

# A novel system for selection of intracellularly active ribozymes using the gene for dihydrofolate reductase (DHFR) as a selective marker in *Escherichia coli*

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## Summary

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 the DHFR expression vector, a sequence encoding either an active or an inactive ribozyme was connected either upstream (5'-connected active or inactive ribozyme) or downstream (3'-connected active or inactive ribozyme) 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). In the case of both 5'-connected and 3'-connected ribozymes, the active ribozyme did indeed cleave the primary transcript *in vivo*, whereas inactive ribozymes had no cleavage activity. We confirmed that cells that harbored the active ribozyme-coding plasmid grew faster in the presence of a fixed concentration of TMP than the corresponding cells that harbored an inactive ribozyme-coding plasmid. Consequently, when cells were transformed with a mixture that consisted of active ribozyme-coding and inactive ribozyme-coding plasmids at a ratio of 1:1, it was mainly the cells that harbored the active ribozyme that survived in the presence of TMP. These results indicated that our positive selection system *in vivo* was functional and that, moreover, if the background "noise" could be removed completely in the future, it might usefully complement existing selection systems *in vitro*.

## I. Introduction

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 (Cech et al., 1981; Guerrier-Takada et al., 1983). 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 (Cameron and Jennings, 1989; Sarver et al., 1990; Uhlmann and Peyman, 1990; Erickson and Izant, 1992; Heidenreich and Eckstein, 1992; Murray, 1992; Ojwang et

al., 1992; Rossi, 1992; Ohkawa et al., 1993). They are expected to be useful in gene therapy for some diseases that are caused by the expression of abnormal mRNA, which include diseases caused by infectious agents such as HIV (Sarver et al., 1990; Heidenreich and Eckstein, 1992; Ojwang et al., 1992; Ohkawa et al., 1993; Leavitt et al., 1994). There are several strategies for inhibiting the expression of specific genes during transcription and translation, as follows. (i) At the DNA level, before transcription from the template DNA, a triple helix or a repressor peptide can be used (Blume et al., 1992; Roy, 1993; Gee et al., 1994; Mayfield et al., 1994; Choo et al., 1994). (ii) After transcription, antisense RNA/DNA or a ribozyme can be used to inhibit translation at the RNA level. (iii) After translation, antibodies or inhibitors can be used at the protein level. 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, it can act as an enzyme. Since the substrate specificity of antisense and ribozyme molecules is high, antisense and ribozyme strategies seem likely to have some value as therapeutic agents (Erickson and Izant, 1992; Murray, 1992; Eckstein and Lilley, 1996).

When the hammerhead ribozyme was engineered such that it could cleave specific RNA sequences "in trans" (Uhlenbeck, 1987; Haseloff and Gerlach, 1988), it was postulated that this ribozyme might be much more effective than simple antisense molecules in several respects (Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Walbot and Bruening, 1988; Maddox, 1989; Inokuchi et al., 1994). However, because the activity and stability of ribozyme are very dependent on the cellular environment (Chen et al., 1997), ribozymes have not yet proven their superiority to antisense molecules and the practical use of ribozymes in therapy has not yet been achieved. There seem to be several reasons for the low activity of ribozymes *in vivo*. (i) Various cellular proteins might exist *in vivo* that inhibit their catalytic activity (Parker et al., 1992; Taira and Nishikawa et al., 1992). (ii) The intracellular concentration of  $Mg^{2+}$  ions is much lower than that used *in vitro* in assays of ribozyme activity (Silver and Clark, 1971; Flatman, 1984; Romani and Scarpa, 1992). (iii) Several cellular RNases contribute to the instability of ribozymes (Olsen et al., 1991; Pieken et al., 1991; Heidenreich and Eckstein, 1992; Paoletta et al., 1992; Taylor et al., 1992; Shimayama et al., 1993). (iv) Unlike the case of some proteinaceous enzymes, 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, the chemical modification and substitution of nucleotides to improve the stability and activity of ribozymes (Thomson et al., 1996).

For the rapid selection of functional sequences *in vitro* from a population of random sequences, Gold, Szostak, and Joyce and their respective groups developed a novel method (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990). This genetic selection system is sometimes called SELEX, an abbreviation for systematic evolution of ligands by exponential enrichment, and the selected nucleic acids are referred to as aptamers. The method takes advantage of a process that mimics evolution, namely, mutation, amplification and selection. A pool of completely random RNAs is subjected to selection. Selected functional RNAs are amplified as double-stranded DNAs and the next generation of RNAs is transcribed from these template DNAs. Then the transcribed RNAs are subjected to selection in the next cycle. In efforts to engineer specific ribozymes, this method was successfully used (Beaudry and Joyce, 1992; Pan and Uhlenbeck, 1992; Lehman and Joyce, 1993; Nakamaye and Eckstein, 1994; Cuenoud and Szostak, 1995; Ishizaka et al., 1995). New functional ribozymes with ligase, kinase, amino-acid cleavage or self-alkylating activities have already been selected by this method (Bartel and Szostak 1993; Lorsch and Szostak 1994; Dai et al., 1995; Wilson and Szostak 1995). One might be able to select very active ribozymes using this method. However, the functional ribozyme selected *in vitro* might not always be the same as 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 (Denman et al., 1994; Kawasaki et al., 1996). Moreover, while the activity of a ribozyme is associated with its specific structure, reverse transcriptase is known to be inhibited by certain secondary structures (Tuerk et al., 1992). Therefore, there is always a risk of missing the most effective ribozymes during selection *in vitro*.

In this report, we describe a novel method for screening *in vivo* that was designed to identify new hammerhead ribozymes with high activity. This method avoids the above mentioned disadvantages of selection *in vitro*. We chose the gene for dihydrofolate reductase (DHFR) as the selective marker. DHFR is an essential proteinaceous enzyme on the pathway to thymidylic acid (Blakley and Benkovic, 1985). 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 (Iwakura et al., 1982; Blakley and Benkovic, 1985; Taira et al., 1987; Taira and Benkovic, 1988). We designed our vector in such a way 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 (Iwakura et al., 1983). Moreover, there is a direct correlation between the level of expression of DHFR and the strength of resistance to TMP. 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 (Fujita et al., 1997).

We placed a ribozyme-encoding sequence either upstream or downstream of the gene for DHFR. We report here that clones that survived at a chosen concentration of TMP harbored mostly active ribozymes.

## II. Results

### A. Design and construction of the screening vector with a 5'-connected ribozyme

As a first step towards our goal of designing a screening system in *E. coli*, we attempted to distinguish between two vectors, one of which contained an active ribozyme and one of which contained 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<sup>5</sup> to A<sup>5</sup> or A<sup>14</sup> to G<sup>14</sup> mutation within the catalytic core of the ribozyme. These mutations completely abolish the activity of the ribozyme (Ruffner et al., 1990; Inokuchi et al., 1994). Because transcription and translation are coupled in *E. coli*, the ribozyme must cleave its target site before completing translation of DHFR. Therefore, in two types of vector, the active ribozyme sequence and the inactive ribozyme sequence were connected, separately, upstream of the gene for DHFR (**Fig. 2**). 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. Then 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, between the ribozyme-coding and DHFR-coding sequences, 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, one of which was the original initiation codon of the DHFR gene itself and the second of which (the pseudo-initiation codon) was located upstream of the original initiation codon to introduce a frame shift. 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 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.

To avoid any readthrough from upstream regions, an "all stop codon" sequence (TAACTAACTAA) was introduced between the ribozyme and the strong SD sequence. In this region, three stop codons should terminate translation in all possible frames. Furthermore, we introduced a terminator sequence (Yanofsky, 1981; Iwakura and Tanaka, 1992) 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.

### B. Discrimination of active ribozymes from inactive ribozymes, connected on the 5' side of the DHFR gene, in the presence of TMP

Taking advantage of the direct relationship between the level of expression of DHFR and the strength of resistance to TMP (Iwakura et al., 1983), we constructed a system for screening active ribozymes. Among several concentrations of TMP tested, we found that, at 70  $\mu$ g of TMP per ml of culture medium, *E. coli* cells that had been transformed with the active ribozyme expression vector grew more rapidly and made larger colonies than cells transformed with the inactive ribozyme expression vector. **Figure 3** shows the difference in growth rates between the active ribozyme- and inactive-ribozyme expressing colonies at 27°C and 37°C. Since *E. coli* strain HB101, which we used in this study, produces a low level of endogenous DHFR, formation of background colonies (noise) could not be avoided. Since the difference in

growth rates between the active ribozyme- and inactive ribozyme-expressing colonies was greater at 27°C than at 37°C (**Fig. 3B**), the selection of active ribozymes described below was made at 27°C in the presence of 70 µg of TMP and 100 µg of ampicillin per milliliter.

Since active ribozyme-expressing colonies grew more rapidly, as expected, than inactive ribozyme-expressing colonies, we performed a random screening assay

according to the procedure outlined in **Figure 4**. In this assay, equimolar amounts of active ribozyme- and inactive ribozyme-coding plasmids were mixed and competent HB101 cells were transformed with the mixture. The transformed cells were divided into two portions and each portion was plated either on an ampicillin-containing plate (100 µg/ml) or on a plate that contained both ampicillin (100 µg/ml) and TMP (70 µg/ml). After incubation for

**Figure 1.** Secondary structures of the 5'-connected ribozyme (**A**) and the 3'-connected ribozyme (**B**). A single point mutation (G<sup>5</sup> to A<sup>5</sup> or A<sup>14</sup> to G<sup>14</sup>; circled) eliminates the ribozyme activity (Ruffner et al., 1990; Inokuchi et al., 1994). Note that the catalytic loop that contains G<sup>5</sup> and A<sup>14</sup> captures Mg<sup>2+</sup> ions since a hammerhead ribozyme is a metalloenzyme (Dahm et al., 1993; Pyle, 1993; Uebayasi et al., 1994; Bassi et al., 1995; Sawata et al., 1995; Amontov and Taira, 1996; Kumar et al., 1996; Orita et al., 1996; Zhou et al., 1996a,b; Zhou et al., 1997).

**Figure 2.** The 5'-connected ribozyme expression vector. The plasmid vector has two ATG codons, one of which is a pseudo-initiation codon, located upstream of the authentic AUG codon, which is the initiation codon for the DHFR gene. If an active ribozyme is introduced upstream of the DHFR-coding region and if, upon transcription, the primary transcript is cleaved by this *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.

**Figure 3. (A)**, Colonies of *E. coli* HB101 cells that had been transformed with the 5'-connected active (left) or inactive (right) ribozyme expression plasmid. In the presence of 70  $\mu\text{g/ml}$  TMP, colonies expressing an active ribozyme (left) grew faster than colonies (right) that expressed an inactive ribozyme. The difference in growth rates between the active ribozyme- and inactive ribozyme-expressing colonies was greater at 27 °C (top) than at 37 °C (bottom).

**(B)**, Distribution of colonies according to their diameters. About 4,000 colonies of the types shown in Figure 3A were divided into 11 classes based on the diameter of colonies. The difference in growth rates between the active ribozyme- and inactive ribozyme-expressing colonies was greater at 27 °C (left) than at 37 °C (right). Since *E. coli* strain HB101 that we used in this study produces a low level of endogenous DHFR, formation of background colonies could not be avoided.

**Figure 4.** Schematic diagram of the *in vivo* selection system. Competent cells are transformed with a mixture of equimolar amounts of active ribozyme- and inactive ribozyme-expressing plasmids. In the absence of selective pressure (Amp plate), both active ribozyme- and inactive ribozyme-expressing colonies are expected to grow at the same rate. By contrast, active ribozyme-expressing colonies are expected to grow faster on the Amp plus TMP plate.

**Table 1.** Numbers of selected colonies with 5'-connected and 3'-connected active and inactive ribozymes on trimethoprim-containing and/or ampicillin-containing plates

	5'-connected ribozyme		3'-connected ribozyme		
	Ampicillin plate	Trimethoprim plate	Trimethoprim plate		
		(70 $\mu\text{g/ml}$ )	(130 $\mu\text{g/ml}$ )	(133 $\mu\text{g/ml}$ )	(140 $\mu\text{g/ml}$ )
"G <sup>5</sup> and A <sup>5</sup> " mixture					
Active ribozyme	29	75	9	7	27

Inactive ribozyme	28	1	0	2	1	3
"A <sup>14</sup> and G <sup>14</sup> " mixture						
Active ribozyme	21	42				
Inactive ribozyme	9	2				

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 they contained 130 µg, 133 µg or 140 µg TMP and 100 µg of Amp per ml for 3'-connected ribozyme screening. Ampicillin plates contained 100 µg Amp per ml without TMP.

one or more days, rapidly growing colonies were picked up at random from both plates. In order to check the reproducibility of our results, we picked up only ten colonies from each plate every day. Then, after minipreparation of plasmid DNA, we determined the sequences of the ribozyme-coding regions of the selected clones. **Table 1** summarizes the sequencing results for the selected clones from more than seven independent experiments. Clones selected in the presence of TMP harbored mainly the active ribozyme: in the case of the "G<sup>5</sup> and A<sup>5</sup>" mixture, only one out of 76 sequences turned out to encode an inactive ribozyme. By contrast, colonies selected in the absence of TMP (in the presence of Amp only) yielded active ribozyme and inactive ribozyme sequences at a ratio of 1:1. Similar results were obtained in the case of the "A<sup>14</sup> and G<sup>14</sup>" mixture.

gene. **Lane 1:** 5'-connected active ribozyme, with G<sup>5</sup> at the catalytic core. **Lane 2:** 5'-connected inactive ribozyme, with A<sup>5</sup> at the catalytic core. The active ribozyme expression vector produced an excised short fragment (lane 1) but there is no truncated fragment in lane 2, which corresponds to the inactive ribozyme expression vector. Lane 1 also shows the intact primary transcript. Lengths of fragments were consistent with the expected values, as estimated from a standard curve of mobilities of RNA size-markers. The numbers indicate the lengths of fragments (in nucleotides) as determined by reference to size-markers (not shown).

In order to confirm that the phenotypic differences shown in **Table 1** really originated from a single base change and not from any other mutations within the DHFR gene, we sequenced several clones in their entirety, including the DHFR-coding region, and we also exchanged the *Hind*III-*Acc*III fragment (see **Fig. 2**) that contained each ribozyme sequence between the selected active and inactive clones. Since (i) no mutation was detected in the DHFR gene and (ii) the exchanged constructs had the opposite respective phenotypes, we were able to conclude that the phenotypic differences shown in Table 1 originated from the single base mutations. Thus, we confirmed that the selection pressure exerted by TMP was useful for identification of a single base change within the catalytic core of the ribozyme, which was correlated with the presence or absence of ribozyme activity, which, in turn, was correlated with the level of expression of DHFR.

### C. Detection by Northern blotting analysis of a fragment cleaved by the 5'-connected ribozyme

In order 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 the 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 (Sioud

**Figure 5.** Northern blotting analysis of the 5'-connected ribozyme expression vector. Ten micrograms of total RNA from *E. coli* cells, transformed with the ribozyme expression vector shown in Figure 2, were subjected to electrophoresis in a 1.8% Metaphor<sup>TM</sup> agarose gel. After transfer to a membrane filter, the RNA was allowed to hybridize with the synthetic oligonucleotide probe (40-mer), which was complementary to part of the DHFR

and Drlica, 1991; Ferbeyre et al., 1995). The results of our Northern analysis are shown in **Figure 5**. As can be seen in lane 1, both the intact primary transcript and the cleaved fragment were detected in the analysis of total RNA extracted from cells that contained the active ribozyme vector. However, no cleavage activity was detected when we analyzed the total RNA extracted from cells that contained the inactive ribozyme vector (lane 2). Although the lane corresponding to the inactive ribozyme (lane 2) appears to include a weak signal with the same mobility as the cleaved fragment, the signal did not represent a cleavage product, as demonstrated below by primer extension analysis (**Fig. 6**). The identification of the bands was based on the mobilities of RNA size-markers.

Why did we detect the cleaved fragments when others have failed? In our case, the target site of the ribozyme was located upstream of the DHFR gene (**Fig. 2**), 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.

**Figure 6.** Primer extension analysis of the 5'-connected ribozyme expression vector. Five micrograms of total RNA were used as template for reverse transcription, with a 5'-end-labeled synthetic oligonucleotide as primer. After transcription, the labeled transcribed product was subjected to electrophoresis on an 8% polyacrylamide gel. Lane 1: active ribozyme with the "all stop codon" was used as template. Lane 2: inactive ribozyme with the "all stop codon" was used as template. Lane 3: active ribozyme without the "all stop codon". Lane 4: inactive ribozyme without the "all stop codon". Both lane 1 and lane 3 include cleaved fragments. On the other hand, no cleaved fragments are visible in lanes 2 and 4. The exact site of cleavage was determined by reference to the sequencing ladders.

#### D. Identification of the cleavage site by primer extension analysis

Although the intact and cleaved mRNAs (**Fig. 5**) were determined to be of the anticipated sizes by reference to RNA size-markers, the exact site of cleavage could not be determined by Northern blotting analysis. In order to confirm that the cleaved fragment shown in **Figure 5** was really produced by the action of the ribozyme, we performed primer extension analysis (**Fig. 6**). In these experiments, we used two different sets of constructs. In one case, we used the plasmids shown in **Figure 2** that contained the "all stop codon" region and either the active (lane 1) or the G<sup>5</sup> to A<sup>5</sup>-mutated inactive (lane 2) ribozyme. In the second case, we used plasmids without the "all stop codon" region but with either an active (lane 3) or the G<sup>5</sup> to A<sup>5</sup>-mutated inactive (lane 4) ribozyme.

As judged from the sequencing ladders that were analyzed simultaneously, exactly the expected target sites were cleaved by the active ribozymes (lanes 1 and 3). By contrast, no cleavage products were detected with the inactive ribozyme constructs (lanes 2 and 4), strongly supporting the conclusion obtained from the results in **Figure 5**. A fragment with one extra base was also observed for each transcript. These fragments can, most probably, be explained by the characteristics of reverse transcriptase, which has a "snap back" feature and which can incorporate one extra nucleotide independently of the template (Frohman et al., 1990). It should be noted that, since the reaction mixture for the reverse transcriptase reaction contained Mg<sup>2+</sup> ions, some of the initial transcripts (intact mRNA) might have undergone ribozyme-mediated cleavage during reverse transcription. However, since there were no products other than the expected ones, we can safely conclude that cleavage occurred specifically at the predetermined target site *in vivo*.

#### E. Construction of a screening system using 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. We examined the 5'-side ribozyme first for the following reasons. 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 was less of a chance that the ribozyme would cleave the target site. Moreover, polysomes could protect a target site, located downstream of a strong SD sequence, from attacks 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 system, regulation of the level of expression of DHFR by a ribozyme seemed easily controllable.

However, there might be a critical defect associated with introduction of a ribozyme upstream of the DHFR gene. During actual screening for active ribozymes on the 5' side from a pool of random sequences, there might be 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 re-initiation 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

**Figure 7.** Construction of the 3'-connected ribozyme expression vector. The ribozyme region and the "all stop codon" between the promoter and SD sequence in the 5'-connected ribozyme expression vector were cut out and then inserted between the gene for DHFR and the terminator sequence. The inactive ribozyme sequence differed from the active sequence by a single G<sup>5</sup> to A<sup>5</sup> mutation within the catalytic core of the ribozyme, as in the 5'-side ribozyme construct. The target site of the 3'-connected ribozyme was the same as that of the 5'-connected ribozyme.

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 obtained with 5'-side ribozymes.

We constructed 3'-connected ribozyme expression vectors that contained either an active ribozyme or an inactive ribozyme sequence. These vectors were based on the 5'-connected ribozyme expression vectors (**Fig. 7**). 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. 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 *Hind*III-*Acc*III region by a linker that had the same length in nucleotides as the corresponding region that contained the ribozyme and the "all stop codon". As a result, the final 3'-connected ribozyme expression vector contained, from the upstream to the downstream region, the promoter, the linker, the 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 (**Fig. 7**). The inactive ribozyme sequence differed from the active one by a single G<sup>5</sup> to A<sup>5</sup> mutation within the catalytic core of the ribozyme as in the 5'-side ribozyme construct. The target site of the 3'-connected ribozyme was exactly the same as that of the 5'-connected ribozyme.

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.

**Figure 8** shows the difference in growth rates between active ribozyme- and inactive ribozyme-expressing colonies at 27°C at 130 µg of TMP per ml of culture

**Figure 8.** Colonies of *E. coli* HB101 cells that had been transformed with the 3'-connected ribozyme expression plasmid. G<sup>5</sup> (Active ribozyme): Active ribozyme. A<sup>5</sup> (Inactive ribozyme): Inactive ribozyme with G<sup>5</sup> replaced by A. C<sup>5</sup> (Inactive ribozyme): Inactive ribozyme with G<sup>5</sup> replaced by C. T<sup>5</sup> (Inactive ribozyme): Inactive ribozyme with G<sup>5</sup> replaced by U. In the presence of 130 µg/ml TMP, colonies expressing the active ribozyme grew more rapidly than colonies that expressed inactive ribozymes.

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 (**Fig. 3**). For some unknown reason, cells harboring the C<sup>5</sup>-inactive ribozyme vector grew more rapidly than cells with the other inactive ribozyme vector. Among the several concentrations of TMP tested, we found that the difference in colony size between active ribozyme- and inactive ribozyme-expressing clones was greatest at a concentration of TMP of 125-140 µg per ml of culture medium. This range of concentrations is higher than the 70 µg of TMP per ml of culture medium used in the assay with the 5' construct. The increased resistance to TMP might have originated from an increased level of the transcript (see **Fig. 9**) and a higher rate of production of DHFR. The level of mRNA might have changed since the sequence of the *Hind* III/*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.

We then carried out a random screening assay for the 3' ribozyme construct, following the procedure used for the 5' ribozyme construct, as outlined in **Figure 4**. *E. coli* cells transformed with the active ribozyme- or inactive ribozyme-coding plasmid were plated on a plate that contained ampicillin (100 µg/ml) or on a plate that contained both ampicillin (100 µg/ml) and TMP (125-140 µl/ml). Faster growing colonies were picked up at random from both plates and ribozyme sequences were confirmed. The results are shown in Table 1. For some unknown reason, the level of background colonies was very sensitive to the concentration of TMP and, therefore, reproducibility was lower 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 1).

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 (**Fig. 9**). As mentioned above, the level of the transcript was higher when the 3'-side ribozyme was used than with the 5'-side ribozyme. As indicated in lanes 2 and 4, both 5'- and 3'-connected ribozymes recognized and cleaved the target site. The cleavage efficiencies were nearly identical for the two types of ribozyme, 24% by the 5'-side ribozyme and 23% by the 3'-side ribozyme. It is noteworthy that polysomes did not seem to inhibit the action of the 3'-connected ribozyme, in our specific construct.

### III. Discussion

Successful selection *in vitro* of tailored RNA has been reported by others and is of considerable current interest (Beaudry and Joyce, 1992; Pan and Uhlenbeck, 1992; Gray and Cedergren, 1993; Lehman and Joyce, 1993; Nakamaye and Eckstein, 1994; Cuenoud and Szostak, 1995; Ishizaka et al., 1995). However, efforts aimed at construction of selection systems *in vivo* have met with only limited success (Ferbeyre et al., 1996; Fujita et al., 1997). For use of ribozymes *in vivo*, we need RNAs that function optimally in the intracellular environment. Tsuchihashi and Herschlag reported that a protein derived from the p7 nucleocapsid (NC) protein of HIV-1 can facilitate cleavage by a ribozyme (Tsuchihashi et al., 1993; Herschlag et al., 1994). Other proteins also probably facilitate ribozyme-catalyzed cleavage (Bertrand and Rossi, 1994). There have been a few reports of the successful ribozyme-mediated inactivation of genes in *Saccharomyces cerevisiae* (Parker et al., 1992; Taira and Nishikawa, 1992; Egli and Braus, 1994; Ferbeyre et al., 1995; Ferbeyre et al., 1996). The difficulties encountered in attempts to characterize ribozyme action in *Saccharomyces cerevisiae* suggest the existence of inhibitory factors in yeast. Under such circumstances, it is obviously desirable to be able to select ribozymes that function in the presence of such putative

**Figure 9.** Northern blotting analysis for comparison of the cleavage efficiency between the 5'-connected ribozyme and the

3'-connected ribozyme. 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 1.8% Metaphor<sup>TM</sup> 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<sup>5</sup> at the catalytic core. Lane 2: 5'-connected active ribozyme with G<sup>5</sup> at the catalytic core. Lane 3: 3'-connected inactive ribozyme, with A<sup>5</sup> at the catalytic core. Lane 4: 3'-connected active ribozyme with G<sup>5</sup> 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). Cleavage efficiencies were the same in lanes 2 and 4.

**Figure 10.** Schematic representation of the design of a plasmid for the *in vivo* selection system. When the ribozyme is active, it can prevent expression of the toxin.

inhibitory factors *in vivo*. To this end, we attempted to construct a positive selection system *in vivo* based on the general scheme shown in **Figure 10**. 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<sub>1</sub> as the gene for the toxin. However, despite considerable effort, we failed to generate any plasmids that corresponded to the one shown in **Figure 10** when RNase T<sub>1</sub> was used as the selective marker. No constructs with a gene for RNase T<sub>1</sub> were rescued from transformed *E. coli* cells. Only frame-shifted constructs, with aborted production of RNase T<sub>1</sub>, were rescued. In this first attempt, we were unable to control the extent of the toxicity of RNase T<sub>1</sub>.

We next chose a potentially more controllable gene as a selective marker, namely, the gene for DHFR (Iwakura et al., 1983; Fujita et al., 1997). As stated in the Introduction, DHFR is essential for DNA synthesis (Blakley and Benkovic, 1985). Moreover, there exists a direct relationship between the level of expression of DHFR and the strength of resistance to TMP (Iwakura et al., 1983). We tested the feasibility of use of the gene for DHFR with the constructs shown in **Figures 2** and **7**. We examined two types of ribozyme, an active and an inactive ribozyme, each located on the 5' or 3' side relative to the target site.

In the case of the 5'-side ribozyme construct, at a fixed concentration of TMP of 70  $\mu\text{g/ml}$ , *E. coli* cells harboring the active ribozyme expression vector grew faster than those harboring the inactive ribozyme expression vector (**Fig. 3**). Then we prepared a mixture of active ribozyme and inactive ribozyme expression vectors in equimolar amounts and plated *E. coli* cells that had been transformed with the mixture on LBM plates that contained TMP at 70  $\mu\text{g/ml}$ . After incubation at 27 °C for 2 to 3 days, colonies were harvested and DNA sequences were examined to determine whether each clone contained the sequence of an active or an inactive ribozyme. In this way, we were able to judge whether our method for selecting active ribozymes had any statistical significance. Since, for the most part, active ribozymes were selected in the presence of TMP (Table 1), DHFR appeared more suitable as a selective marker than RNase T<sub>1</sub>. We also demonstrated, by Northern blotting and primer extension analyses (**Figs. 5** and **6**), that the active ribozymes were fully functional *in vivo*; they cleaved the primary transcript of the DHFR gene specifically and at the predetermined site only. In both of these analyses (**Figs. 5** and **6**), the mutant ribozyme (G<sup>5</sup> to A<sup>5</sup>) had no cleavage activity. Another change, that eliminated ribozyme activity was the single base change at A<sup>14</sup>. With the A<sup>14</sup>/G<sup>14</sup> system, we were also able to select active ribozymes in the presence of TMP (Table 1). Taking all our results into account, we can conclude that the difference in phenotypes of the clones originated from only the single base mutation at the catalytic core of the hammerhead ribozyme (**Fig. 1**).

Our analysis of the construct shown in **Figure 2** confirmed the possibility of selecting active ribozymes *in vivo* with DHFR as a selective marker. However, in its present form, the method for selection of an active mutant ribozyme from a completely randomized large pool is inadequate: the background noise might easily obscure identification of an active mutant from a large pool of inactive molecules. Furthermore, randomized sequences on the 5' side might influence the levels of transcription and translation (data not shown). 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 (**Fig. 7**). In

the case of the 3' ribozyme constructs, we had to use a higher concentration of TMP because of the higher level of transcription. At concentrations of TMP of 125-140  $\mu\text{g/ml}$ , *E. coli* cells harboring the active ribozyme expression vector grew faster than those harboring the inactive ribozyme expression vector (**Fig. 8**). We prepared a mixture of active ribozyme and inactive ribozyme expression vectors and applied the same random screening procedure as in the case of the 5'-connected ribozyme. The *E. coli* cells transformed with the mixture were plated on LBM plates that contained TMP at 125-140  $\mu\text{g/ml}$ . We picked up rapidly growing colonies at random and examined the ribozyme sequences of selected colonies (Table 1). As compared with the noise with the 5' construct, the background noise in the case of 3'-connected ribozyme could not be reduced, even though, for the most part, active ribozymes could also be selected in the presence of TMP.

Comparison of the efficiency of ribozyme-mediated cleavage *in vivo* between 5'- and 3'-connected ribozymes by Northern blotting analysis revealed that the efficiencies of cleavage were identical (**Fig. 9**) 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 (Zhang et al, 1997). 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.

We have not yet optimized our positive selection system *in vivo*. We know that the cleavage activity of the ribozyme depends strongly on the target site. Among several possible target sites, we chose arbitrarily, in this study, one target site close to the initiation codon. Genes other than the gene for DHFR might also be more suitable as selective markers (the general positive selection system shown in **Figures 2** and **7** might be applicable to genes other than the gene for DHFR). We are now trying to improve this system by removing the "noise", using several strategies that include the use of a DHFR-null strain. Nevertheless, as a first step toward the construction of a positive selection system *in vivo*, the present system allowed us successfully to identify a single base change that was associated with a change in ribozyme activity. While a bacterial *cis*-acting system is described in this report, it is clear that our approach might be adaptable to a *trans*-acting eukaryotic system, which would be of value for the development of ribozyme-mediated gene therapies for human diseases.

#### IV. Experimental procedures

### A. Bacterial strains and plasmids

*E. coli* HB101 (*recA13, supE44*; Takara Shuzo Co., Kyoto) was used as the recipient for transformation. Several ribozyme expression vectors were constructed by modifying the DHFR expression vector pTZDHFR20 (Iwakura et al., 1995).

### B. Synthesis of oligonucleotides and construction of plasmids

We prepared ten kinds of oligodeoxynucleotide for construction of 5'-connected ribozyme expression vectors [active-ribozyme linkers (forward, 5'-AGC TTA ACT AAT TGA ATT CCT GAT GAG TCC CTA GGG ACG AAA CCA TGG ACT AAC TAA CTA AT-3'; and reverse, 5'-CCG GAT TAG TTA GTT AGT CCA TGG TTT CGT CCC TAG GGA CTC ATC AGG AAT TCA ATT AGT TA-3'), inactive-ribozyme (G<sup>5</sup> to A<sup>5</sup>) linkers (forward, 5'-AGC TTA ACT AAT TGA ATT CCT AAT GAG TCC CTA GGG ACG AAA CCA TGG ACT AAC TAA CTA AT-3'; and reverse, 5'-CCG GAT TAG TTA GTT AGT CCA TGG TTT CGT CCC TAG GGA CTC ATT AGG AAT TCA ATT AGT TA-3'), inactive-ribozyme (A<sup>14</sup> to G<sup>14</sup>) linkers (forward, 5'-AGC TTA ACT AAT TGA ATT CCT GAT GAG TCC CTA GGG ACG AGA CCA TGG ACT AAC TAA CTA AT-3'; and reverse, 5'-CCG GAT TAG TTA GTT AGT CCA TGG TCT CGT CCC TAG GGA CTC CTT AGG AAT TCA ATT AGT TA-3'), pseudo-ATG linkers (forward, 5'-CCG GAA AAG GAG GAA CTT CCA TGG TCG AAT TCA ACC TAT ATG ATC AGT CTG ATT GCG GCG-3'; and reverse, 5'-CTA GCG CCG CAA TCA GAC TGA TCA TAT AGG TTG AAT TCG ACC ATG GAA GTT CCT CCT TTT-3'), and 3'-terminator linkers (forward, 5'-TCG AGC GTC GTT AAA GCC CGC CTA ATG AGC GGG CTT TTT TTT TTA G-3'; and reverse, 5'-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 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'), 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<sup>5</sup> to A<sup>5</sup>, or A<sup>14</sup> to G<sup>14</sup>) was introduced within the catalytic core of the active ribozyme (Fig. 1). These changes had been shown previously to destroy cleavage activity (Ruffner et al., 1990; Inokuchi et al., 1994). Ribozyme linkers were

"tailed" with a recognition sequence for restriction endonuclease *Hind*III at the 5' end and with one for *Acc*III at the 3' end. Pseudo-ATG linkers were tailed with a recognition sequence for restriction endonuclease *Acc*III at the 5' end and with one for *Nhe*I at the 3' end. 3'-Terminator linkers were tailed with a recognition sequence for restriction endonuclease *Xho*I at the 5' end and with one for *Bam*HI at the 3' end. Each oligonucleotide linker was denatured at 95°C in a water bath and then gradually cooled to room temperature in TE buffer. After annealing, each linker set was then ligated to the digested vector pTZDHFR20 via its restriction sites and the tailed cohesive ends of the synthetic oligonucleotide linkers, for construction of 5'-connected ribozyme expression vectors (Fig. 2).

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 with one for *Bam*HI in the case of the reverse primer. Linkers instead of a ribozyme were tailed with a recognition sequence for restriction endonuclease *Hind*III at the 5' end and with one for *Acc*III at the 3' end. For the construction of 3'-connected ribozyme expression vectors, the region that contained 5'-connected ribozymes were cut out from 5'-connected ribozyme vectors by restriction enzymes *Hind*III and *Acc*III and a linker was ligated to the digested vector, replacing the ribozyme portion. 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. Then, these fragments were ligated to the digested ribozyme-free vector via the *Xho*I and *Bam*HI restriction sites.

### C. Composition of culture media

Plates of LB-modified (LBM) medium, containing polypeptone, yeast extract, NaCl, and 16 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 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 (70 µg/ml).

### D. Northern blotting analysis

Plasmid vector pTZDHFR, harboring both a 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 Co., Toyama) from 2 ml of a culture of cells in 2x YT medium. Ten micrograms of total RNA per sample were denatured in glyoxal and dimethyl sulfoxide, subjected to electrophoresis in 1.8% Metaphor™ agarose gel (FMC Inc., Rockland), and transferred to a Hybond-N™ nylon membrane (Amersham Co., 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 <sup>32</sup>P by T4 polynucleotide kinase (Takara Shuzo Co., Kyoto). Prehybridization and hybridization were performed in the same solution (5x SSPE, 50% formamide, 5x Denhardt's solution, 0.5% SDS, 150 mg/ml calf thymus DNA). Final washing was performed in 0.1x SSPE, 0.1% SDS at 70 °C for 30 min.

### E. Primer extension analysis

Total RNA was isolated from *E. coli* cells. An aliquot of 0.2 pmol of [<sup>32</sup>P]-labeled oligonucleotide primer (5'-GCC GAT AAC GCG ATC TAC-3'; complementary to DHFR mRNA) was allowed to hybridize to 5 µg of an RNA sample with heating at 65 °C for 90 min and gradual cooling to room temperature in 15 µl of a solution of 10 mM Tris-HCl (pH 8.3), 0.15 M KCl, and 1 mM EDTA. Then 15 µl of 2x RT reaction mixture, which contained 30 mM Tris-HCl (pH 8.3), 15 mM MgCl<sub>2</sub>, 8 mM DTT, 0.8 mM each dNTP, 6 units of human placental ribonuclease inhibitor, and 80 units of SuperScript RNaseH reverse transcriptase (Gibco BRL, Gaithersburg, MD) were added. The reverse transcriptase (RT) reactions were allowed to proceed at 42 °C for 60 min to avoid any influence of the secondary structure of the mRNA. After the RT reaction, 2 µl of stop solution, containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol, were mixed with 3 µl of the reaction mixture and the resulting sample was fractionated on a 7 M urea-8% polyacrylamide gel. Four ddNTP sequencing reactions with the same [<sup>32</sup>P]-labeled primer were fractionated together, creating sequencing ladders as markers.

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