other than that for DHFR by the use of 3′-connected ribozymes.

REFERENCES

1. Cech, T.R., Zang, A.J., and Grabowski, P.J. (1981) *In vitro* splicing of the ribosomal RNA precursor of *Tetrahymena*: involvement of a guanosine nucleotide in the excision of the intervening sequence. *Cell* **27**, 487-496
2. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* **35**, 849-857
3. Cameron, F.H. and Jennings, P.A. (1989) Specific gene suppression by engineered ribozymes in monkey cells. *Proc. Natl. Acad. Sci. USA* **86**, 9139-9143
4. Sarver, N., Cantin, E.M., Chang, P.S., Zaia, J.A., Ladne, P.A., Stephens, D.A., and Rossi, J.J. (1990) Ribozymes as potential anti-HIV-1 therapeutic agents. *Science* **247**, 1222-1225
5. Uhlmann, E. and Peyman, A. (1990) On antisense oligonucleotides including synthesis, properties, control of gene expression, toxicity, and application. *Chem. Rev.* **90**, 544-584
6. Erickson, R.P. and Izant, J.G., eds. (1992) *Gene Regulation*, Biology of Antisense RNA and DNA, Raven Press, New York
7. Heidenreich, O. and Eckstein, F. (1992) Hammerhead ribozyme-mediated cleavage of the long terminal repeat RNA of human immunodeficiency virus type 1. *J. Biol. Chem.* **267**, 1904-1909
8. Murray, J.A.H., ed. (1992) *Antisense RNA and DNA*, Wiley-Liss, New York
9. Ojwang, J.O., Hampel, A., Looney, D.J., Wong-Staal, F., and Rappaport, J. (1992) Inhibition of human immunodeficiency virus type 1 expression by a hairpin ribozyme. *Proc. Natl. Acad.*

Sci. USA **89**, 10802-10806
10. Rossi, J.J. (1992) Ribozymes. *Curr. Opin. Biotechnol.* **3**, 3-7
11. Altman, S. (1993) RNA enzyme-directed gene therapy. *Proc. Natl. Acad. Sci. USA* **90**, 10898-10900
12. Bratty, J., Chartrand, P., Ferbeyre, G., and Cedergren, R. (1993) The hammerhead RNA domain, a model ribozyme. *Biochim. Biophys. Acta* **1216**, 345-359
13. Ohkawa, J., Yuyama, N., Takebe, Y., Nishikawa, S., and Taira, K. (1993) Importance of independence in ribozyme reactions: kinetic behavior of trimmed and of simply connected multiple ribozymes with potential activity against human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **90**, 11302-11306
14. Leavitt, M.C., Yu, M., Yamada, O., Kraus, G., Looney, D., Poeschla, E., and Wong-Staal, F. (1994) Transfer of an anti-HIV-1 ribozyme gene into primary human lymphocytes. *Hum. Gene Ther.* **5**, 1115-1120
15. Dahm, S.C., Derrick, W.B., and Uhlenbeck, O.C. (1993) Evidence for the role of solvated metal hydroxide in the hammerhead cleavage mechanism. *Biochemistry* **32**, 13040-13045
16. Pyle, A.M. (1993) Ribozymes: a distinct class of metalloenzymes. *Science* **261**, 709-714
17. Uebayasi, M., Uchimaru, T., Koguma, T., Sawata, S., Shimayama, T., and Taira, K. (1994) Theoretical and experimental considerations on the hammerhead ribozyme reactions-divalent magnesium ion-mediated cleavage of phosphorus-oxygen bonds. *J. Org. Chem.* **59**, 7414-7420
18. Sawata, S., Komiyama, M., and Taira, K. (1995) Kinetic evidence based on solvent isotope effects for the nonexistence of a proton-transfer process in reactions catalyzed by a hammerhead ribozyme: implication to the double-metal-ion mechanism of catalysis. *J. Am. Chem. Soc.* **117**, 2357-2358
19. Amontov, S.V. and Taira, K. (1996) Hammerhead minizymes with high cleavage activity: a dimeric structure as the active conformation of minizymes. *J. Am. Chem. Soc.* **118**, 1624-1628
20. Kumar, P.K.R., Zhou, D.-M., Yoshinari, K., and Taira, K. (1996) Mechanistic studies on hammerhead ribozymes in *Catalytic RNA* (Eckstein, F. and Lilley, D.M.J., eds.) Nucleic Acids and Molecular Biology, Vol. 10, pp. 217-230, Springer-Verlag, Berlin
21. Orita, M., Vinayak, R., Andrus, A., Warashina, M., Chiba, A., Kaniwa, H., Nishikawa, F., Nishikawa, S., and Taira, K. (1996) Magnesium-mediated conversion of an inactive form of a hammerhead ribozyme to an active complex with its substrate. An investigation by NMR spectroscopy. *J. Biol. Chem.* **271**, 9447-9454
22. Zhou, D.-M., Kumar, P.K.R., Zhang, L.-H., and Taira, K. (1996) Ribozyme mechanism revisited-evidence against direct coordination of Mg^{2+} ion with the Pro-R oxygen of the scissile phosphate in the transition state of a hammerhead ribozyme-catalyzed reaction. *J. Am. Chem. Soc.* **118**, 8969-8970
23. Zhou, D.-M., Usman, N., Wincott, F.E., Matulic-Adamic, J., Orita, M., Zhang, L.-H., Komiyama, M., Kumar, P.K.R., and Taira, K. (1996) Evidence for the rate-limiting departure of the 5′-oxygen in nonenzymatic and hammerhead ribozyme-catalyzed reactions. *J. Am. Chem. Soc.* **118**, 5862-5866
24. Zhou, D.-M., Zhang, L.-H., and Taira, K. (1997) Explanation by the double-metal-ion mechanism of catalysis for the differential metal ion effects on the cleavage rates of 5′-oxy and 5′-thio substrates by a hammerhead ribozyme. *Proc. Natl. Acad. Sci. USA* **94**, 14343-14348
25. Eckstein, F. and Lilley, D.M.J., eds. (1996) *Catalytic RNA, Nucleic Acids and Molecular Biology,* Vol. 10, Springer-Verlag, Berlin
26. Uhlenbeck, O.C. (1987) A small catalytic oligoribonucleotide. *Nature* **328**, 596-600
27. Haseloff, J. and Gerlach, W.L. (1988) Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* **334**, 585-591
28. Walbot, V. and Bruening, G. (1988) Plant development and ribozyme for pathogens. *Nature* **334**, 196-197
29. Maddox, J. (1989) The great gene shears story. *Nature* **342**, 609-613
30. Inokuchi, Y., Yuyama, N., Hirashima, A., Nishikawa, S., Ohkawa, J., and Taira, K. (1994) A hammerhead ribozyme inhibits the proliferation of an RNA coliphage SP in *Escherichia coli. J. Biol. Chem.* **269**, 11361-11366
31. Chen, H., Ferbeyre, G., and Cedergren, R. (1997) Efficient hammerhead ribozyme and antisense RNA targeting in a slow ribosome *Escherichia coli* mutant. *Nature Biotech.* **15**, 432-435
32. Parker, R., Muhlrad, D., Deshler, J.O., Taylor, N., and Rossi, J.J. (1992) Chimeric antisense RNAs in *Catalytic RNA* (Eckstein, F. and Lilley, D.M.J., eds.) Nucleic Acids and Molecular Biology, Vol. 10, pp. 183-195, Springer-Verlag, Berlin
33. Taira, K. and Nishikawa, S. (1992) Construction of several kinds of ribozyme: their reactivities and utilities in *Gene Regulation* (Erickson, R.P. and Izant, J.G., eds.) Biology of Antisense RNA and DNA, pp. 35-54, Raven Press, New York
34. Silver, S. and Clark, D. (1971) Magnesium transport in *Escherichia coli. J. Biol. Chem.* **246**, 569-576
35. Flatman, P.W. (1984) Magnesium transport across cell membranes. *J. Membr. Biol.* **80**, 1-14
36. Romani, A. and Scarpa, A. (1992) Regulation of cell magnesium. *Arch. Biochem. Biophys.* **298**, 1-12
37. Pieken, W.A., Olsen, D.B., Benseler, F., Aurup, H., and Eckstein, F. (1991) Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes. *Science* **253**, 314-317
38. Paolella, G., Sproat, B.S., and Lamond, A.I. (1992) Nuclease-resistant ribozymes with high catalytic activity. *EMBO J.* **11**, 1913-1919
39. Taylor, N.R., Kaplan, B.E., Swiderski, P., Li, H., and Rossi, J.J. (1992) Chimeric DNA-RNA hammerhead ribozymes have enhanced *in vitro* catalytic efficiency and increased stability *in vivo*. *Nucleic Acids Res.* **20**, 4559-4565
40. Shimayama, T., Nishikawa, F., Nishikawa, S., and Taira, K. (1993) Nuclease-resistant chimeric ribozymes containing deoxyribonucleotides and phosphorothioate linkages. *Nucleic Acids Res.* **21**, 2605-2611
41. Pingoud, A. and Jeltsch, A. (1997) Recognition and cleavage of DNA by type-II restriction endonucleases. *Eur. J. Biochem.* **246**, 1-22
42. Thomoson, J.B., Tuschl, T., and Eckstein, F. (1996) The hammerhead ribozyme in *Catalytic RNA* (Eckstein, F. and Lilley, D.M.J., eds.) Nucleic Acids and Molecular Biology, Vol. 10, pp. 173-196, Springer-Verlag, Berlin
43. Ellington, A.D. and Szostak, J.W. (1990) *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818-822
44. Robertson, D.L. and Joyce, G.F. (1990) Selection *in vitro* of an RNA enzyme that specifically cleaves single-stranded DNA. *Nature* **344**, 467-468
45. Tuerk, C., MacDougal, S., and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505-510
46. Beaudry, A.A. and Joyce, G.F. (1992) Directed evolution of an RNA enzyme. *Science* **257**, 635-641
47. Pan, T. and Uhlenbeck, O.C. (1992) *In vitro* selection of RNAs that undergo autolytic cleavage with Pb^{2+}. *Biochemistry* **31**, 3887-3895
48. Lehman, N. and Joyce, G.F. (1993) Evolution *in vitro* of an RNA enzyme with altered metal dependence. *Nature* **361**, 182-185
49. Nakamaye, K.L. and Eckstein, F. (1994) AUA-cleaving hammerhead ribozymes: attempted selection for improved cleavage. *Biochemistry* **33**, 1271-1277
50. Cuenoud, B. and Szostak, J.W. (1995) A DNA metallo-enzyme with DNA ligase activity. *Nature* **375**, 611-614
51. Ishizaka, M., Ohshima, Y., and Tani, T. (1995) Isolation of active ribozymes from an RNA pool of random sequences using an anchored substrate RNA. *Biochem. Biophys. Res. Commun.* **21**, 403-409
52. Bartel, D.P. and Szostak, J.W. (1993) Isolation of new ribozyme from a large pool of random sequences. *Science* **261**, 1411-1418
53. Lorsch, J.R. and Szostak, J.W. (1994) *In vitro* evolution of new ribozyme with polynucleotide kinase activity. *Nature* **371**, 31-36
54. Dai, X., De Mesmaeker, A., and Joyce, G.F. (1995) Cleavage of an amide bond by a ribozyme. *Science* **267**, 237-240
55. Wilson, C. and Szostak, J.W. (1995) *In vitro* evolution of a

self-alkylating ribozyme. *Nature* **374**, 777-782
56. Denman, R.B., Smedman, M., Ju, W., Rubenstein, R., Potempska, A., and Miller, D.L. (1994) Ribozyme-mediated degradation of beta-amyloid peptide precursor mRNA in COS-7 cells. *Nucleic Acids Res.* **22**, 2375-2382
57. Kawasaki, H., Ohkawa, J., Tanishige, N., Yoshinari, K., Murata, T., Yokoyama, K.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
58. Fujita, S., Kogma, T., Ohkawa, J., Mori, K., Kohda, T., Kise, H., Nishikawa, S., Iwakura, M., and Taira, K. (1997) Discrimination of a single base change in a ribozyme using the gene for dihydrofolate reductase as a selective marker in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **94**, 391-396
59. Blakley, R.L. and Benkovic, S.J., eds. (1985) *Folates and Pterins*, Vol. 2, Wiley Interscience, New York
60. Iwakura, M., Shimura, Y., and Tsuda, K. (1982) Cloning of dihydrofolate reductase gene of *Escherichia coli* K12. *J. Biochem.* **91**, 1205-1212
61. Iwakura, M., Shimura, Y., and Tsuda, K. (1983) Construction of plasmid vectors for cloning of promoters using the dihydrofolate reductase gene of *Escherichia coli* K-12. *J. Biochem.* **93**, 927-930
62. Taira, K., Fierke, C.A., Chen, J.-T., Johnson, K.A., and Benkovic, S.J. (1987) On interpreting the inhibition of and catalysis by dihydrofolate reductase. *Trends Biochem. Sci.* **12**, 275-278
63. Taira, K. and Benkovic, S.J. (1988) Evaluation of the importance of hydrophobic interactions in drug binding to dihydrofolate reductase. *J. Med. Chem.* **31**, 129-137
64. Iwakura, M., Jones, B.E., Luo, J., and Matthews, C.R. (1995) Strategy for testing the suitability of cysteine replacements in dihydrofolate reductase from *Escherichia coli*. *J. Biochem.* **117**, 480-488
65. Ruffner, D.E., Stormo, G.D., and Uhlenbeck, O.C. (1990) Sequence requirements of the hammerhead RNA self-cleavage reaction. *Biochemistry* **29**, 10695-10702
66. Yanofsky, C. (1981) Attenuation in the control of expression of bacterial operons. *Nature* **289**, 751-758
67. Iwakura, M. and Tanaka, T. (1992) Dihydrofolate reductase gene as a versatile expression marker. *J. Biochem.* **111**, 31-36
68. Sioud, M. and Drlica, K. (1991) Prevention of human immunodeficiency virus type 1 integrase expression in *Escherichia coli* by a ribozyme. *Proc. Natl. Acad. Sci. USA* **88**, 7303-7307
69. Ferbeyre, G., Bratty, J., Chen, H., and Cedergren, R. (1995) A hammerhead ribozyme inhibits ADE1 gene expression in yeast. *Gene* **155**, 45-50
70. Ferbeyre, G., Bratty, J., Chen, H., and Cedergren, R. (1996) Cell cycle arrest promotes *trans*-hammerhead ribozyme action in yeast. *J. Biol. Chem.* **271**, 19318-19323
71. Zhang, S., Stancek, M., and Isaksson, L.A. (1997) The efficiency of a *cis*-cleaving ribozyme in an mRNA coding region is influenced by the translating ribosome *in vivo*. *Nucleic Acids Res.* **25**, 4301-4306