Construction of Rational Screening System for Selection of Functional RNA and Protein

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Doctoral Program in Engineering
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Contents

Chapter 3

Chapter 4

Abbreviations

A	adenine, adenosine
A, Ala	alanine
a.a.	amino acids
AIDS	acquired immune deficiency syndrome
Amp	ampicillin
Amp^r	ampicillin resistance
ATP	adenosine triphosphate
C	cytosine, cytidine
C	cysteine
cDNA	complementary DNA
CTP	cytidine triphosphate
D, Asp	aspartic acid
dA	deoxyadenosine
DDS	drug delivery system
dG	deoxyguanosine
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dsDNA	double stranded DNA
dT	deoxythymidine
DTT	dithiothreitol
dUMP	deoxyuridine 5'-monophosphate
E	glutamic acid
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
F, Phe	phenylalanine
G	guanine, guanosine
G	glycine
GTP	guanosine triphosphate
H	histidine
HDV	hepatitis delta virus
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus-type1

HPLC	high performance liquid chromatography
I	isoleucine
IPTG	isopropyl-1-thio-β-D-galactoside
K	lysine
kb	kilo base
K_d	equilibrium dissociation constant
K_{rel}	relative equilibrium constant
k_{off}	dissociation rate constant
k_{on}	association rate constant
L	leucine
LB	Luria-Bertanis medium
LBM	LB modified
M	methionine
min	minute(s)
mRNA	messenger RNA
MTX	methotrexate
N	asparagine
NADPH	nicotinamide adenine dinucleotide phoshate, reduced form
NC	nucleocapsid
nt	nucleotide(s)
NTP	ribonucleoside triphosphate
P	proline
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Q	glutamine
R	arginine
RE peptide	Tat derived peptide containing basic region
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
S	serine
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
SELEX	systematic evolution of ligands by exponential enrichment
ssDNA	single strand DNA

T	thymine
T	threonine
TAR	*trans*-activating response region
Tat	*trans*-activation protein
TE	Tris-EDTA
TFIIIA	transcription factor IIIA
TMP	trimethoprim
Tris	tris(hydroxymethyl) aminomethane
tRNA	transfer RNA
U	uracil, uridine
UTP	uridine triphosphate
V	valine
W	tryptophan
Y	tyrosine

Chapter 1

General Introduction

Functional RNAs and Proteins

Inter- and intra-cellular diverse functional molecules relate to the many reactions involving the regulation of metabolism, reproduction, transcription, translation and signal transfer for maintaining the organism. Almost functional molecules are synthesized in according to the information of the genes. In general, almost organisms have deoxyribonucleic acid (DNA) as the component of gene except ribonucleic acid virus (RNA virus). The information of DNA is transcribed to ribonucleic acid (RNA), and then RNA is translated to protein or peptide based on amino acids (hereafter, because both protein and peptide are formed by poly-amino acids, the term peptide is included in protein, unless otherwise noticed). It was accepted as central dogma that all the intermediate reactions to synthesize proteins were catalyzed by proteinaceous enzymes for a long time. Protein have a role as not only the structural framework of the organism but also the functional molecules regulating biological reaction. Enzyme and antibody, that belong to the kinds of proteins, are typical functional molecules in the cells. Enzyme catalyzes various chemical reaction and antibody recognizes antigen molecule. In principle, these molecules are constructed by amino acids except gluco-proteinaceous and phosphorylated ones and so on. Hence, these molecules are translated from RNA according to the information of gene. As a another functional molecules, there are hormone, local chemical mediator and neurotransmitter at inter- or intra-cellular environment. Some of these materials consist of protein and another consist of derivatives of amino acids. These materials act as chemical signal for inter- or intra-cellular transport of the information by binding to ligand or cell-surface-receptor. The point is that most of *in vivo* functional factor containing enzyme, antibody, hormone, local chemical mediator and neurotransmitter are formed by protein, peptide amino acid, these derivatives.

On the other hand, It had been believed that nucleic acids, that is DNA and RNA, provided no function but information as genome and the only proteinaceous enzymes were the functional motifs. However, in recent year, some of functional nucleotides including catalytic RNA and DNA, and artificial binding motif to ligand (aptamer) were discovered. Catalytic RNAs, known collectively as ribozyme, were discovered in the early 1980s in the group I intron of *Tetrahymena* by Cech's group and as the RNA subunit of ribonuclease P (RNase P) by Altman's group (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 (HDV) ribozymes; and ribosomal RNA (Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Altman, 1989; Cech, 1989; Symons, 1992; Gesteland et al., 1993). All natural ribozymes have highly specific endoribonuclease activities. Moreover, in 1990s, Gold, Szostak, and Joyce and their respective groups developed the method for finding artificial functional DNA and RNA (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold,

1990). This method is called SELEX (Systematic Evolution of Ligands by Exponential enrichment) or *in vitro* genetic selection (Gold et al., 1995; Lorsch and Szostak, 1996; Williams and Bartel, 1996; Breaker, 1997a; Osbirne and Ellington, 1997). Some artificial catalytic DNA and RNA were developed by this method (Beaudry and Joyce, 1992; Bartel and Szostak, 1993; Lorsch and Szostak, 1994; Cuenoud and Szostak, 1995; Dai et al., 1995; Wilson and Szostak, 1995; Lohse and Szostak, 1996; Carmi et al., 1996; Santoroand Joyce, 1997). And artifical DNA and RNA motifs which bind to ligand specifically are discovered by SELEX and these motifs were named aptamer (Ellington and Szostak, 1990; Tuerk and Gold, 1990). In present, these functional RNA motifs has received much attention.

Application of the Functional Protein and RNA

It has been expected that functional protein and RNA are applied to industry including medicine, agriculture and chemistry. As a example of the application for medicine, these protein and RNA are useful for gene therapy and drug delivery system (DDS). The methods for the transfection of the gene using adenovirus and retrovirus vectors have been developed in recent year (Ariga and Sakiyama, 1995). The genes of vectors introduced to cell by the methods express drugs consisting of protein and RNA continuously. Their methods have advantage over usual chemical drug, because medication need not be given one after another for continuous expression of drug *in vivo*. Moreover, functional RNA and protein that can recognize cell-surface-receptor will be expected as the application of DDS. It is possible for DDS to apply to an agricultural chemical. In the example of the application for the field of chemistry, the engineering of catalytic RNA (ribozyme) and protein (enzyme) purposed to bio-degradate environmental hormones, plastics and the materials of the destruction of the environment has been expected. It might be possible that engineered enzyme degrades plastics to non-toxic materials without burning or chemical treatment, because nucleic acids and amino acid are non-toxic materials to environment.

When functional proteins and RNAs are applied to industry, at first I must find or create various functional proteins and RNAs in response to various purposes. Now although gene therapy using already-known molecules has been performed, discovery of unknown functional molecules would progress the possibility of gene therapy further. In fact, the enzymes can degrade plastics, efficiently, constructed by nylon and polystyrene are not found now and there are no enzyme that have the strong power of oxdization or reduction to bio-degradate environmental hormone including dioxine. However, to find aiming novel functional molecules from the would of nature is a hard task. And the probability that a given random sequence polynucleotide or protein chain will fold to form a stable three-dimensional structure with a given ligand binding or catalytic activity is unknown, but is generally thought to

be very low. Accordingly, It is important to make the easy system for screening functional proteins and polynucleotides (DNA, RNA) from a pool of various sequences in the laboratory.

Screening system for RNAs and DNAs

Molecular screening procedures, is essential to the concept of Darwinian evolution, require the integration of two chemical process inevitably; amplification and selection (Figure 1). "Evolutionary molecular engineering" using self-replicative RNAs was proposed in 1984 (Eigen and Gardiner, 1984), and then in 1990, the procedure, based on the concept, for selection of functional RNA sequences *in vitro* from a pool of random sequences had been developed by Gold's, Szostak's, and Joyce's groups at the same time, because RNA, by virtue of its genotypic and phenotypic properties, is a suitable substrate for molecular evolution in the laboratory (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990, Figure 2A). Gold's group isolated high-affinity nucleic acid ligand (that is aptamer) for T4 DNA polymerase and named this procedure SELEX. On the other hand, Szostak's group isolated RNA molecules that bind specifically to a variety of organic dyes from a population of random sequence RNA molecules and call the procedure *in vitro* genetic selection. Joyce's group suggested the simple concept of the method at 1 year before (Joyce, 1989; Robertson and Joyce, 1990). The SELEX and *in vitro* genetic selection methods (hereafter, I calls the method SELEX) allows rapid screening of, typically, $10^{14\text{-}15}$ oligonucleotide sequences to identify rare molecules with suitable binding or catalytic activities against target molecules (Gold et al., 1995; Lorsch and Szostak, 1996; Williams and Bartel, 1996; Breaker, 1997a; Osbirne and Ellington, 1997). 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 cycles. Within the following a few years, various aptamers are derived by SELEX and these ligands often have K_d values in the range 10 pM to 100 pM (Eaton, 1995; Green, 1995 and Yamamoto, 1998). Furthermore, although all wild type ribozymes, including Group I and Group II intron; RNA subunit of RNase P; ribosomal RNA; hammerhead, hairpin and Hepatitis delta virus (HDV) ribozymes, was known as the end-ribonuclease, SELEX promoted the development of novel functional catalytic RNAs (Cech et al., 1981; Guerrier-Takada et al., 1983; Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Altman, 1989; Cech, 1989; Symons, 1992; Gesteland et al., 1993). SELEX provided various functional ribozymes with acyl transferase activity, cleavage activity of amino bond, ligase activity, self-alkyle transferase activity, kinase activity and so on (Beaudry and Joyce, 1992; Bartel and Szostak, 1993; Lorsch and Szostak, 1994; Dai et al., 1995; Wilson and Szostak

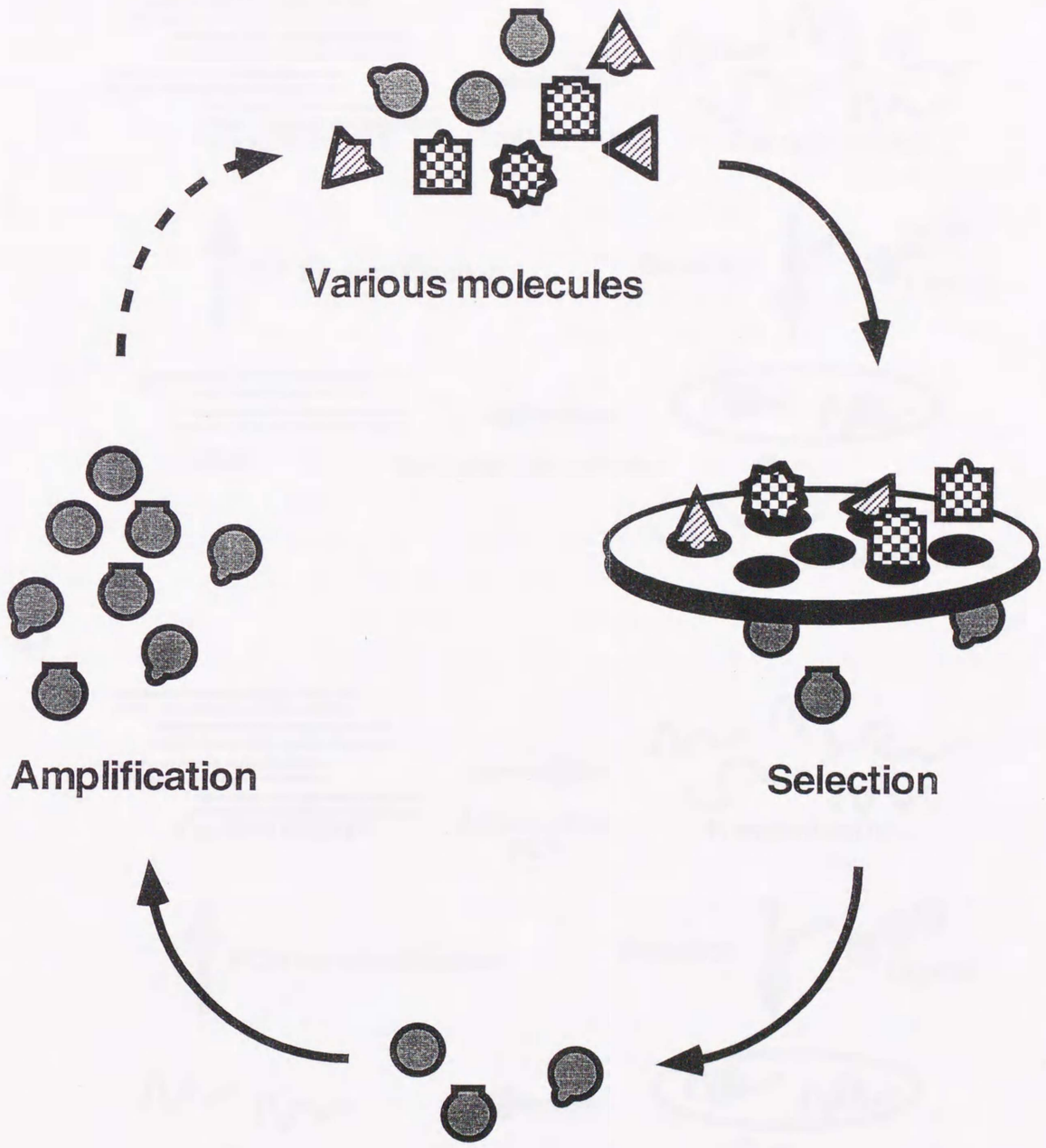


Figure 1. The Concept of the Molecular Evolution. This concept, is essential to the Darwinian evolution, requires the integration of two chemical process ineviably, namely amplification and selection.

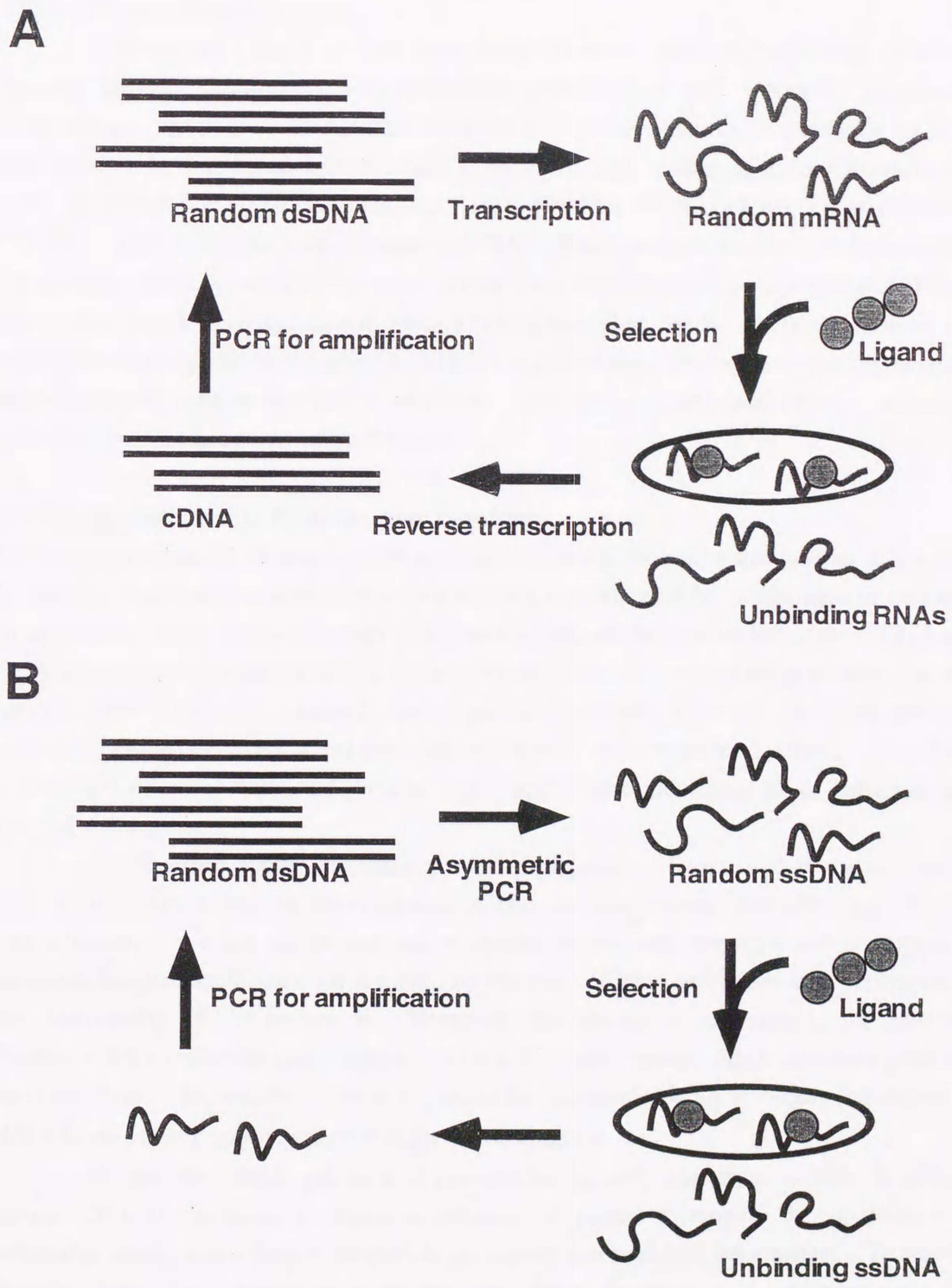


Figure 2. (A) Schematic diagram of *in vitro* genetic selection for RNA. (B) for ssDNA.

1995; Lohse and Szostak, 1996).

Whereas DNA has long been regarded as a passive molecule, identically suited for carrying genetic information but structurally monotonous and therefore functionally impoverished. However, DNA molecules have almost same chemical composition as RNA molecules, and a single-strand DNA could, in principle, approximate the tertiary structure of a RNA (Khan and Roe, 1988; Perreault et al., 1989; Breaker, 1997b). Thus, it was postulated that DNA might form functional structure as RNA. Based on these concept, several attempts to generate single strand DNA enzymes by *in vitro* selection have been successful (Figure 2B). Catalytic DNAs (DNA enzyme) with DNA ligase activity, with self-cleaving activity and with RNA cleaving activity is derived by SELEX and all of these DNAzymes form single strand DNA (Cuenoud and Szostak, 1995; Carmi et al., 1996; Santoro and Joyce, 1997). Almost all DNA enzymes are known as artificial motifs.

Screening System for Proteins and Peptides

Protein and peptide are expected as the excellent functional molecules that avail to diagnostic, therapeutic, and industrial applications, because they consisting of the combination of 20 kinds of amino acids have more complexity and diversity than another molecules, such as DNA and RNA are compose of only 4 kinds of nucleotides, and carry out a wider range of structural and catalytic roles in biology. Indeed, almost cellular functional molecules, including enzyme, antibody, cell-surface-receptor and transcriptional factor, are constructed by them. Therefore, a discovery of novel functional proteins and peptides have the advantage of industrial and medical developments.

As described above, molecular screening procedures, is essential to the concept of Darwinian evolution, require the integration of two chemical process inevitably; amplification and selection. In using the procedures, molecular having both genotype and phenotype is essential, because amplification is achieved by the copy of gene and selection is performed in the functionality of the molecules. Therefore, the procedures are suitable for selecting functional RNA molecules easily against protein and peptide, because RNA have both genotype and phenotype. Indeed, the systems applied to the selection of RNA is already introduced as SELEX and *in vitro* genetic selection at previous paragraph.

On the other hand, selection of proteins having only phenotype is difficult without device. It is the common problems in selection of protein to amplify the information of selected proteins, return them to the pool in next cycles and read their information. To resolve these problems, it is important to couple genotype, that is ribonucleic acid, and phenotype, that is protein, to select proteins. So far the various screening systems to select the specific functional proteins from the pool of them having random sequences of amino acids have been

developed.

Until recently, most approaches to this problem have involved a step in which the DNA is transcribed and translated in *vivo*, and the resulting protein is expressed in such away as to remain physically linked to the encoding nucleic acid, which then is recovered for amplification and further selection. As one of the such approaches, phage display method is famous (Smith, 1985; Harrison et al., 1996; Smith and Petrenko, 1997) (Figure 3). In the case of phage display, the foreign gene sequence coding peptide or protein domain is spliced into the gene for one of the phage coat protein, so that the foreign amino acid sequence is genetically fused to the endogenous amino acids of the coat protein to make a hybrid "fusion" protein. Phages inserted such foreign sequence infect *Escherichia coli* (*E. coli*) cells, and then when they are released from the cell, the hybrid coat protein is incorporated into phage particles, so that the foreign peptide or protein domain is displayed in the outer surface. Because this phage have both DNA and displayed peptide coding the DNA, that is both genotype and phenotype, DNA can be collected after selection against displayed peptide. Another example of *in vivo* approaches include plasmid display, and completely *in vivo* genetic approaches (Schatz et al., 1996; Moore and Arnold, 1996; Harada et al., 1996; Boder and Wittrup, 1997; Zhang et al., 1997). However, the most of systems are not able to retain the diversity of pool, because they have the process based on living cells in order to link phenotype to genotype. In the case of phage display, diversity of pool of proteins is about only population of 9 orders.

More recently, two types of selection techniques of special protein (peptides or antibody) *in vitro* (cell-free) system, so-called ribosome display (polysome display) system and protein-RNA covalent binding system, have been reported (Mattheakis et al., 1994; Mattheakis et al., 1996; Hanes and Pluckthun, 1997; He and Taussig, 1997; Nemoto et al., 1997; Roberts and Szostak, 1997; Hanes et al., 1998; Makeyev et al., 1999; Hanes et al., 1999) (Figure 4). In these approaches, multiple rounds of selection cycles, that DNA library is transcibed, and the RNA is translated, protein with RNA having the information of amino acid sequences is selected, selected RNA is reverse transcribed and amplified, are repeated to enrich functional proteins. These approaches can follow amino acid sequences of population of 12 orders in theory. In the former case, ribosome forms the complex along with protein and ribonucleic acid, and complex is retained by eliminating stop codon. In the latter case, protein binds covalently to ribonucleic acid which is ligated to DNA labeled by puromysin at 3' end. In both cases, protein-RNA complex must be formed stably and largely *in vitro* to combine genotype and phenotype, thereby selecting functional proteins efficiently.

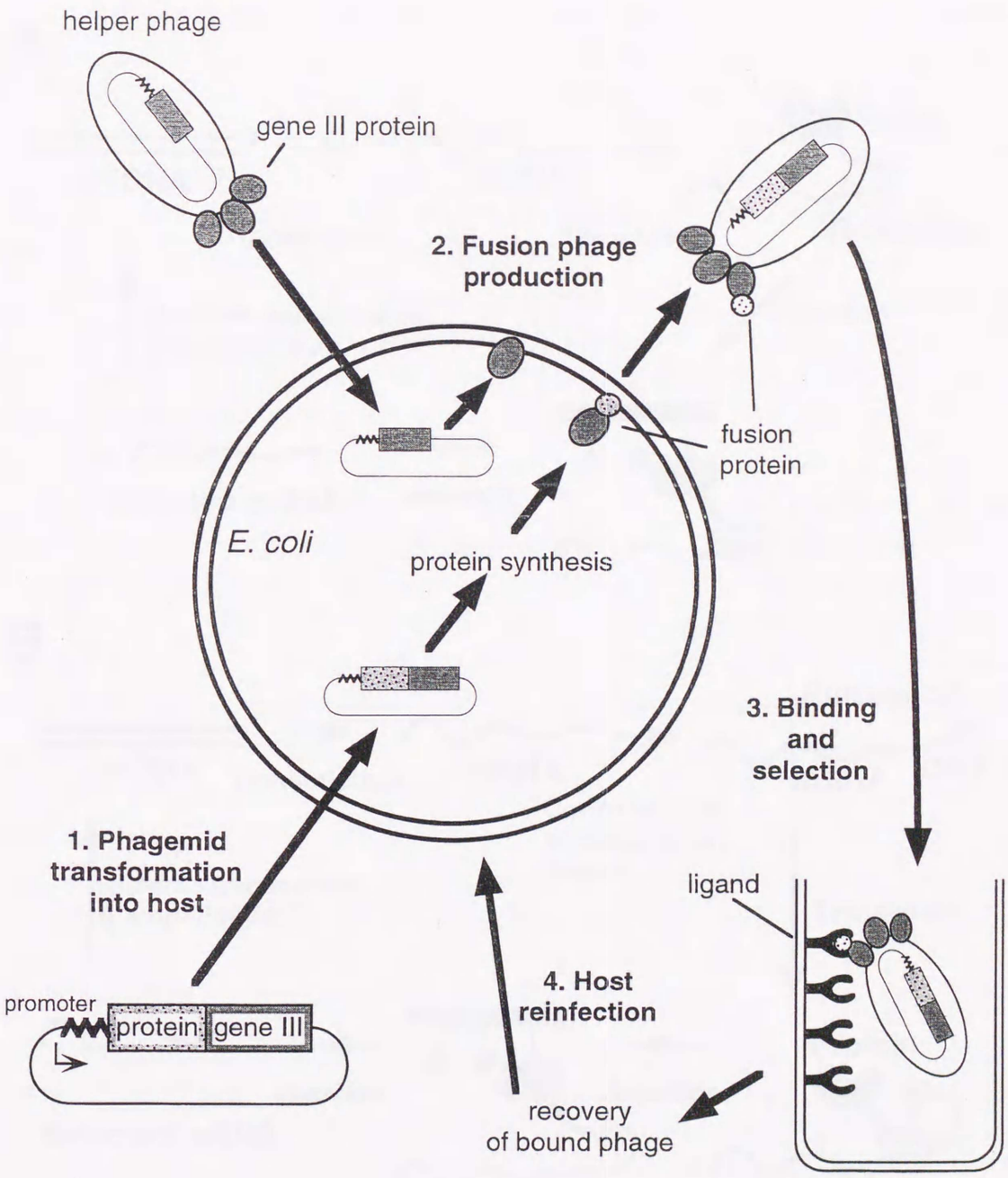


Figure 3. Schematic diagram of phage display method. A combinatorial library encoding the protein is inserted into a phagemid vector such that the resulting protiens become fused to the gene III coat protein ot filamentous bacteriophage. The library is transformed into an *E. coli* host. A pool of phage displaying is generated by infectingthe host with helper phage. This phage pool is allows to bind to ligand. Phage bound to the ligand are used to infect *E. coli*, allowing the cycle of amplification and selection to be repeated.

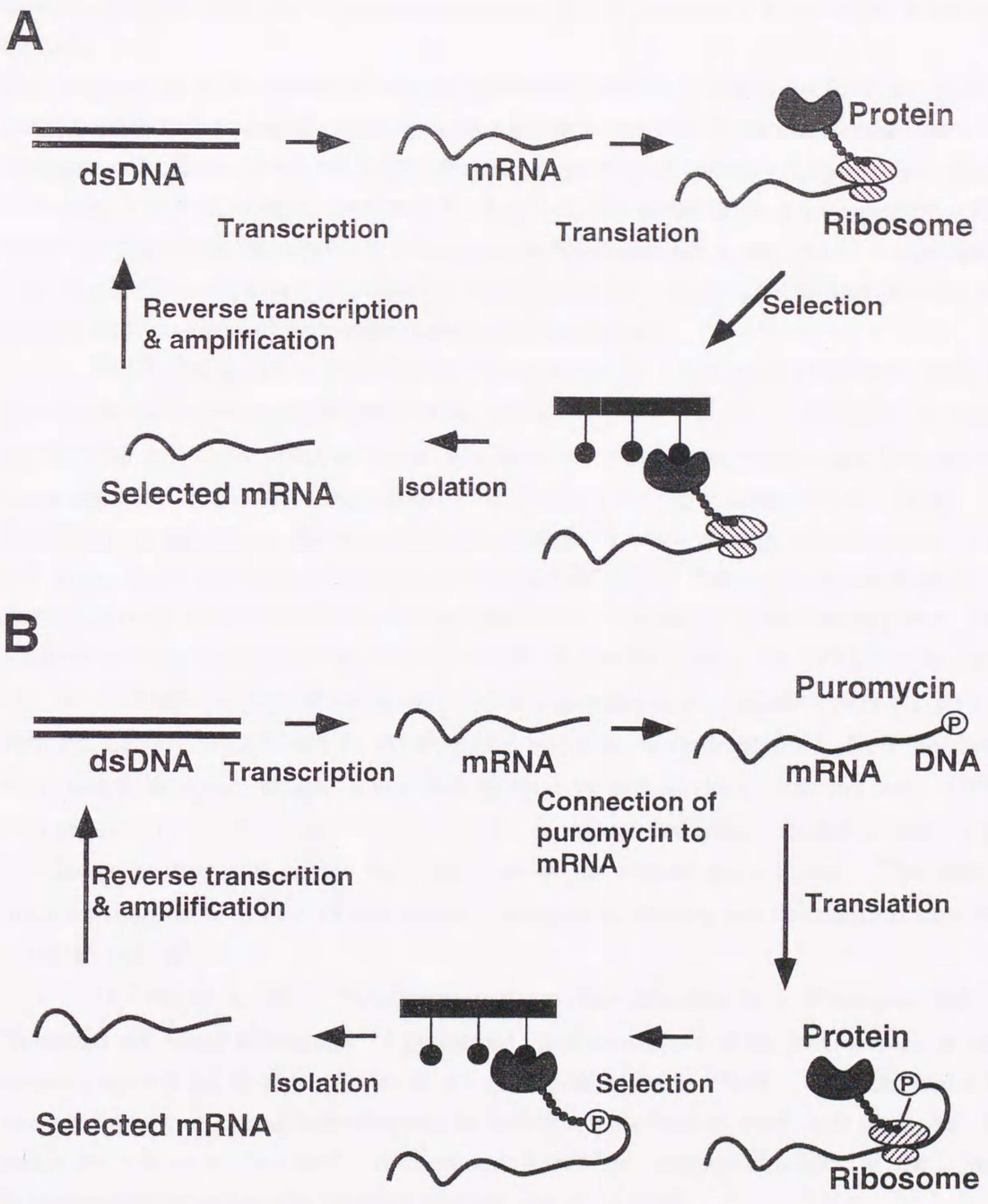


Figure 4. Schematic diagrams of screening system for protien *in vitro*. Multiple rounds of selection cycles, as shown upper diagrams, are repeated to enrich fouctional proteins. (A) Ribosome display system. (B) protein-RNA covalent binding system.

Introduction of Rational Screening Systems for Selection of Functional RNA and Protein

The thesis describes the rational screening system for selection of functional RNA and protein. Functional RNA and protein (peptide) motifs including ribozyme, aptamer, enzyme, antibody, hormone and cellular factor are useful for the application of industry, as described above. Especially, I expects to apply functional RNA and protein as the drug of gene therapy. It is advantage that vector introduced to cell expresses functional RNA and protein continuously. Accordingly, It is important to make the easy system for screening functional proteins and nucleotides from a pool of various sequences in the laboratory.

In Chapter 2, title is "Discrimination of a single base change in a ribozyme using the gene for dihydrofolate reductase (DHFR) as a selective marker in *E. coli*., I describes the model of screening system for RNA motif *in vivo* that was designed to identify new hammerhead ribozymes with high activity (Fujita et al., 1997; Fujita et al., 1998; Hamada et al., 1999). As describe above, ribozyme, that is one of functional RNA, were discovered in the early 1980s and various types of ribozyme have been identified from now. Natural ribozymes have RNA-cleavage activity and exhibit high substrate specificity. Therefore, ribozymes appear to have potential as tools for suppressing the expression of specific genes. In 1990, for the rapid selection of functional sequences *in vitro* from a population of random sequences, a novel methods, namely SELEX and *in vitro* genetic selection, were developed. New functional ribozymes with ligase, kinase, amino-acid cleavage or self-alkylating activities have already been selected by this procedure. However, the functional ribozymes selected *in vitro* might not always be the same as the best ribozyme in the cellular environment. Therefore, I constructed the novel *in vivo* system which is designed to identify new hammerhead ribozyme with high affinity.

In Chapter 3, title is "Comparison of *In vivo* Activities of 5'-Connected and 3'-Connected *cis*-Acting Ribozymes", I performed the improvement of the previous our *in vivo* screening system for ribozyme (Fujita et al., 1998; Hamada et al., 1998). I constructed a 3'-connected ribozyme system and compared its activities with those of the 5'-side ribozyme, that means the system in Chapter 2, to examine whether the cleavage efficiency of the 3'-side ribozyme might be affected by potential protection by polysomes.

In Chapter 4, title is "Extremely high affinity between tandemly connected, Tat-derived peptides and Tat aptamers", I describes the development of the adapter for interaction motifs to link RNA and protein (Fujita et al., Submitted) The development of adapter for strongest interaction between mRNA and protein is inevitable to construct our rational protein selection *in vitro*, described in Chapter 5. To construct a strong binding mediator, it is effective to link

plural protein motifs and plural RNA motifs respectively. In previous study, our laboratory found a novel artificial RNA motif, namely Tat-aptamer, that binds specifically to trans-activator protein (Tat) of Human Immunodeficiency Virus Type 1 (HIV-1) with high affinity (Yamamoto et al., 1997; Yamamoto et al., 1998). Because the interaction between Tat-aptamer to Tat protein is extremely high, this interaction might be useful for linking strongly between protein and RNA. Moreover, I examined tandem linking of plural Tat-aptamers and plural Tat-derived peptide containing arginine-rich basic region in order to emphasize the interaction.

In Chapter 5, title is "Approach for *in vitro* rational protein selection using dihydrofolate reductase (DHFR) as model protein", I suggests the rational protein screening system for protein and peptide *in vitro*. As described above, 2 kinds of *in vitro* screening methods for protein, namely ribosome display and protein-RNA covalent binding systems, already have been developed. In the former case, ribosome forms the complex along with protein and ribonucleic acid, and complex is retained by eliminating stop codon. In the latter case, protein binds covalently to ribonucleic acid which is ligated to DNA labeled by puromysin at 3' end. In these approaches, multiple rounds of selection cycles, that DNA library is transcibed, and the RNA is translated, protein with RNA coding the information of amino acid sequences is selected, selected RNA is reverse transcribed and amplified, are repeated to enrich functional proteins. Namely, when protein is selected, mRNA coding the information of the protein is collected too. RNA is reverse-transcribed and amplified for reading the information of the selected protein.

In the selection of protein, selection from the pool containing diverse amino acid sequences is needed. These *in vitro* methods are better than the some *in vivo* methods, because the extension of the diversity of the pool is possible. These methods can follow amino acid sequences of population of 12 orders in theory, although the diversity of *in vivo* system is 9 orders. But, each *in vitro* system have another defects. In ribosome display, the retaining of the RNA-ribosome-protein complex is difficult. In protein-RNA covalent binding systems, in each cycles, synthesis for linking between puromycin and mRNA is difficult.

In our study, I engineered novel RNA and peptide motif. peptide motif binds to RNA motif with high affinity and specificity. In Chapter 5, I suggests that these motifs are used as adapter to form strong protein-RNA linkage without chemical synthesis for the selection of protein. The advantage of our method is that (i) Synthetic step is none, because the tools for linking between RNA and protein are ribonucleic acid and amino acid perfectly and that (ii) RNA-protein directly complex is formed.

Chapter 2

A Novel System for Selection of Intracellularly Active Ribozyme Using the Gene for Dihydrofolate Reductase (DHFR) as a Selective Marker in *Escherichia Coli*

2.1 Summary

For use of ribozymes *in vivo*, selection of functional ribozymes in the cellular environment (in the presence of inhibitory factors and limited concentrations of mandatory Mg^{2+} ions, etc.) is desirable. As a first step towards this goal, I developed a new screening system for detection *in vivo* of an active ribozyme from pools of active and inactive ribozymes using the gene for dihydrofolate reductase (DHFR) as a selective marker. In our DHFR expression vector, the sequence encoding either the active or the inactive ribozyme was connected to the DHFR gene. The plasmid was designed such that, when the ribozyme was active, the rate of production of DHFR was high enough to endow resistance to trimethoprim (TMP). I demonstrated that the active ribozyme did indeed cleave the primary transcript *in vivo* whereas the inactive ribozyme had no cleavage activity. 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 the inactive-ribozyme-coding plasmid. Consequently, when cells were transformed by a mixture that consisted of active- and inactive-ribozyme-coding plasmids at a ratio of 1:1, (i) mainly those cells that harbored active ribozymes survived in the presence of TMP and (ii) both active- and inactive-ribozyme-harboring cells grew at an identical rate in the absence of TMP, a demonstration of a positive selection system *in vivo*. If the background "noise" could be removed completely in the future, it might usefully complement existing selection systems *in vitro*.

2.2 Introduction

Ribozyme and antisense technologies appear to have potential as methods for suppressing the expression of specific genes (Cameron and Jennings, 1989; Sarver et al., 1990; Uhlmann and Peyman, 1990; Erickson and Izant, 1992; Gray and Cedergren, 1992; Heidenreich and Eckstein, 1992; Murray, 1992; Ojwang et al., 1992; Rossi, 1992). Therefore, it is hoped that they will be powerful tools in gene therapy for some diseases caused by aberrant gene expression, including diseases caused by infectious agents such as HIV (human immunodeficiency virus) (Sarver et al., 1990; Heidenreich and Eckstein, 1992; Ojwang et al., 1992; Leavitt et al., 1995). There are several strategies for inhibition of the expression of specific genes during transcription and translation (Blume et al., 1992; Roy, 1993; Choo et al., 1994; Gee et al., 1994; Mayfield et al., 1994). The hammerhead ribozyme belongs to the class of molecules known as antisense RNAs (hereafter, the term ribozymes refers exclusively to hammerhead ribozymes unless otherwise noted) (Pyle, 1993; Dahm et al., 1993; Uebayasi et al., 1994; Sawata et al, 1995; Kumar et al., 1996; Amontov and Taira 1996; Zhou et al., 1996a; Zhou et al., 1996b; Bassi et al., 1995; Orita et al., 1996). However, because of 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 for therapeutic purposes (Sarver et al., 1990).

When the hammerhead ribozyme was engineered in such a way that it can cleave a specific RNA sequence "in *trans*" (Uhlenbeck, 1987; Haseloff and Gerlach, 1988), it was postulated that it 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 of their instability and their lower than expected activities *in vivo*, ribozymes have not yet proven their superiority to antisense molecules. There seem to be several reasons for their low activity *in vivo*: (i) there may be many cellular proteins *in vivo* that inhibit their catalytic activity (Parker et al., 1992; Taira and Nishikawa 1992); (ii) the intracellular concentration of Mg^{2+} ions is much lower than that used *in vitro* for testing the ribozyme activity (Silver and Clark, 1971; Flatman, 1984; Romani and Scarpa, 1992); and (iii) several cellular RNases contribute to their instability (Olsen et al., 1991; Pieken et al., 1991; Heidenreich and Eckstein, 1992; Paolella et al., 1992; Taylor et al., 1992; Shimayama et al., 1993). In order to overcome some of these problems, many approaches have been made, including some attempts to select active ribozymes *in vitro* (Beaudry and Joyce, 1992; Pan and Uhlenbeck, 1992; Lehman and Joyce, 1993; Nakamaye and Eckstein, 1994; Cuenoud and Szostak, 1995; Ishizaka et al., 1995). The drawback to selection *in vitro* is that the activity *in vitro* does not always reflect the activity *in vivo* (Denman et al., 1994).

Moreover, selection systems *in vitro* always involve reverse transcription. The activity of the ribozyme is associated with its specific structure but reverse transcriptase activity is known to be inhibited by some secondary structures (Tuerk et al., 1992). Therefore, there is always a risk of missing the most effective ribozymes during selection *in vitro*.

Because of these limitations to screening systems *in vitro*, I needs to design a screening system *in vivo* whereby selection can be made under the cellular conditions under which ribozymes must be active. As a first step towards the development of a screening system *in vivo*, I used the gene for dihydrofolate reductase (DHFR) as a selective marker in *Escherichia coli (E. coli)*. The addition by DHFR of a methyl group to deoxyuridine 5'-monophosphate (dUMP) to form thymidylic acid is an important reaction in DNA synthesis (Blakley and Benkovic, 1985). Since DNA synthesis is required by all proliferating cells, inhibition of DNA synthesis is one of the most effective ways of controlling cell division. Several drugs, such as trimethoprim (TMP) and methotrexate (MTX), 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). When an inhibitor of DHFR, such as TMP, is present in the culture medium at a certain concentration, DHFR-producing clones, which have had already been transfected by a DHFR-expressing vector, are expected to survive and grow more rapidly than non-expressing clones (Iwakura et al., 1983). Therefore, if I can control the level of expression of the DHFR gene by a ribozyme, I should be able to determine the activities of ribozymes in terms of resistance to TMP (TMP^r). Namely, TMP^r should be a function of ribozyme activity that can, in turn, be estimated from the concentration of TMP in the culture medium. I reports here that clones that survived at a fixed concentration of TMP harbored mostly active ribozymes. Moreover, this selection system successfully identified a single base change *in vivo* and, therefore, to the best of our knowledge, this is the first report that suggests the possibility of positive selection *in vivo* of functional ribozymes.

2.3 Materials and Methods

Bacterial Strains and Plasmids.

E. coli HB101 (*rec*A13, *sup*E44; Takara Shuzo Co., Kyoto) was used as a recipient for transformation. Several ribozyme expression vectors were constructed by modifying the DHFR expression vector pTZDHFR20 (Iwakura et al., 1995).

Synthesis of Oligonucleotides and Construction of Plasmids.

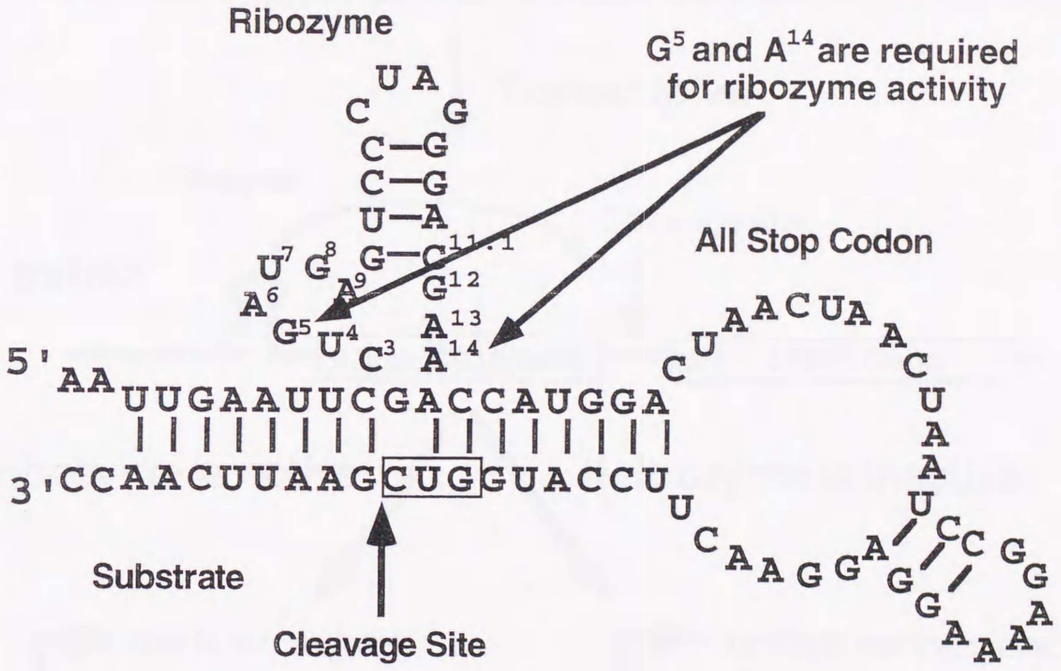
Oligodeoxynucleotides [active-ribozyme linkers (forward, 5'- AGC TTA ACT AAT TGA ATT CCT GAT GAG TCC CTA GGG ACG AAA CCA TGG ACT AAC TAA CTA AT -3'; and the corresponding reverse sequence), pseudo-ATG linkers (forward, 5'- CCG GAA AAG GAG GAA CTT CCA TGG TCG AAT TCA ACC TAT ATG ATC AGT CTG ATT GCG GCG -3'; and reverse), 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)] were synthesized with a DNA synthesizer (model 392; Applied Biosystems, Foster City, CA) and purified by chromatography. Single base change (G^5 to A^5, or A^{14} to G^{14}) was introduced within the active-ribozyme catalytic core (Figure 1). These changes had been already shown to destroy cleavage activity (Ruffner et al., 1990; Inokuchi et al., 1994). Each linker was "tailed" with a recognition sequence for an appropriate restriction endonuclease. Each oligonucleotide linker was denatured at 95℃ 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 (Figure. 2).

Composition of Culture Medium.

Luria-Bertani's broth-modified (LBM) plates, containing polypeptone, yeast extract, NaCl, and 16 mM $MgSO_4$, were used for experiments to check the growth rate of individual clones. For the incubation of transformed *E. coli* cells on LBM plates, the medium contained ampicillin (Amp) (100 μg/ml) and/or TMP (70 μg/ml).

Northern Blot Analysis.

Plasmid vector pTZDHFR harboring both a ribozyme and a DHFR gene was used to transform *E. coli* HB101. After overnight incubation at 37 ℃, total RNA was isolated with ISOGEN™ (Nippongene Co., Toyama) from 2 ml of cell culture in 2x YT medium. Ten micrograms of total RNA per sample were denatured in glyoxal/dimethyl sulfoxide, subjected to electrophoresis in a 1.8 % Metaphor™ agarose (FMC Inc.) gel, and transferred to a Hybond-N™ nylon membrane (Amersham Co.) (Sambrook et al., 1989). 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 has been labeled with ^{32}P using T4 Polynucleotide kinase (Takara Shuzo). Prehybridization and hybridization were performed in the same solution (5x SSPE, 50% formamide, 5x Denhardt's solution, 0.5% SDS, 150 μg/ml calf thymus DNA). Final washing was performed in 0.1x SSPE, 0.1% SDS at 70 ℃ for 30 min.



Active Ribozyme

Figure 1. Secondary structure of an active ribozyme. A single point mutation (G^5 to A, or A^{14} to G; circled) eliminates the ribozyme activity. It is to be noted that the catalytic loop containing G^5 and A^{14} captures Mg^{2+} ions since a hammerhead ribozyme is a metalloenzyme.

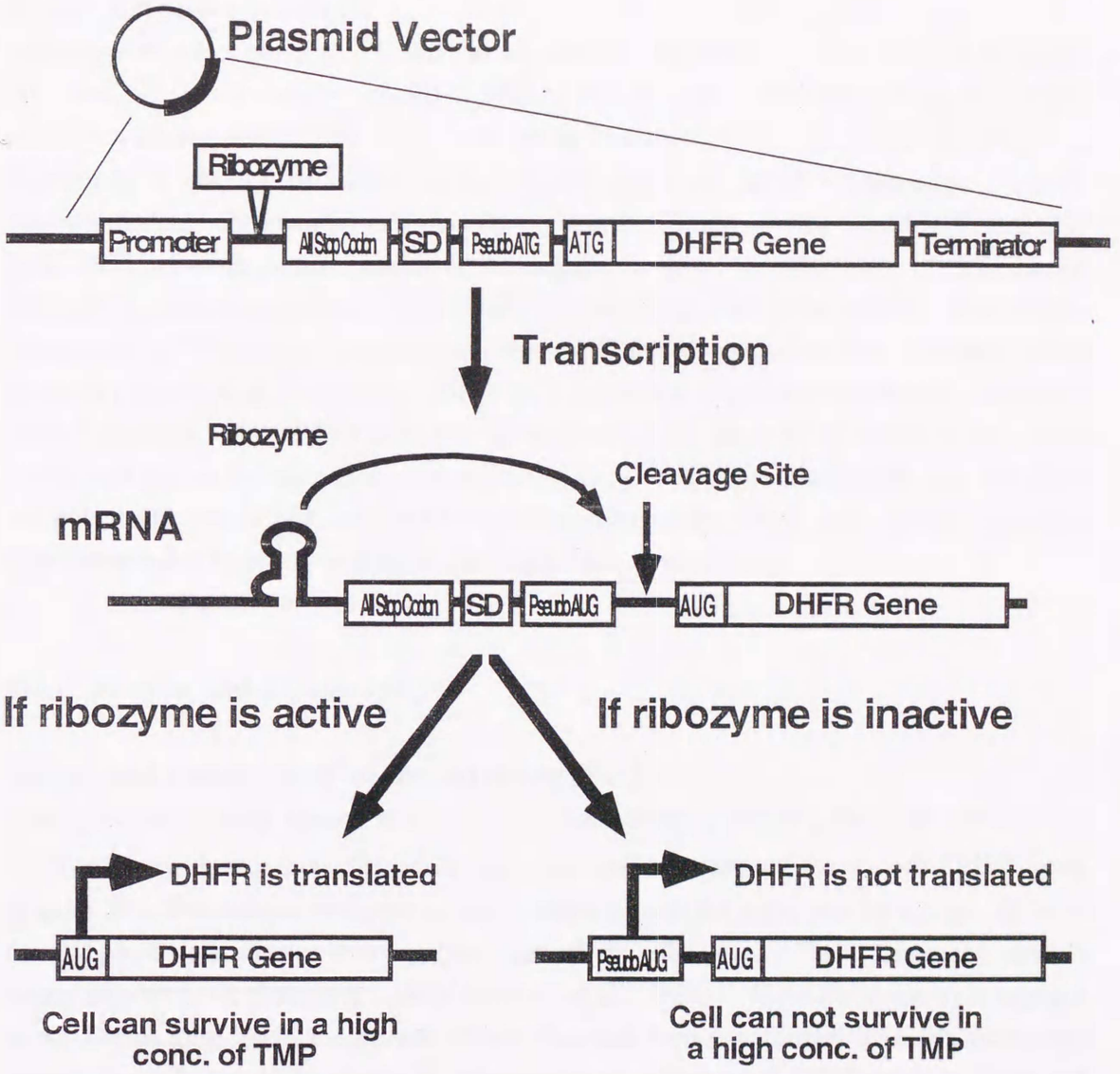


Figure 2. The ribozyme-connected DHFR expression vector. The plasmid vector has two ATG codons, one of which is a pseudo-initiation codon, located upstream of the authentic ATG which is the initiation codon for the DHFR gene. If an active ribozyme is introduced upstream of the DHFR-coding region and 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 primary transcript starts translation at the pseudo-initiation codon, which is associated with a strong Shine-Dalgano (SD) sequence and is out of frame with respect to the DHFR gene.

Primer Extension Analysis.

An aliquot of a 0.2 pmol of [^{32}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 by heating at 65 ℃ 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 containing 30 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 8 mM DTT, 0.8 mM each dNTP, 6 units human placental ribonuclease inhibitor, and 80 units of SuperScript RNaseH$^-$ reverse transcriptase (Gibco BRL, Gaithersburg, MD) were added. The reverse transcription (RT) reactions were carried out at 42 ℃ for 60 min to avoid the 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 from the same [^{32}P]-labeled primer were fractionated together, creating sequencing ladders as markers.

2.4. Results and Discussion

Design and Construction of the Screening Vectors.

I designed a screening system in *E. coli*. In our screening vectors, either an active or an inactive ribozyme sequence (Figure 1) was connected upstream of the *E. coli* DHFR gene (Figure 2). The inactive ribozyme sequence differed from the active one by a single G^5 -› A (or A^{14} -› G) mutation within catalytic core of the ribozyme. These mutations abolish ribozyme activity (Ruffner et al., 1990; Inokuchi et al., 1994). If the ribozyme were targeted to the DHFR gene itself, the growth of cells that had been transformed by active-ribozyme-coding plasmids should be slower in the presence of inhibitors of DHFR such as TMP and MTX. Then, clones surviving in the presence of TMP or MTX would turn out to have inactive-ribozyme-coding sequences, with resultant negative selection. Since it is desirable to select colonies that possess active ribozymes (positive selection), when I designed our vectors, the ribozyme was not targeted to the DHFR gene itself but to the region, designated the interspace, between two ATG codons (Figure 2), one of which was the original initiation codon of the DHFR gene itself and the second one of which was located upstream of the original initiation codon. The second ATG was associated with a strong Shine-Dargarno (SD) sequence and was out of frame relative to the DHFR gene. Therefore, because of the strong SD sequence associated with the upstream pseudo-ATG, the primary transcript would not produce a significant amount of DHFR. Only when the active ribozyme had cleaved the

sequence between the two ATG codons, would the control of translation by the pseudo-ATG with a strong SD sequence be abolished and the original ATG would lead to production of DHFR.

In order to avoid any readthrough from the upstream regions, an "all stop codon" sequence (TAA CTA ACT AA) was introduced between the ribozyme and SD sequences. In this region, a stop codon would be encountered in all three possible frames. Furthermore, in order to facilitate the analysis of transcripts, a terminator sequence (Yanofsky, 1981; Iwakura and Tanaka, 1992) was introduced downstream of the DHFR gene. Then, if the active ribozyme were to attack the so-called interspace and cleave the primary transcript, which would consist of both the ribozyme and the DHFR genes connected in tandem, the resulting truncated (ribozyme-free) DHFR mRNA would potentially be detectable by Northern blot analysis.

Discrimination of Active Ribozymes from Inactive Ribozymes 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), I constructed an active ribozyme-screening system. Among several concentrations of TMP tested, I found that at 70 μg TMP/ml culture medium, *E. coli* clones that had been transformed with the active- ribozyme-expression vector grew more rapidly and made larger colonies as compared to the clones with the inactive ribozymes. Figure 3 shows the difference in growth rates between the active- and inactive-ribozyme-expressing colonies at 27 ℃ and 37 ℃. Since the *E. coli* strain HB101 used in this study produces a low level of endogenous DHFR, formation of background colonies could not be avoided. Since the difference in growth rates between the active- and inactive-ribozyme-expressing colonies was greater at 27 ℃ than at 37 ℃ (Figure 3B), selection of active ribozymes described below was made at 27 ℃ in the presence of 70 μg TMP and 100 μg Amp per milliliter.

Since active-ribozyme-expressing colonies grew more rapidly, as expected, than inactive-ribozyme-expressing colonies, I carried out a random screening assay according to the procedure outlined in Figure 4. In this assay, equimolar amounts of active- 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 Amp- (100 μg/ml) containing plate or on an Amp- (100 μg/ml) and TMP- (70 μg/ml) containing plate. After incubation for one or more days, rapidly growing colonies were picked up at random from both plates. In order to check the reproducibility, I picked up only 10 colonies from each plate per day. Then, after minipreparation of plasmid DNA, sequences of the ribozyme regions of the selected clones were determined. Table 1 summarizes the

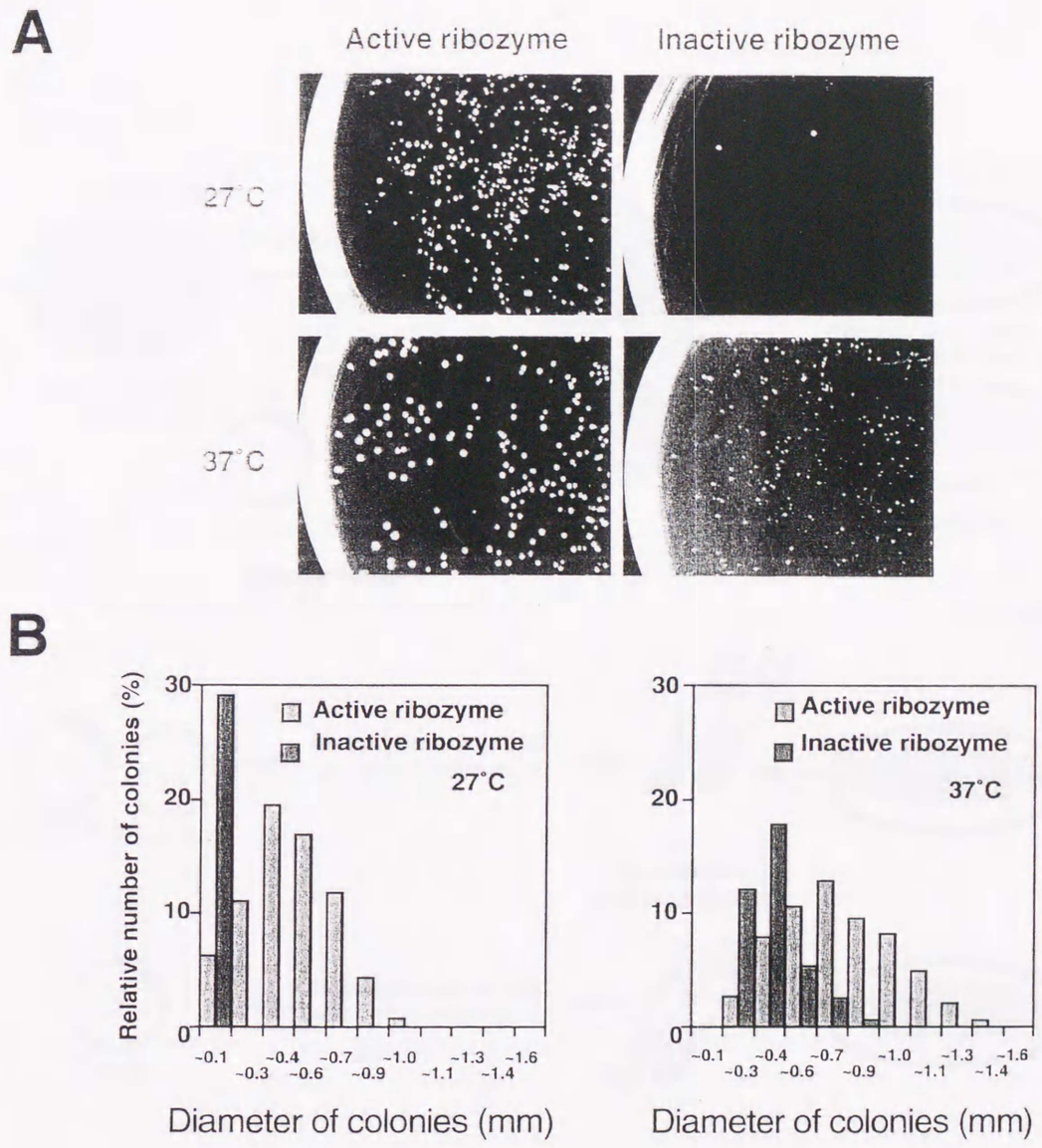


Figure 3. (A), Colonies of *E. coli* HB101 cells that were transformed with the active- (right) or inactive- (left) ribozyme-expression plasmid. In the presence of 70 mg/ml TMP, colonies expressing active ribozymes (right) grew faster than colonies (left) that expressed inactive ribozymes. The difference in growth rates between the active- and inactive-ribozyme-expressing colonies was greater at 27 °C (top) than at 37 °C (bottom). (B), Distribution of colonies according to their colony-size. About 4,000 colonies appeared in Figure 3A were classified into 11 classes based on the diameter of colonies. The difference in growth rates between the active- and inactive-ribozyme-expressing colonies was greater at 27 °C (left) than at 37 °C (right). Since the E. coli strain HB101 used in this study produces a low level of endogenous DHFR, formation of background colonies could not be avoided.

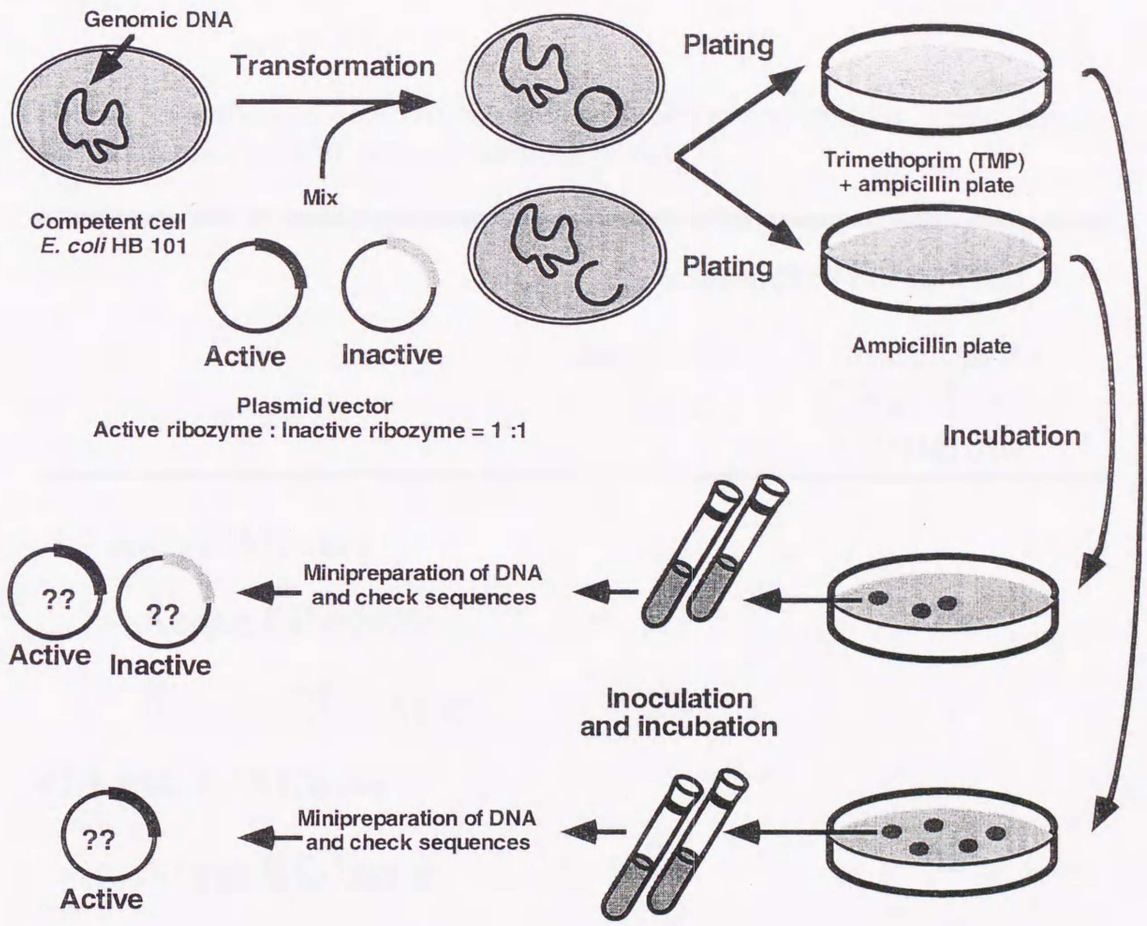


Figure 4. Schematic diagram of the *in vivo* selection system. Competent cells are transformed with a mixture of equimolar amounts of active- and inactive-ribozyme-expression plasmids. In the absence of selection pressure (Amp plate), both active- and inactive-ribozyme-expressing colonies are expected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

Table 1. Numbers of selected colonies with active and inactive ribozymes on TMP-containing and/or Amp-containing plates.

	5'- Connected Ribozyme	
	Ampicillin Plate	Trimethoprim (TMP) Plate (70 μg/ml)
G^5 and A^5 Mixture		
Active RIbozyme	29	75
Inactive RIbozyme	28	1
G^{14} and A^{14} Mixture		
Active RIbozyme	21	42
Inactive RIbozyme	9	2

Plates were incubated at 27 °C for 2-3 days, then larger colonies were picked up at random. TMP plates contained 70 μg of TMP and 100 μg of Amp per milliliter, and Amp plates contained 100 μg of Amp per milliliter without TMP.

sequencing results for the selected clones from more than seven independent experiments. Clones selected in the presence of TMP harbored mainly active ribozymes: in the case of G^5 and A^5 mixture, only one out of seventy-six sequences turned out to be an inactive ribozyme sequence. By contrast, clones selected in the absence of TMP (in the presence of only Amp) yielded active and inactive sequences at a ratio of 1:1. Similar results were obtained in the case of A^{14} and G^{14} mixture.

In order to confirm that the phenotypic difference shown in Table 1 really originated from a single base change and not from any other mutations within the DHFR gene, I sequenced several clones in their entirety, including the DHFR region and I further exchanged the *Hin*dIII-*Acc*III fragment (see Figure 2) that contained the ribozyme sequence between the selected active and inactive clones. Since (i) no mutation was detected in the DHFR gene and (ii) the exchanged construct had the opposite phenotype, I could conclude that the phenotypic difference presented in Table 1 originated from a single base mutation. Therefore, I confirmed that the selection pressure of TMP was useful for identification of a single base change within the catalytic core of the ribozyme, which was correlated with ribozyme activity, which, in turn, was correlated with the level of expression of DHFR.

Detection of a Cleaved Fragment by Northern Blot Analysis.

In order to confirm that the phenotypic difference was associated with the cleavage activity of the ribozyme, Northern blot analysis was carried out with total RNA from *E. coli* HB101 cells that had been transfected with ribozyme expression vectors. Northern blot analysis is the most direct method for identifying cleavage activities of ribozymes *in vivo*. However, since cleaved fragments tend to undergo rapid degradation *in vivo*, Northern blot analysis failed in the past to detect cleaved fragments (Sioud and Drlica, 1991; Ferbeyre et al., 1995). Our results of 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 I analyzed the total RNA extracted from cells that contained the inactive ribozyme vector (lane 2). Although the inactive ribozyme lane (lane 2) appears to show a weak signal at the size of the truncated fragment, this is not the cleavage product as will be evidenced by the primer extension analysis (Figure 6). The identification of the bands was based on mobility of RNA size-markers.

Why did I detect the cleaved fragments when others have failed? In our case, the ribozyme target site was located upstream of the DHFR gene (Figure 2). Therefore, the DHFR mRNA itself remained intact before and after the ribozyme-mediated cleavage. Thus, the

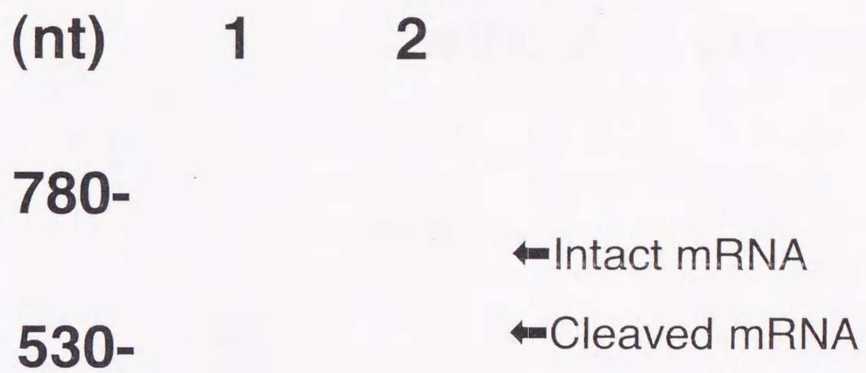


Figure 5. Northern blot analysis. Ten micrograms of total RNA from *E. coli* cells, transformed with the ribozyme-DHFR expression vector shown in Figure 2, were subjected to electophoresis in 1.8% Metaphor™ agarose. After transfer to a membrane filter, the RNA was allowed to hybridize with the synthetic oligonucleotide probe (40-mer), which was complementary to part of the DHFR gene. Lane 1: active ribozyme, with G^5 at the catalytic core. Lane 2: inactive ribozyme, with A^5 at the catalytic core. The active ribozyme expression vector produced the excised short fragment (lane 1) but there was no truncated fragment in lane 2, which originated from the inactive ribozyme expression vector. Lane 1 also shows the intact primary transcript. Fragment sizes were consistent with the expected lengths, estimated from a standard curve for mobilities of RNA size-markers. The numbers indicate the length of fragments in nucleotides determined by using size markers (not shown).

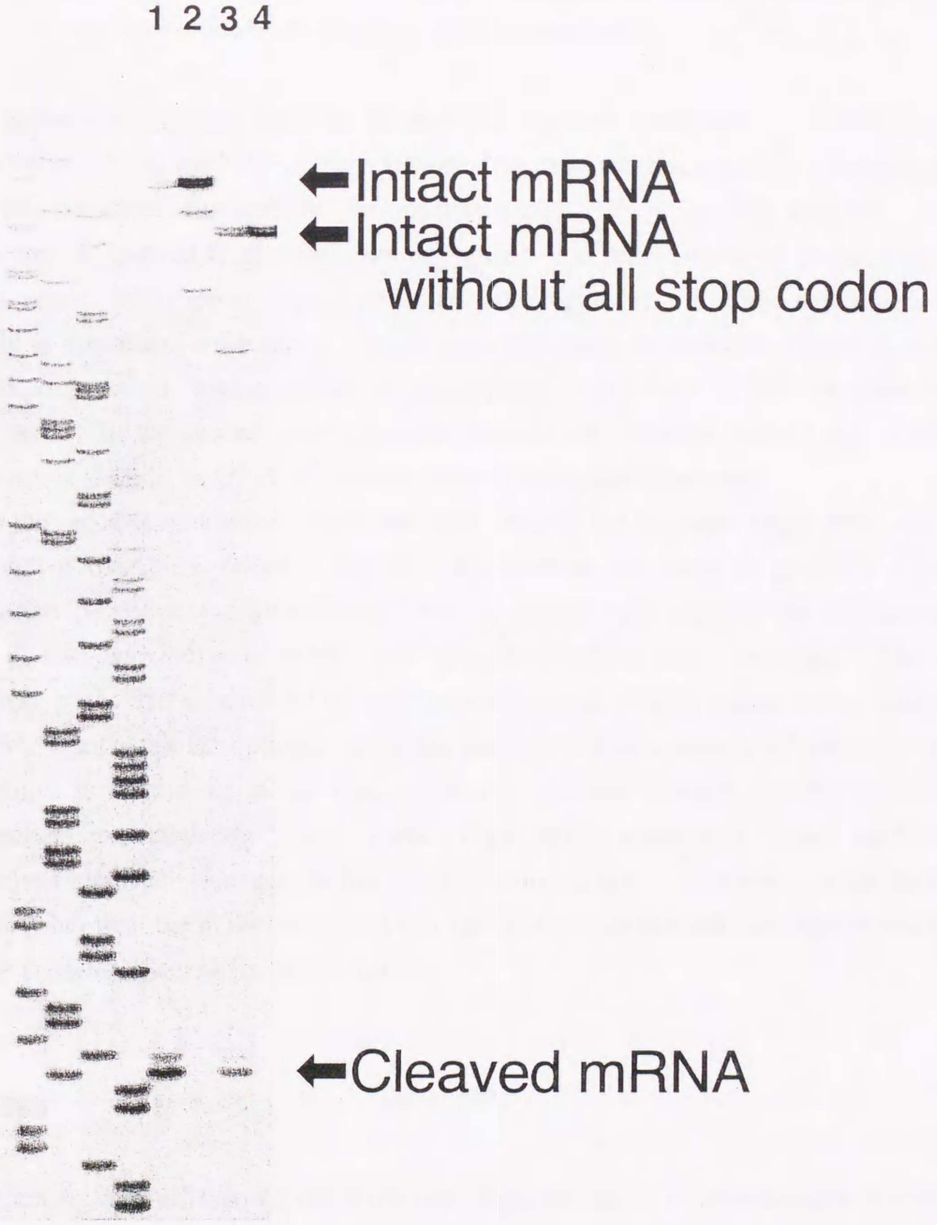


Figure 6. Primer extension analysis. Five micrograms of total RNA were used as template for reverse transcription, with a 5'-end-labeled synthetic oligonucleotide primer. After transcription, the labeled transcribed product was subjected to electrophoresis on an 8% polyacrylamide gel. Lane 1: active 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 seen in lane 2 and lane 4. The exact site of cleavage was determined by reference to the sequencing ladders.

protection from digestion by RNases "stored" within the sequence of DHFR mRNA, did not change after the cleavage (protection by the binding of ribosomes, etc.).

Identification of the Cleavage Site by Primer Extension Analysis. Although the sizes of intact and cleaved mRNAs (Figure 5) were determined to be correct by reference to RNA size-markers, the exact cleavage site was not determined by Northern blot analysis. In order to confirm that the cleaved fragment shown in Figure 5 was really produced by the action of the ribozyme, primer extension analysis was carried out (Figure 6). In these experiments, two different sets of constructs were used. In one case, the plasmids shown in Figure 2, that contained the "all stop codon" region and either active (lane 1) or G^5 -> A^5 inactive (lane 2) ribozyme were used. In the second case, plasmids without the "all stop codon" region but having either an active (lane 3) or G^5 -> A^5 inactive (lane 4) ribozyme were used.
As judged from the sequencing ladders on the left side, exactly the expected target sites were cleaved by the active ribozymes (lanes 1 and 3). By contrast, no cleavage products were detected with inactive ribozyme constructs (lanes 2 and 4), this strongly supports the conclusion for Figure 5. A one-base-longer fragment 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 incorporates one extra nucleotide independently of the template (Frohman, 1990). It should be noted that, since the reaction mixture for the reverse transcriptase reaction contained Mg^{2+} ions, parts of the initial transcripts (intact mRNA) underwent ribozyme-mediated cleavage during reverse transcription. However, since there were no products other than the expected ones, I can safely conclude that the cleavage occurred specifically at the predetermined target site *in vivo*.

2.5 Conclusion

Successful selection *in vitro* of tailored RNA has been reported 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, to our knowledge, no such selection system exists *in vivo*. When ribozymes are to be used *in vivo*, I need to select the RNA that functions best in the cellular environment. Tsuchihashi and Herschlag reported that a protein derived from the p7 nucleocapsid (NC) protein of HIV-1 can facilitate ribozyme cleavage (Tsuchihashi et al., 1993; Herschlag et al., 1994). Other proteins also probably facilitate ribozyme cleavage (Bertrand and Rossi, 1994). By contrast, there are few reports of successful ribozyme-mediated gene

inactivation 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 in characterizing ribozyme action *in vivo* may hint at the existence of cellular inhibitory factors. Under such circumstances, it is desirable to be able to select functional ribozymes in the presence of such putative inhibitory factors *in vivo*. To this end, I first attempted to construct a positive selection system *in vivo* based on the general scheme shown in Figure 7. 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 toxin mRNA, then cells harboring active ribozymes should survive and should form colonies. Consequently, all surviving colonies should hold information about active ribozyme sequences. In our first attempt, the toxin gene selected was the gene for Ribonuclease T_1 (RNase T_1). However, despite some considerable effort, I failed to generate any plasmids that corresponded to the one shown in Figure 7, when RNase T_1 was used as a selective marker. No constructs with a gene for RNase T_1 were rescued from transformed *E. coli* cells. Only frame-shifted constructs, with aborted production of RNase T_1, could be rescued. In this first attempt, I could not control the extent of the toxicity of RNase T_1.

I, next, chose a potentially more controllable gene as a selective marker, namely, the gene for DHFR (Iwakura et al., 1983). 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). As a first step towards constructing an *in vivo* screening system, I tested the feasibility of use of DHFR gene with a construct shown in Figure 2. I initially examined two types of ribozyme, an active and an inactive ribozyme, in our initial test of the system.

At a fixed concentration of TMP of 70 μg/ml, *E. coli* cells harboring the active ribozyme expression vector grew faster than those harboring the inactive ribozyme expression vector (Figure 3). Then I prepared a mixture of active and inactive ribozyme expression vectors in equimolar amounts and plated the transformed *E. coli* cells with the mixture on LBM plates that contained TMP at 70 μg/ml. After incubation at 27 ℃ for 2 to 3 days, clones were harvested and their DNA sequences were examined to determine whether the clone contained the sequence of an active or an inactive ribozyme. In this way, I could judge whether there was any statistical significance to our method for selecting active ribozymes. Since for the most part, active ribozymes could be selected in the presence of TMP (Table 1), DHFR appeared more suitable as a selective marker than RNase T_1. I also demonstrated, by Northern blot and primer extension analyses (Figure 5 and 6), that the active ribozymes were fully functional *in vivo*, cleaving the primary mRNA of DHFR specifically at the predetermined site only. In

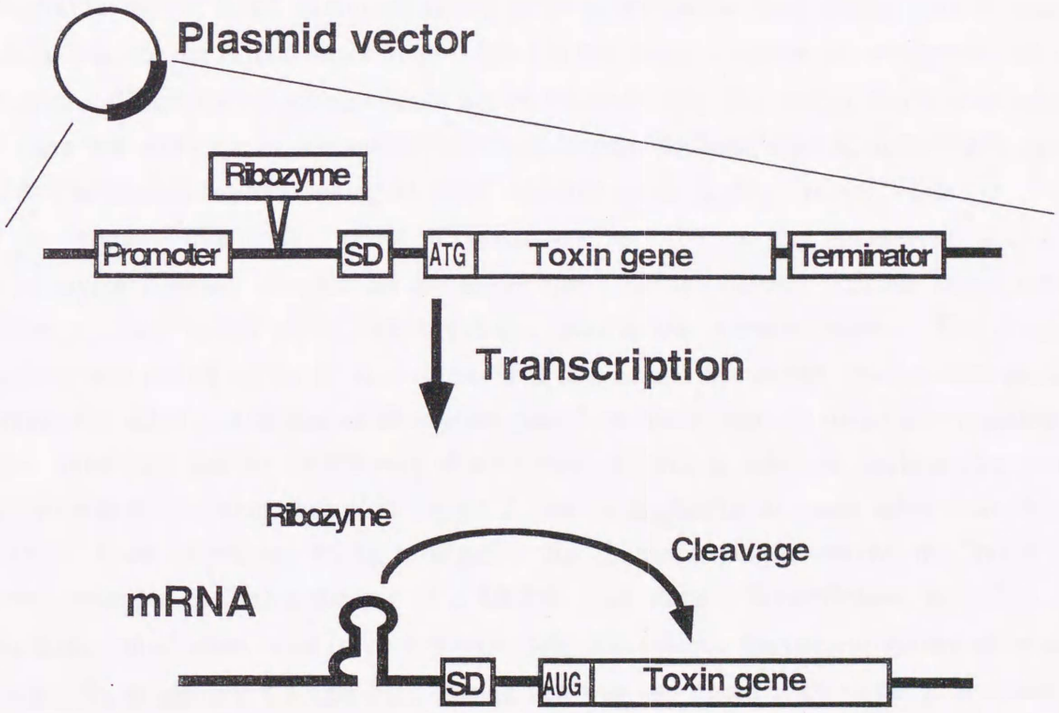


Figure 7. Schematic representation of a plasmid for the *in vivo* selection system. When the ribozyme is active, it can prevent expression of the toxin.

both of these analyses (Fig. 5 and 6), the mutant ribozyme (G_5 -> A) did not have any cleavage activity. Another change, that eliminates ribozyme activity is a single base change at A14 (Rufner et al., 1990). With this A_{14}/G_{14} system, for the most part, active ribozymes could be selected in the presence of TMP (Table 1). Taking all these results into accounts, I can conclude that the difference in phenotypes of these clones originated from only a single-base mutation at the catalytic core of the hammerhead ribozyme (Figure 1).

The examination of the construct shown in Figure 2 revealed the possibility of selecting active ribozymes *in vivo* using DHFR as a selective marker. However, in its present form, the background "noise" could obscure selection of an active mutant from a large pool of inactive molecules (since the *E. coli* strain HB101 used in this study produces an endogenous DHFR, formation of background colonies could not be avoided): This is a preconstruction experiment and there was an escape of one inactive ribozyme among 76 clones selected in the G_5/A_5 system and two inactive ribozymes among 44 clones selected in the A_{14}/G_{14} system (Table 1). I have not yet optimized this positive selection system *in vivo*. I know that the cleavage activity of the ribozyme depends strongly on the target site. Among several possible target sites, I arbitrarily chose, in this study, one target site close to the initiation codon. The ribozyme sequence was placed on the 5' side of the DHFR gene and no attempt has yet been made to compare the activity with that of ribozymes placed on the 3' side (to avoid any reinitiation). Genes other than that for DHFR may also be more suitable as selective markers (the general positive selection system shown in Figure 2 may be applicable to genes other than that for DHFR). I are, at present, trying to improve this system (trying to remove the "noise") by several strategies including the use of a DHFR- null strain. Nevertheless, as a first step toward the construction of an *in vivo* positive selection system, 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 the approach might be adapted to a *trans*-acting eukaryotic system which would be of value for the development of ribozyme gene therapies for human disease.

Chapter 3

Comparison of *In Vivo* Activities between 5'-Connected and 3'-Connected *Cis*-Acting Ribozymes

3.1 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*. I has 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 (E. coli)* to TMP. Therefore, I newly constructed a 3'-connected ribozyme system, and activities *in vivo* of 5'-connected and 3'-connected ribozymes were compared. I 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.

3.2. 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; Guerruer-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, 1983; Sarver et al., 1990; Uhlmann and Peyman, 1990; Erickson and Izant, 1992; Heudebreuch and Eckstein, 1992; Murray, 1992; Ojwang et al., 1992; Rossi, 1992; Altman, 1993; Bratty et al., 1993; 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, including diseases caused by infectious agents such as human immunodeficienfy virus (HIV) (Sarver et al., 1990; Heidenreich and Eckstein, 1992; Ojwand et al., 1992; Ohkawa et al., 1993; Leavitt et al., 1994). 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 (Dahm et al., 1993; Pyle, 1993; Uebayasi et al., 1994; Sawata et al., 1995; Amontov and Taira, 1996; Kumar et al., 1996; Orita et al., 1996; Zhou et al., 1996a; Zhou et al., 1996b; Zhou et al., 1997). Since the substrate specificity of ribozyme molecules is high, ribozyme strategies seem likely to have some value as therapeutic agents (Erickson and Izant, 1992; Murray, 1992; Eckstein and Lilly, 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 ribozymes are highly dependent on the cellular environment (Chen et al., 1997), ribozymes have not yet proven their significant superiority to antisense molecules. 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, 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 (Heidebreich and Eckstein, 1992; Pieken et al., 1991; Paolella et al., 1992; Taylor et al., 1992; Shimayama et al., 1937).

(iv) Unlike certain proteinaceous enzymes (Pingoud and Jeltsch, 1997), 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 (Pieken et al., 1991; Paolella et al., 1992; Taylor et al., 1992; Shimayama et al., 1937; Thomoson et al., 1996). Selection *in vitro* of functional nucleic acids is also of considerable current interest (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk et al., 1990). This method has successfully been used to engineer specific ribozyme (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). 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 (Denman et al., 1994; Kawasaki et al., 1996).

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, I 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, I selected the gene for Ribonuclease T_1 (RNase T_1) as the gene for the toxin. However, despite considerable effort, I 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 *E. coli* cells. Only frame-shifted constructs, with aborted production of RNase T_1, were rescued. In this first attempt, I was unable to control the extent of the toxicity of RNase T_1.

I next chose a potentially more controllable gene as a selective marker, namely, the gene for dihydrofolate reductase (DHFR) (Fujita et al., 1997). DHFR is an essential proteinaceous enzyme in 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; Iwakura et al., 1983; Blakley and Benkovic, 1985; Taira et al., 1987; Taira and Benkovic, 1988). I 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, I can regulate the toxicity of TMP by changing its concentration. Therefore, if I can control the level of expression of the gene for DHFR, which depends on the activity of a ribozyme, I should be able to select ribozymes, that are active in the cellular environment by monitoring resistance to TMP (Fujita et al., 1997).

In our previous study, a ribozyme-encoding sequence was placed upstream of the gene for DHFR for the following reasons (Fujita et al., 1997). 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 Shine-Dalgarno (SD) sequence from attack by ribozymes. Therefore, I 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, I 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.

3.3. Materials and Methods

Bacterial Strains and Plasmids

E. coli HB101 (*rec*A13, *sup*E44; 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).

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 (Fujita et al., 1997). I 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 (Figure 1). These changes had been shown previously to destroy cleavage activity (Ruffner et al. 1990; Inokuchi et al. 1994).

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 (Figure 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. Then, 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 Luria-Bertani's broth-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

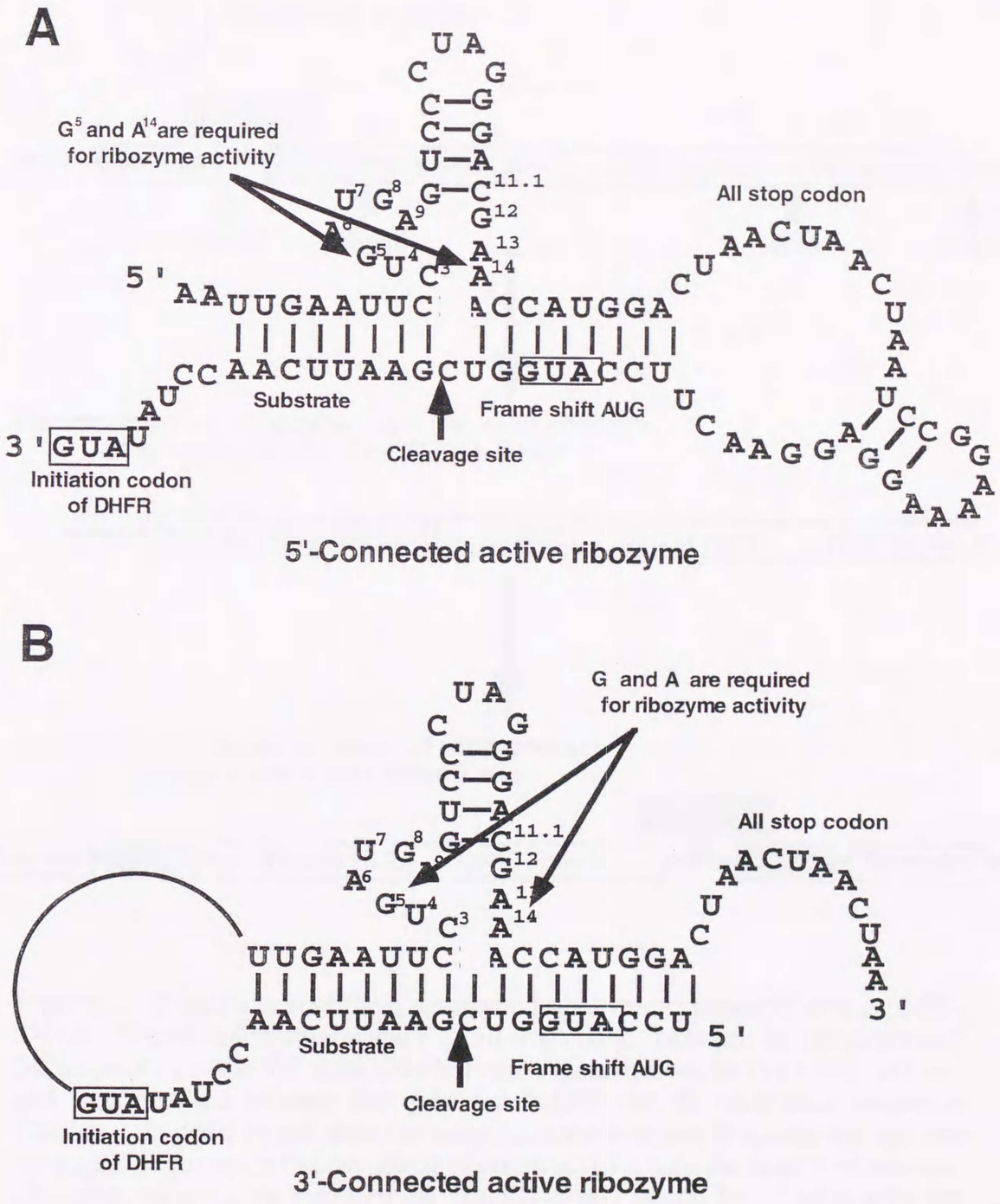


Figure 1. Secondary structures of the 5'-connected ribozyme (A) and the 3'-connected ribozyme (B). A single point mutation (G^5 to A^5 or A^{14} to G^{14}; circled) eliminates the ribozyme activity.

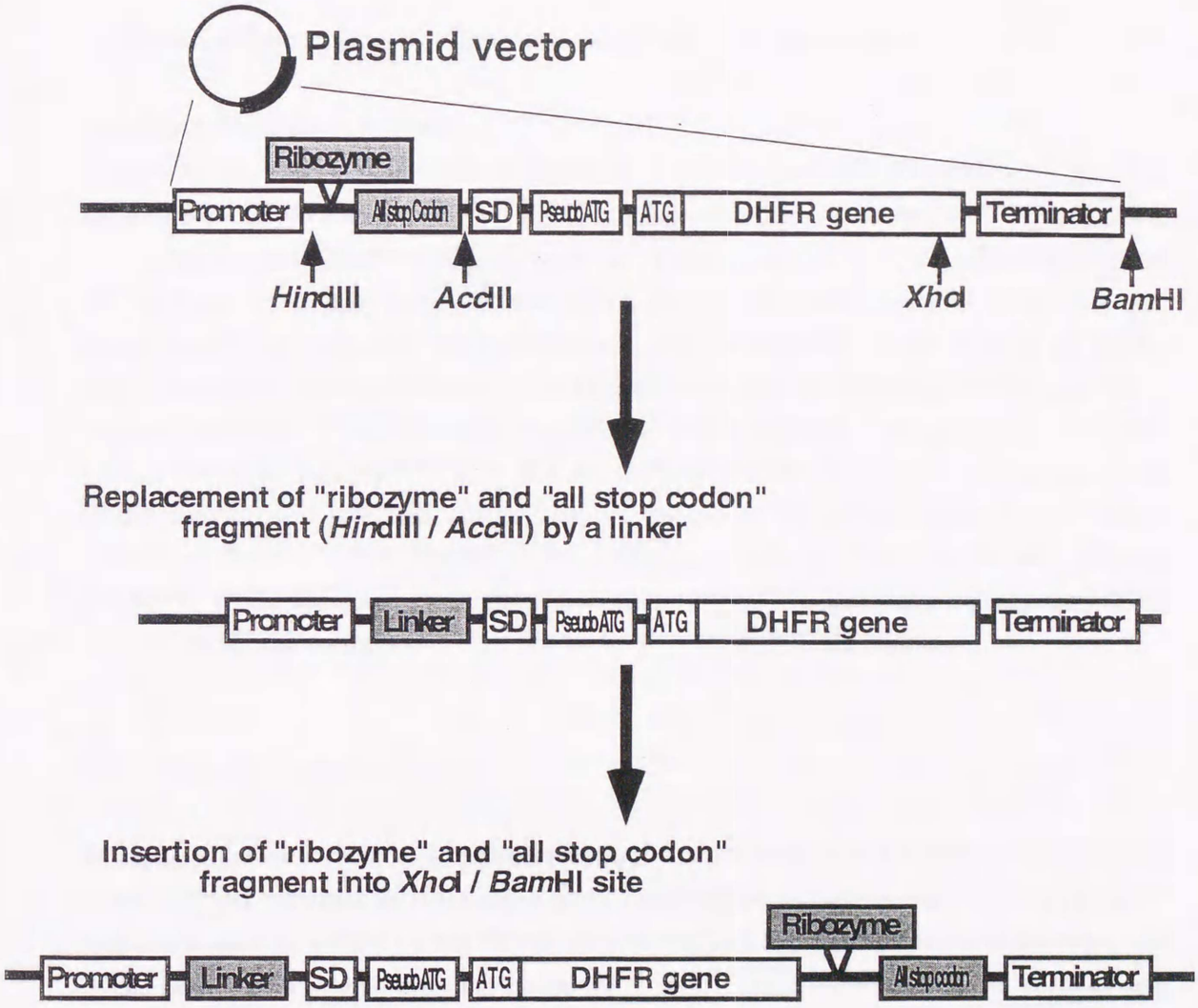


Figure 2. Construction of the 3'-connected ribozyme expression vector, pMH-3'-RIB. The ribozyme region and the "all stop codon" between the promoter and SD sequences in the 5'-connected ribozyme expression vector (top) were cut out and then inserted between the gene for DHFR and the terminator sequence (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 (Figure 1). The target site of the 3'-connected ribozyme was the same as that of the 5'-connected ribozyme (see Figure 3).

supplemented with ampicillin (100 μg/ml) and/or TMP (130- 140 μg/ml).

Northern Blotting Analysis

Plasmid vector pMH-3'-RIB (refer to Figure 2), harboring both a 3' ribozyme and a gene for DHFR, was used to transform *E. coli* HB101. After overnight incubation at 37 ℃, 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 2.5% NuSieve (3:1)™ 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 ^{32}P by use of 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 ℃ for 30 min.

3.4. 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*, I 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 (Figure 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 (Figure 1A). These mutations completely abolish the activity of the ribozyme (Ruffner et al. 1990; Inokuchi et al. 1994). 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 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, I 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 (Hamada et al., 1999; 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, I felt that it was worth examining 3'-side ribozymes in prokaryotic cells also to determine whether I could achieve the same or greater selective power than that obtained with 5'-side ribozymes.

I constructed 3'-connected ribozyme expression vectors containing either an active ribozyme or an inactive ribozyme sequence (Figure 1B). These vectors were based on the 5'-connected ribozyme expression vectors (Figure 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), I 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 Figure 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 (Figure 1B). The target site of the 3'-connected ribozyme was exactly the same as that of the 5'-connected ribozyme (Figure 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. I needs a positive selection method to find active ribozyme sequences. Therefore, I took advantage of a frame shift in the AUG codon. I introduced, from the upstream to the downstream direction, an efficient SD sequence, a frame shift initiation codon that was out of frame relative to the gene for DHFR, a target site for the

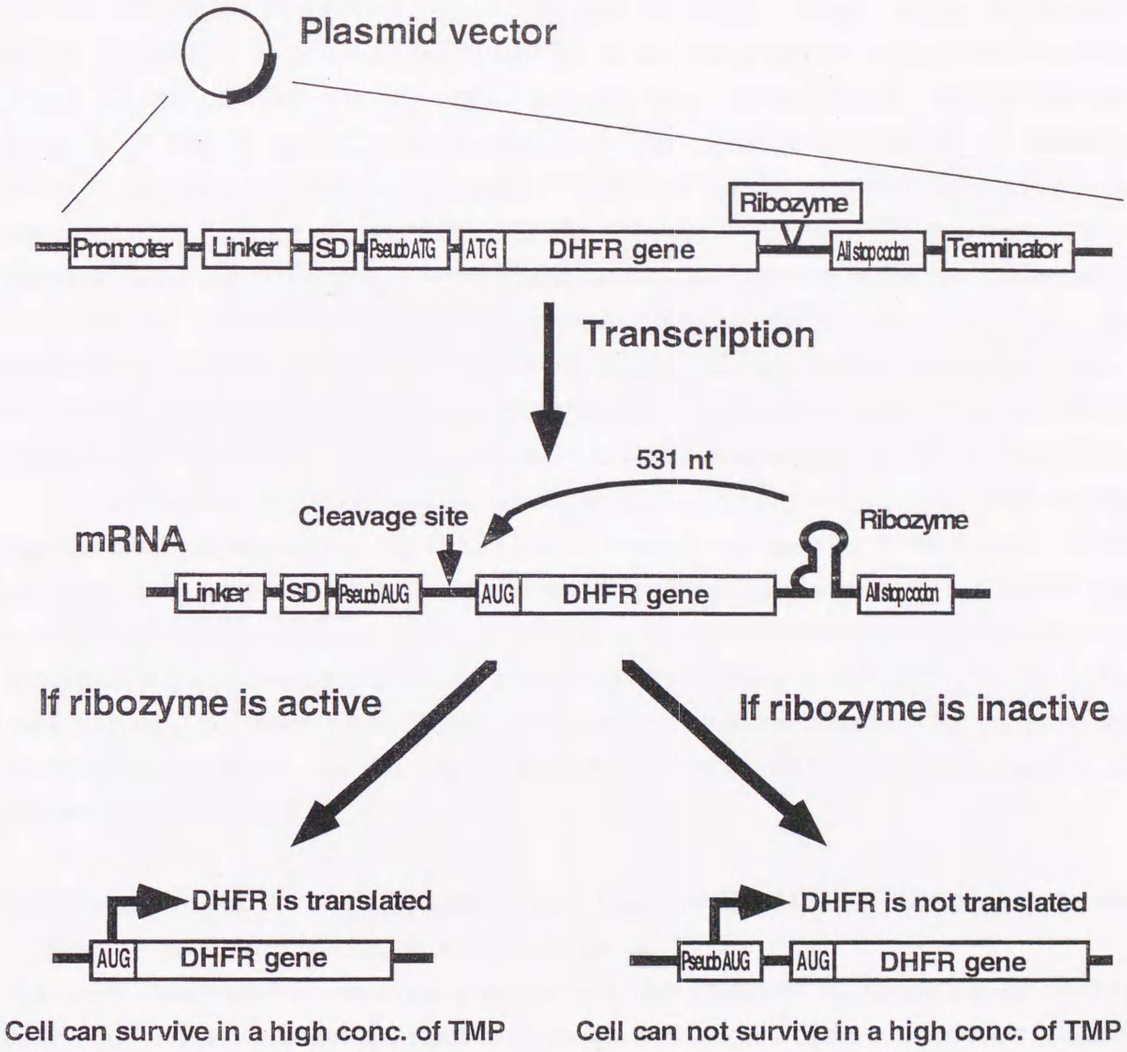


Figure 3. The 3'-connected ribozyme expression vector. The plasmid vector has two ATG codons, one of which is a pseudo-initiation codon, located upstream of the authentic ATG codon, which is the initiation codon for the DHFR gene. If, upon transcription, the primary transcript is cleaved by the cis-acting 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 (refer to Chaper 2) and 531 nt for the 3' ribozyme.

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 (Figure 1), 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 (Figure 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 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 (Figure 3).

Furthermore, I 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, I 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 (TAA CTA ACT AA) had been introduced between the ribozyme and the strong SD sequence. In this region, three stop codons should terminate translation in all possible frames (Fujita et al., 1997).

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 (Iwakura et al., 1983). 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 I had observed previously with the 5'-connected ribozyme construct (Fujita et al., 1997). 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, I 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 (Fujita et al., 1997). The increased

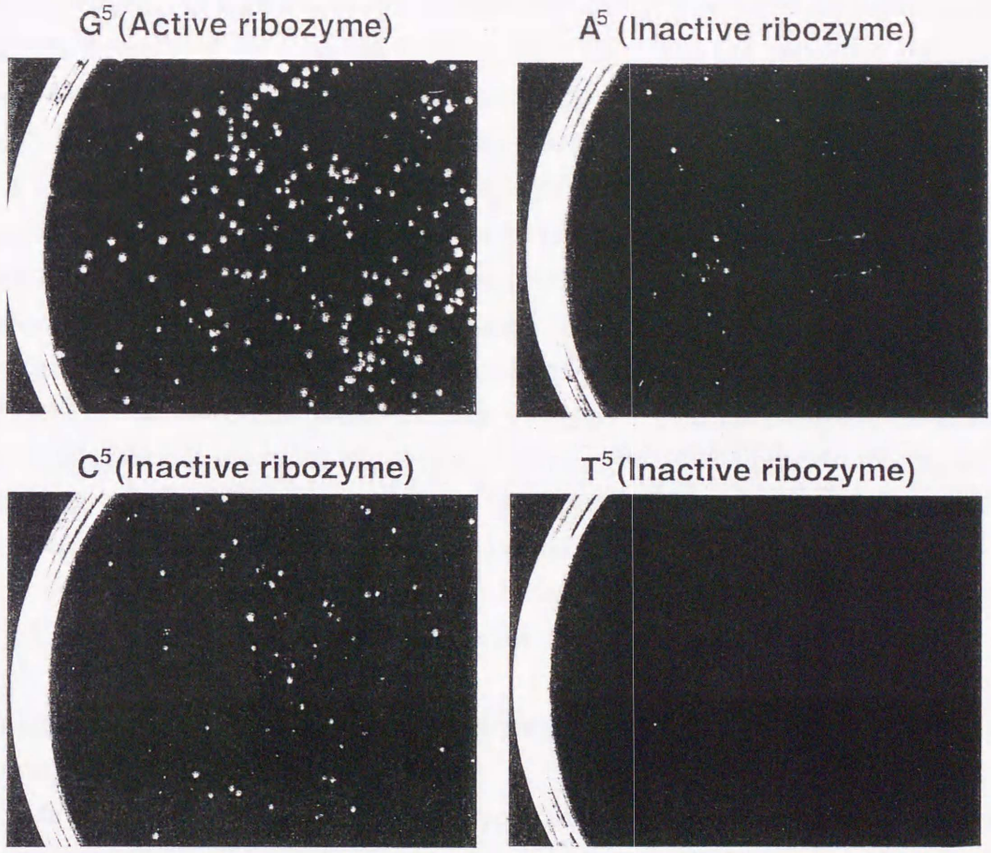


Figure 4. Colonies of *E. coli* HB101 cells that had been transformed with the 3'-connected ribozyme expression plasmid. G^5 (Active ribozyme): Active ribozyme. A^5 (Inactive ribozyme): Inactive ribozyme with G^5 replaced by A. C^5 (Inactive ribozyme): Inactive ribozyme with G^5 replaced by C. T^5 (Inactive 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 Amp (100 µg/ml) alone or both Amp (100 µg/ml) and TMP (130 µg/ml). The same numbers and sizes of colonies were detected on 2 plates containing Amp (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.

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*d III/*Acc* III region strongly influenced the rate of transcription, as confirmed in experiments with different kinds of linker (data not shown). I 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.

I then carried out a random screening assay for the 3' ribozyme construct, following the procedure used for the 5' ribozyme construct (see Figure 4) (Fujita et al., 1997). *E. coli* cells 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 1, together with the previously obtained results with the 5' ribozyme construct (Fujita et al., 1997). 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, I did achieve limited success even though I could not eliminate the background colonies (Table 1).

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, I 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 (Sioud and Drlica, 1991; Ferbeyre et al., 1995). 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, I performed Northern blotting analysis for both types of construct under identical conditions (Figure 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 (Figure 3) As indicated in lanes 2 and 4, both 5'- and 3'-connected ribozymes recognized and cleaved specifically the target site. Most importantly, the cleavage efficiencies were nearly identical for the two types of ribozyme, 24 % for the 5'-side ribozyme

Table 1. Numbers of selected colonies with 5'-connected and 3'-connected active and inactive ribozymes on trimethoprim (TMP) -containing and/or ampicillin (Amp) -containing.

G^5 and A^5 Mixture	5'- Connected Ribozyme		3'- Connected Ribozyme			
	Amp Plate	TMP Plate	Amp Plate	TMP Plate		
(μg/ml)		70		130	133	140
Active Ribozyme	29	75	8	9	7	27
Inactive Ribozyme	28	1	11	0	2	3

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 μg, 133 μg 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 Chapter 2 for the purpose of comparison.

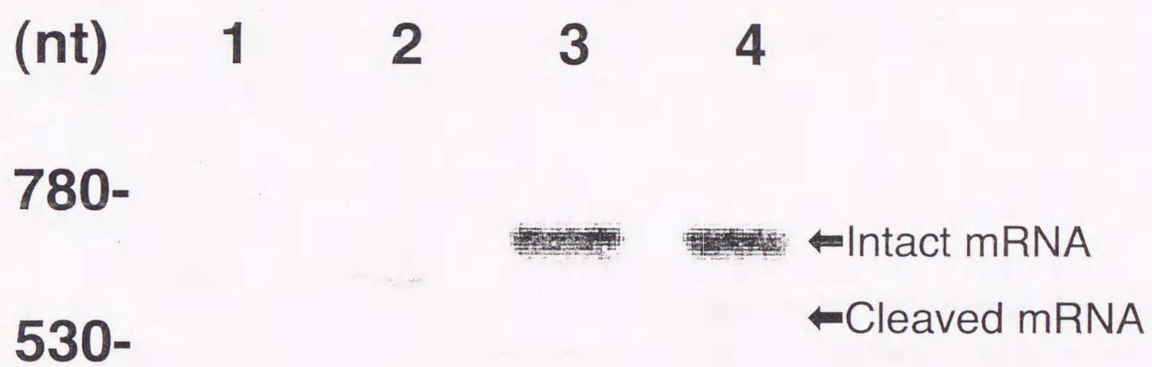


Figure 5. Northern blotting analysis (Pictrographic printout) for comparison of the cleavage efficiencies of the 5'-connected and 3'-connected ribozymes. Ten micrograms of total RNA from *E. coli* cells transformed with the 5'-connected or 3'-connected ribozyme expression vector were subjected to electrophoresis 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.

and 23% for the 3'-side ribozyme. (The extent of cleavage was determined by quantity 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 I 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 (Figure 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.

3.5. Conclusion

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; Bratty et al., 1993; Lehman and Joyce, 1993; Nakamaye and Eckstein, 1994; Cuenoud and Szostak, 1995; Ishizaka et al., 1995). However, efforts to construct selection systems *in vivo* have met with only limited success (Ferbeyre et al., 1996). For use of ribozymes *in vivo*, I needs RNAs that function optimally in the intracellular environment. To this end, I 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, I 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, I 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 1). 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, I 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 (Zhang et al., 1997).

Northern blotting analysis revealed that the efficiencies of ribozyme- mediated cleavage *in vivo* were identical for 5' - and 3' -connected ribozymes (Figure 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. I 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 (Figure 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, I 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.

Chapter 4

Extremely High Affinity between Tandemly Connected, Tat-Derived Peptides and Tat Aptamers

4.1. Summary

The strong binding mediators were constructed to develop the technique for linking between functional RNA and protein. By combining function of RNA and protein, it is expected that functional protein or RNA is transported to the suitable part of cell. Furthermore, it might be possible to construct the novel protein selection system *in vitro*. I suggested that tandem linking of multiple Tat-aptamers $(Apt)_n$ and multiple Tat-derived peptide (RE peptide) are useful as the mediator for linking between RNA and protein. At first, I investigated that the dissociation constant between Tat aptamer and RE peptide. Tat aptamer derived by *in vitro* selection bound to RE peptide with high affinity and specificity. Moreover, I succeeded to enhance the binding affinity between protein and RNA by using tandem repeated aptamers and tandem repeated RE peptide as mediator. As the model, dissociation constant between dihydrofolate reductase-RE-peptides-fusion-protein; DHFR-$(RE)_3$, having 3 tandem repeated RE peptides, and $(Apt)_3$, having 3 tandem repeated aptamers, was less than 16 pM, and was over 50 fold lower than the dissociation constant between RE peptide and aptamer. I confirmed that the strongest interaction between tandem aptamer and tandem RE peptide is useful as mediator to linking functional protein and RNA.

4.2. Introduction

The development of the technique for linking between functional RNA and functional protein is important for a transport and a recruitment of RNA or protein at the target site. By combining ribozyme with RNA helicase which slides RNA strand and unwinds the second structure of RNA, ribozyme might be recruited to the target site efficiently (Warashina et al., in preparation). Ribonuclease H (RNase H) which is linked to antisense RNA by a mediator is carried to the specific site of RNA of an abnormal gene, such as oncogene or human immunodeficiency virus (HIV) genome (Maitra et al., 1995; Cirino et al., 1997; Player et al., 1998). Binding of a functional RNA to transport protein including importin and exportin is advantage for transport of functional RNA. Furthermore, it might be possible to construct the novel protein selection system *in vitro* by extremely strong interaction between protein and template mRNA coding the proteins (Mattheakis et al., 1994; Hanes et al., 1997; He and Taussig, 1997; Nemoto et al., 1997; Roberts and Szostak, 1997; Hanes et al., 1998; Hanes et al., 1999; Makeyev et al., 1999; Roberts, 1999). For these purpose, it is important to construct the strong binding mediator for linking RNA and protein (Figure 1).

To construct a strong binding mediator, it is effective to link multiple protein motifs and multiple RNA motifs respectively. In principle, The Gibbs free energy change for binding of protein having two binding blocks (A-B) is the sum of intrinsic Gibbs free energy changes for binding of its component blocks (A and B) and the loss of internal entropy of A-B by approaches for binding ($\Delta G^0_{AB}=\Delta G^i_A+\Delta G^i_B+\Delta G_S$) (Jencks, 1981). It is expected to exponential decrease of the equilibrium dissociation constant (K_d) of A-B in compare with that of each block (A and B). In recent study, it was reported that the equilibrium dissociation constant (K_d) of artificial ligand consisting of two blocks that have been optimized for binding to different subsites of the protein become low drastically (Shuker et al., 1996; Hajduk et al., 1997). In nature, various zinc finger proteins, that perform diverse biological functions in regulation of cell growth and differentiation through DNA-, RNA-, and protein-protein interactions, have tandem zinc finger motifs to increase binding affinity (Miller et al., 1985; Joho et al., 1990; Pavletich and Pabo, 1991; Pavletich and Pabo, 1993; Klug and Rhodes, 1987).

In previous study, I found a novel artificial RNA motif, namely Tat-aptamer that binds specifically to *trans*-activator protein (Tat) of HIV-1 with high affinity (Yamamoto et al., 1998; Yamamoto et al., in preparation). The expression of genes encoded by HIV-1 is regulated by Tat protein with several regulatory factors in the long terminal repeat of HIV-1 (Gaynor, 1992). Tat binds to one of the regulatory region in the long terminal repeat (LTR) of mRNA, namely *trans*-activating region (TAR) (Rosen et al., 1985). Tat protein which binds to TAR region of

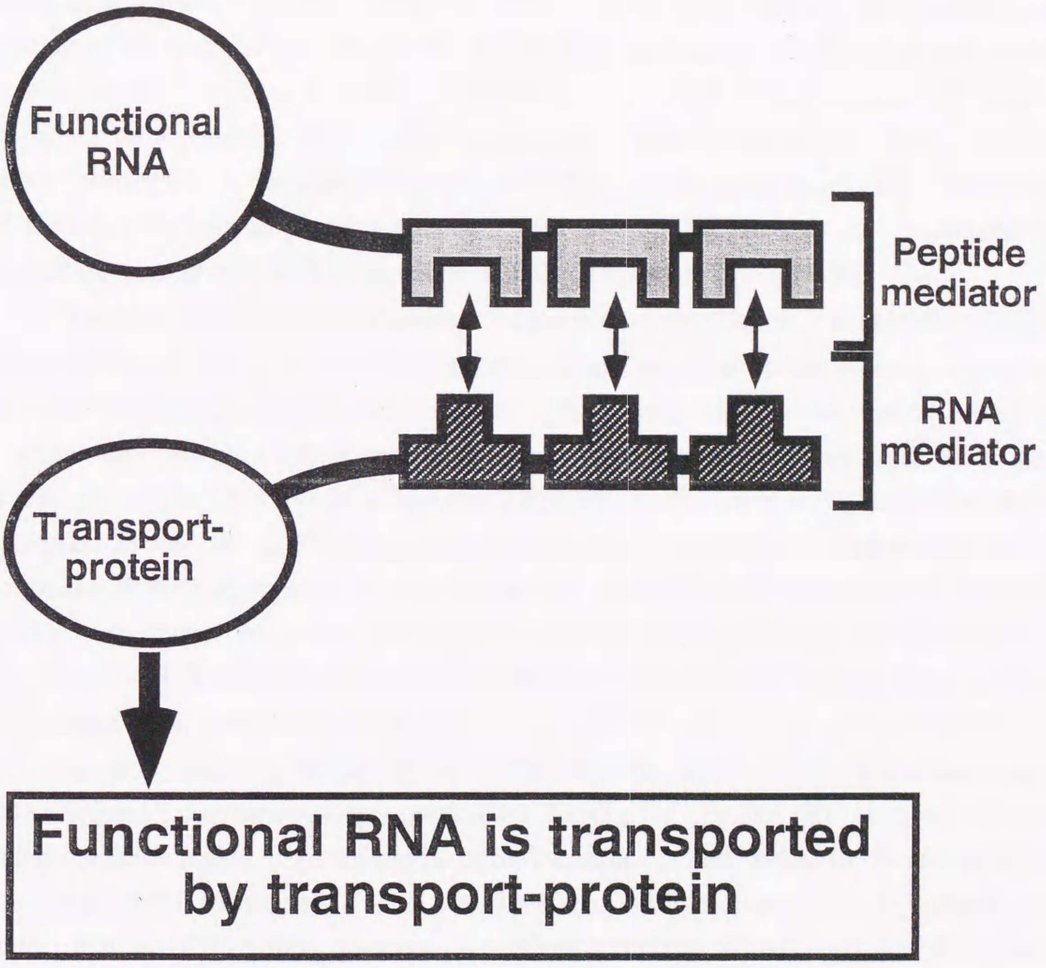


Figure 1. The concept of the strong binding mediator between RNA and protein. Functional protein or RNA is conbined to RNA or protein correlated to transport or recruitment by mediator. Functional protein or RNA is carried and concentrated to the part of cell by transporter. In our case, Tat aptamer and Tat derived peptide (RE peptide) were used as mediator. Tat aptamer binds to RE peptide with high affinity. As a result, protein with RE peptide bind to RNA with Tat aptamer.

mRNA of HIV-1 stimulates the elongation properties of RNA polymerase II (Cullen, 1986; Peterline et al., 1986; Rice and Mathews, 1988). Tat is small cysteine-rich nuclear protein consisting of 86 amino acids. It has two major domain, a cysteine-rich region and arginine-rich basic region (Arya et al., 1985; Sodroski et al., 1985). The cysteine-rich region is essential for the function of this protein (Garcia et al., 1988; Kubota et al., 1988). The basic region is responsible for the specific binding to TAR RNA (Weeks et al., 1990). Moreover, I confirmed that Tat derived peptide containing Tat basic domain binds to our Tat aptamer with higher affinity and specificity in compare with TAR (Yamamoto et al., 1998).

The second structure of Tat aptamer which is discovered by *in vitro* genetic selection is resemble to that of TAR. I confirmed that the second structure of Tat-aptamer contains two TAR core motifs and the affinity of Tat-aptamer to Tat protein is higher than that of TAR. And the interaction between Tat-aptamer and Tat-derived peptide was stronger than almost interactions between RNA motifs and protein (peptide) -motifs, such as the interaction between transcription factor IIIA (TFIIIA) having nine tandemly repeated zinc fingers and 5S RNA (Ryan et al., 1998; Yamamoto et al., in preparation). Because interaction between Tat-aptamer and Tat is very strong, this interaction might be useful for linking strongly between protein and RNA. Moreover, I think it is important to improve the interaction to keep more stable and stronger complex of protein and RNA.

As one of the approaches to solve this problem, in this study, I examined tandem linking of plural Tat-aptamers, $(Apt)_n$, and plural Tat-derived peptides (RE peptides) containing arginine-rich basic region. As the model of the functional protein linked the functional RNA, I chose dihydrofolate reductase (DHFR), and therefore DHFR is fused to the N-terminal of RE peptides, that is DHFR-$(RE)_n$ proteins. I confirmed binding affinity between these tandem motifs in order to develop strong mediators.

4.3. Materials and Methods

Construction and Preparation of the Labeled Tandem Tat-Aptamers, $(Apt)_n$, and TAR RNA for Gel Mobility Shift Assay

For preparation of tandem Tat-aptamers, $(Apt)_n$, the sequence of Tat aptamer was isolated by *in vitro* genetic selection in previous study (Yamamoto et al., 1998). Sense and anti-sense template oligonucleotides, 5'- AAA AAA CGA AGC TTG ATC CCG TTT GCC GGT CGA TCG CTT CG -3'; 5'- TTT TTT CGA AGC GAT CGA CCG GCA AAC GGG ATC AAG CTT C -3'; corresponded to Tat-aptamer with the 6 oligonucleotides of adenosine linker, that need to construct tandem Tat-aptamers, were synthesized by DNA synthesizer (model 392A; Applied

Biosystems). To construct several tandem Tat aptamer motifs $(Apt)_n$, 100 pmol of annealed double strand Tat aptamer with linker was ligated by DNA Ligation Kit ver.2 (Takara). The reaction was carried out in a 20 μl mixture that containing 5 μl of solution I and 10 μl of solution II. To obtain various tandem number of aptamers and promote trans-reaction, I performed reaction at 26 °C for 10 min. After ligation, all ligated sample except enzyme by phenol/chloroform and ethanol precipitation was polymerized by rTaq polymerase (Takara) to elongate and add projecting adenosine at 3' end at 68 °C for 10 min. All PCR and polymerizations were performed in 100 μl scale containing 2.5 U of rTaq or ExTaq polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and each 200 μM dNTP mixture. Sample containing various tandem aptamers with projecting adenosine at each 3' end inserted to pCR vector (Invitrogen) by ligation-protocol of TA Cloning Kit (Invitrogen). 10 μl of pCR vector was transfected to *Esterichia coli* (*E. coli*) (One Shot cells, Invitrogen) for separating genotype, and pCR vectors containing various number of tandem aptamers are purified by minipreparation and checked sequences using DNA sequencer. I obtained 3 kinds of pCR vectors including 1 aptamer $(Apt)_1$, 2 tandem aptamer $(Apt)_2$ and 3 tandem aptamer $(Apt)_3$. Each pCR vectors were amplified by upper and lower PCR primers 5'- AGT AAT ACG ACT CAC TAT AGG GCG AAG CTT GAT CCC GTT T -3'; 5'- CGA AGC GAT CGA CCG GCA -3'; to add T7 promoter for transcription. Purified each template double strand DNAs for aptamers by gel electrophoresis using 1 % Sea Kem agarose (FMC) were transcribed by T7 RNA polymerase *in vitro* at 37 °C for 2 hours using a T7 Ampliscribe kit (Epicentre Technologies). Transcribed each aptamers were isolated using 15 % denaturing polyacrylamide gel (PAGE) and dephosphorylated at 5'-end using alkaline phosphatase (*E. coli* A19) (Takara) at 37 °C for 1 hour. After that, aptamers were labeled by γ^{32}P-ATP using T4 polynucleotide kinase at 37 °C for 1 hour and were purified by 15 % PAGE.

For template of tandem TAR RNA, sense strand DNA containing the sequences of T7 promoter and antisense strand DNA were synsesized by DNA synthesizer. 100 pmol of annealed double strand DNA was polymerized by Taq polymerase and purified by phenol/chloroform and ethanol precipitation. After that, I prepared labeled TAR RNA same as preparation of Tat aptamers.

Construction and Preparation of Dihydrofolate Reductase-RE Peptide-Fusion-Proteins; DHFR-$(RE)_n$

For preparation of DHFR-$(RE)_n$ fusion proteins, at first, 90 oligonucleotide of Template sense oligonucleotide, 5'- ATG CAT ATG GTC GTA AAA AAC GTC GTC AAC GTC GTC GTC CTC CTC AAG GTT CTC AAA CTC ATC AAG TTT CTT TAT CTA AAC AAC CTA CTT -3'; containing upstream sequences of RE peptide of HIV-1, 40 oligonucleotide of annealing

region against antisense oligonucleotide and antisense oligonucleotide, 5'- CTG CAG CGC CAC CGC CGC CTT CTT TAG GAC CAG TAG GAT CAC CAC GAG ATT GAG AAG TAG GTT GTT TAG ATA AAG AAA CTT GAT GAG TTT -3'; containing down stream sequences of RE peptide of HIV-1, 40 oligonucleotide of annealing region against sense oligonucleotide, sequences for linker and *Eco*T22I site at 5' end and *Pst*I site at 5' end were synthesized by DNA synthesizer (model 392; Applied Biosystems, Foster City, CA). 2 kinds of oligonucleotides were annealed, polymerized and inserted to pCR vector according to the protocols described upper section (I named RE peptide inserted plasmid pCR-RE).

The gene for DHFR with *Pst*I site at 3' end that derived from plasmid pTZDHFR20 (Iwakura et al., 1995; Fujita et al., 1997; Fujita et al., 1998) was inserted to plasmid pET30a (Novagen) that was optimized for protein expression and purification (I named DHFR inserted pET30a plasmid pETDHFR). RE peptide with linker cut from pCR-RE plasmid by *Pst*I and *Eco*T22I was inserted to 3' side of DHFR gene at pETDHFR plasmid. Because *Pst*I and *Eco*T22I restriction sites can be connected and restriction site is deleted, I can connect RE peptide to 3' side of DHFR gene at pETDHFR continuously. I constructed 3 kinds of DHFR-RE fusion expression plasmids, namely pETDHFR-$(RE)_1$, pETDHFR-$(RE)_2$ and pETDHFR-$(RE)_3$ (Refer to Figure 5 about the structure of 3 kinds of fusion proteins).

Purification of each DHFR-RE Peptide Fusion Proteins

Each DHFR-RE peptide fusion protein expression plasmids pETDHFR-$(RE)_n$ were transfected to *E.coli*. strain NovaBlue (DE3), which harbors an isopropyl-1-thio-β-D-galactoside (IPTG) - inducible T7 RNA polymerase gene (Novagen) and *E. coli* containing pETDHFR-$(RE)_n$ was pre-incubated in 5 ml of Luria-Bertani's medium (LB) at 37 °C for 1 day and incubated in 500 ml of LB media at 30 °C. 1 mM of is added to medium at the period of logarithmic multiplication of *E.coli* and media. were incubated at 30 °C for 4 hours. These proteins were expressed from pET plasmids as polyhistidine tagged molecules.

Cells were harvested in 25 ml of the buffer containing 20 mM Na phosphate buffer (pH 7.4), 10 mM imidazole, 0.5 M NaCl and disrupted by French press. The extract was clarified by centrifugation and 0.22 μm filter (Millipore) and passed over 1 ml of Hi Trap chrating column (nickel agarose column) (Pharmacia). The column was washed with 20 ml of wash buffer (20 mM Na phosphate buffer (pH 7.4), 60 mM imidazole, 0.5 M NaCl) and proteins eluted by the elution buffer (20 mM Na phosphate buffer (pH 7.4), 0.5 M imidazole, 0.5 M NaCl). To remove RE peptide fraction of degraded proteins mainly cut at site of linker and except imidazole, proteins passed over gel filtration column (HiTrap Desalting; Pharmacia). Furthermore, to remove DHFR fraction of degraded proteins, cation exchange chromatography

using Hi Trap SP (Pharmacia) was performed using high performance liquid chromatography (HPLC). Purity of protein were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Concentration of purified proteins were measured by BCA assay kit (Pierce).

Measurement of Dissociation Constant by Gel Mobility Shift Assay

In gel mobility shift assay for measurement of dissociation constant between RNA; $(Apt)_1$ or $(Apt)_2$ or TAR, and peptide; RE peptide, or protein, DHFR-$(RE)_n$, a fixed concentration (30 pM or 1 nM) of 5'-end labeled aptamers or TAR with tRNA were denatured in Tat binding buffer; 10 mM Tris HCl (pH 8.0), 70 mM NaCl, 2 mM EDTA and 0.1 % Nonidet P-40, at 95 °C for 3 min for restructure and then the mixtures were incubated with a range of excess concentration of Tat derived peptide (RE peptide) or DHFR-$(RE)_n$ protein (30 pM-64 nM) in Tat binding buffer in a total volume of 10 μl at 30 °C for over 1 hours. Free and protein or peptide bound RNA were separated by non-denaturing gel electrophoreses through 5-15 % polyacrylamide, 0.5x TBE buffer and were analyzed.

In competitive binding assay, labeled 4 nM aptamer $(Apt)_1$ and 4 nM RE peptide were pre-mixed with tRNA in Tat binding buffer after denature and restructure same as normal binding assay. After incubation for binding, 0.5 nM-8 nM of tandem aptamer as a competitor; $(Apt)_2$ or $(Apt)_3$, were added to pre-mixture and these mixture were incubated in Tat binding buffer in a total volume of 10 μl at 30 °C for over 1 hours. Free and protein or peptide bound RNA were separated and were analyzed in same condition to normal binding assay.

Equilibrium dissociation constants were determined by calculation of theoretical curve of free RNA concentration or complex RNA via peptide concentration using the least-squares methods.

4.4. Results and Discussion

Comparison of K_d Values of TAR, $(Apt)_1$ and $(Apt)_2$ by the Gel Mobility Shift Assay

To form strong interaction between mRNA and protein, I suggested to use HIV-1 Tat (trans-activation protein) aptamer and HIV-1-Tat derived peptide (RE peptide) as mediator (Figure 1). I constructed several plasmids coding RNA motifs based on Tat aptamer (Figure 2). RNA motifs connected the sequences coding T7 promoter at 5' end were inserted to the multicloning sites of pCR plasmids (Invitrogen). Linear double strand DNA coding TAR was synthesized by DNA synthesizer.

At first, I compared dissociation constant of Tat aptamer to that of TAR by gel

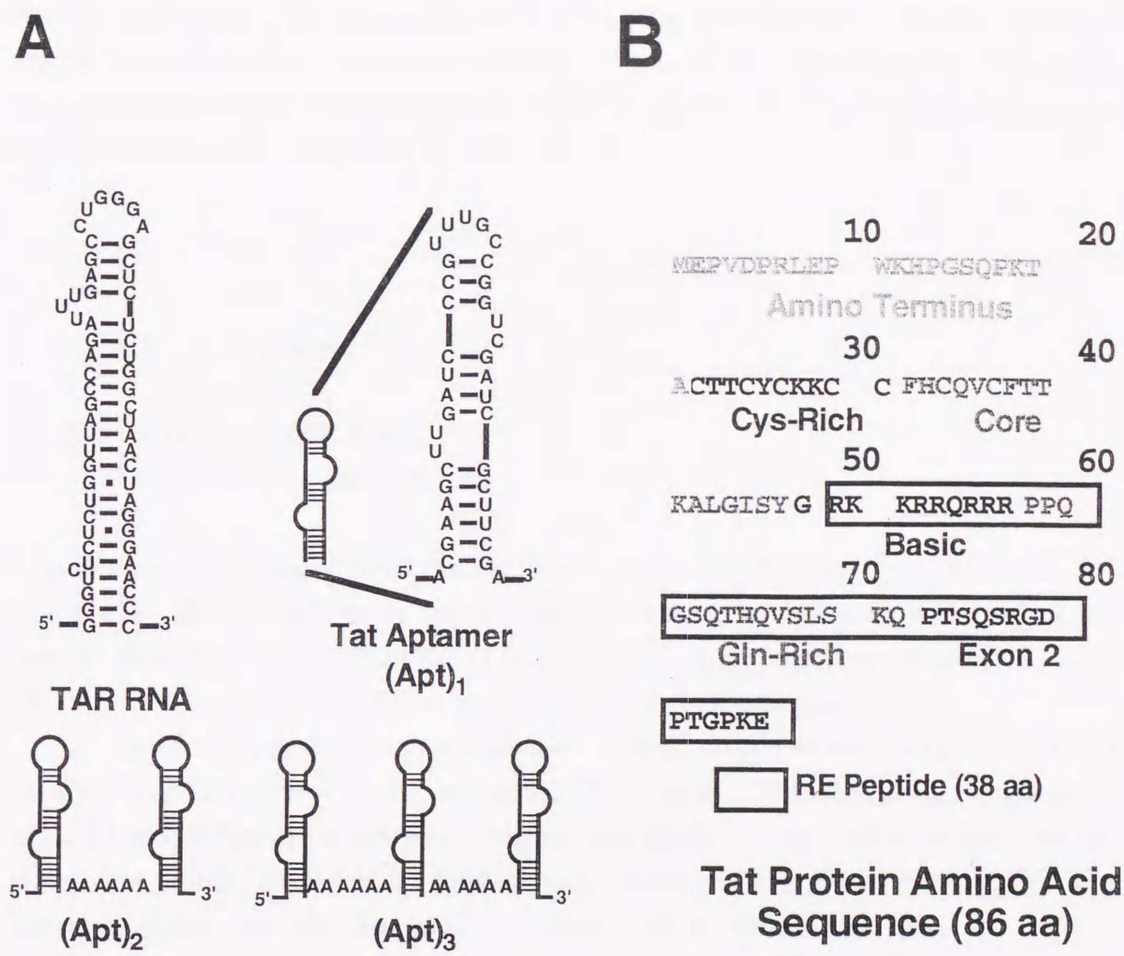


Figure 2. Schenatic drawing of mediators for linking between functional RNA and protein. (A) Schenatic drawing of Tat aptamer, 2 and 3 tandemly repeated Tat aptamers and trans-activating region (TAR) RNA. The second structure of Tat aptamer which was finded by in vitro genetic selection is resemble to that of TAR. Tat aptamer contains two TAR core motif, namely two bulge-loops. (Apt)2 and (Apt)3 were linked by 6 mer poly adenisine nucleotide linkers. (B) Schenatic drawing of Tat protein and RE peptide. Tat is small cysteine-rich nuclear protein consisting of 86 amino acids. It has two major domain, a cysteine-rich region and arginine-rich basic region. The basic region is responsible for the specific binding to TAR RNA. RE peptide which is surrounded by square in figure is the region containing arginine-rich basic region, glutamine rich region and exon 2 (49 aa - 86 aa).

mobility shift assay. Tat aptamer and TAR RNA were transcribed by T7 polymerase and RE peptide was synthesized by peptide synthesizer. When The follow equations was defined, dissociation constant of Tat aptamer and TAR RNA against Tat derived peptide were 1.5 nM and 30 nM respectively (Figures 3A, 3B).

$$(Apt)_1 + RE \rightleftarrows (Apt)_1 \cdot RE$$

$$K_d = [(Apt)_1] \cdot [RE] / [(Apt)_1 \cdot RE]$$

$$Y_{free}(\%) = K_d / (K_d + [RE]) \text{ X } 100$$
$$Y_{complex}(\%) = [RE] / (K_d + [RE]) \text{ X } 100$$

It shows that Tat aptamer have binding to RE peptide 20 fold stronger than TAR RNA. Nevertheless, Interaction between aptamer and peptide is too weak in compared with interaction between general antibody and antigen to maintain complex for long time (Although K_d value is low, k_{on} and k_{off} are each fast. Therefore, protein is easy to release from RNA).

In next approach for strong combining between protein and mRNA (DHFR-$(RE)_n$), I designed tandem aptamers, $(Apt)_n$, and tandem Tat peptides. In recent study, it was reported when 2 kinds of ligands, which were designed individually and can bind to the different site of same target protein, are linked by covalent bond, binding affinity between linked ligands and target was higher drastically than individual binding affinity between each ligand and target. At first, to confirm whether tandem aptamer can bind Tat peptide with high affinity, I investigated binding affinity between tandem aptamers $(Apt)_n$ and RE peptide by gel mobility shift assay. I compared 2 kinds of tandem aptamers, $(Apt)_n$,which are formed by tandem 2; $(Apt)_2$ or 3 aptamers; $(Apt)_3$, to normal aptamer; $(Apt)_1$. Each tandem aptamers were linked by 6 mer poly adenosine nucleotide linkers in order to estimate that Poly A not very affect the second structure of aptamer.

In gel mobility shift assay for RE peptide and $(Apt)_2$, 2 mobility shift bands were detected (Figure 3C). It means that Tat peptide binds to 2 parts of $(Apt)_2$. When I presumed the following equations of the equiblium constant, I could explain Figure 3C.

$$(Apt)_2 + RE \rightleftarrows (Apt)_2 \cdot RE$$
$$(Apt)_2 \cdot RE + RE \rightleftarrows (Apt)_2 \cdot RE \cdot RE$$

$$K_{d1} = [(Apt)_2] \cdot [RE] / [(Apt)_2 \cdot RE]$$

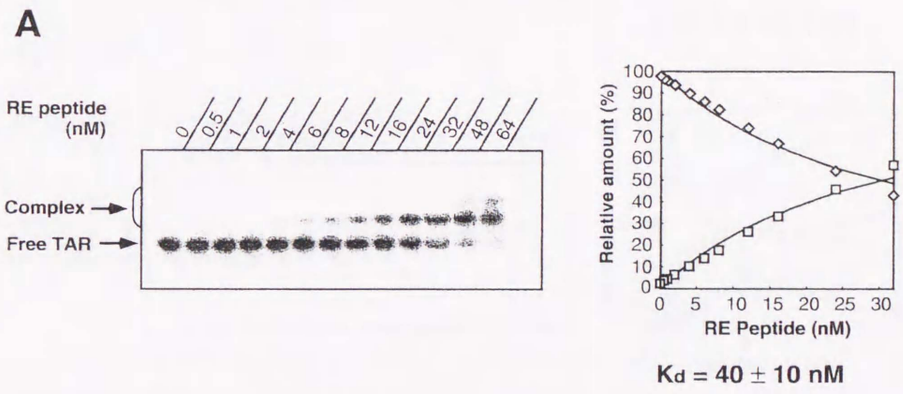


Figure. 3A. TAR binding to RE peptide. The mixtures of 5'-end labeled RNA and RE peptide were incubated at 30 °C for over 1 hour in Tat binding buffer. Free and peptide bound RNA were separated by non-denatureing gel electrophoreses through 15 % poluacrylamide. The scheme and the graph of gel mobility shift assay showing the formation of complex between TAR and RE peptide. Binding reactions contained 5'-end labeled TAR RNA (1 nM, 1000 cpm) and various concentration of RE peptides. ◇ shows the relative amount of free TAR (%). □ shows the relative amount of complex between TAR (%). K_d value of TAR is 30 nM.

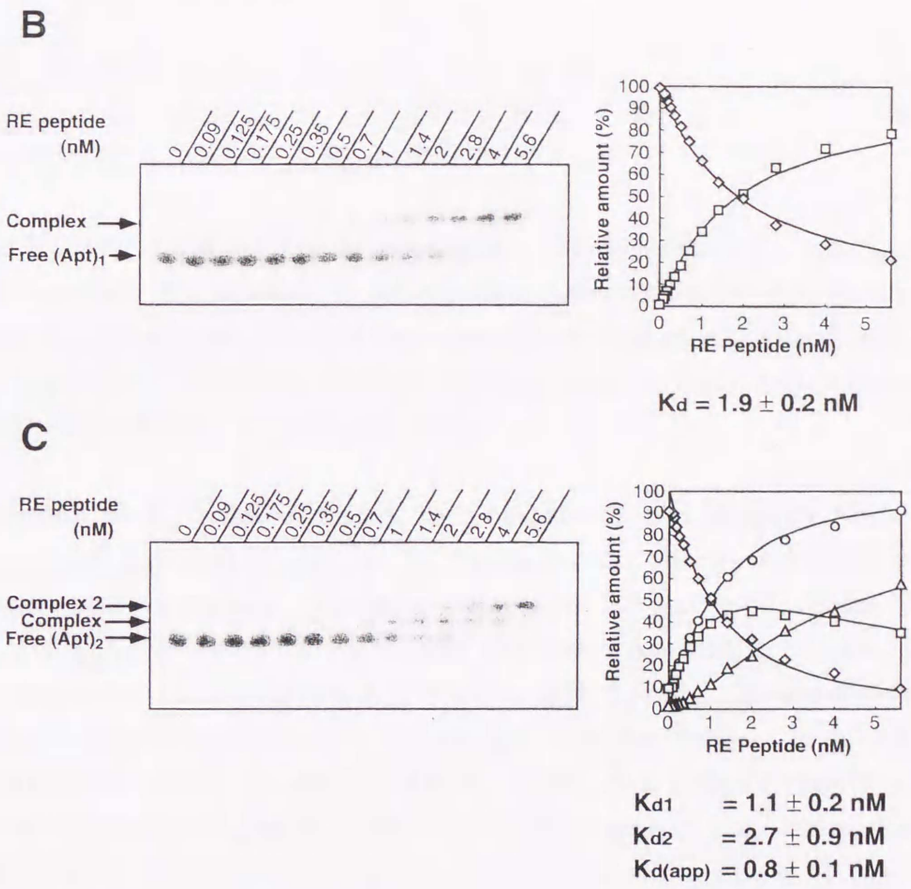


Figure. 3B,C. Tat aptamer $(Apt)_1$ or tandem Tat aptamer $(Apt)_2$ binding to RE peptide. The mixtures of 5'-end labeled RNA and RE peptide were incubated at 30 °C for over 1 hour in Tat binding buffer. Free and peptide bound RNA were separated by non-denatureing gel electrophoreses through 15 % poluacrylamide. The scheme and the graph of gel mobility shift assay showing the formation of complex between (B) $(Apt)_1$, or (C) $(Apt)_2$ RNA and RE peptide. Binding reactions contained 5'-end labeled (B) $(Apt)_1$ (30 pM, 100 cpm) or (C) $(Apt)_2$ (30 pM, 100 cpm) and various concentration of RE peptides. ◇ shows the relative amount of free $(Apt)_1$ (%) or $(Apt)_2$ (%). □ shows the relative amount of complex between $(Apt)_1$ or $(Apt)_2$ and RE peptide (%). In the case of $(Apt)_2$, because $(Apt)_2$ have two binding site to RE peptide, complex between $(Apt)_2$ and two RE peptides was formed (complex 2). Δ shows the relative amount of complex 2 between $(Apt)_2$ and two RE peptides (%). O shows the sum of relative amount of complex (complex 1 (%) + complex 2 (%). K_d value of $(Apt)_1$ are and 1.8 nM. In the case of $(Apt)_2$, K_d value on the first binding of RE peptide is 1.2 nM and K_d value on the next binding of RE peptide to complex is 3.6 nM. Apperent K_d value on the formation of complex is 0.9 nM.

K_{d2}= [$(Apt)_2$·RE]·[RE]/ [$(Apt)_2$·RE·RE]

$Y_{free}(\%) = K_{d1} K_{d2} / (K_{d1} K_{d2} + K_{d2} [RE] + [RE]^2) \times 100$
$Y_{complex1}(\%) = K_{d2} [RE] / (K_{d1} K_{d2} + K_{d2} [RE] + [RE]^2) \times 100$
$Y_{complex2}(\%) = [RE]^2 / (K_{d1} K_{d2} + K_{d2} [RE] + [RE]^2) \times 100$

K_{d1} and K_{d2} are 1.2 nM and 3.6 nM respectively, when I calculated K_{d1} and K_{d2} values based on these equation. K_{d1} of $(Apt)_2$ to RE peptide is lower than K_d of Aptamer to RE peptide. Apparent K_d value of $(Apt)_2$ is 2-fold lower than that of Aptamer. K_{d2} values hardly changed by linking 2 aptamers. Therefore, when Tat peptide is linked tandemly, I can expect to drastically reduce K_d value of $(Apt)_2$ to tandem RE peptide.

Comparison of K_d Value of $(Apt)_3$ by Competitive Gel Mobility Shift Assay

I can not detect K_d values of $(Apt)_3$ to Tat peptide in order that gel shift bands did not isolate each other (Data not shown). Therefore, I investigated K_{rel} of $(Apt)_3$ against $(Apt)_1$ (K_{rel} = $K_{d(Apt)3}$ / $K_{d(Apt)1}$) by competitive gel mobility shift assay. Apparent K_d of $(Apt)_3$ is derived by K_{rel}. As control, I also measured K_{rel} of $(Apt)_2$ against $(Apt)_1$. I measured the amount of $(Apt)_2$ or $(Apt)_3$ in order to dissociate 50% of $(Apt)_1$ from RE peptide. In this cases, I can not measure exact K_d values. To measure exact K_d values, each Aptamer must be added in large excess in comparison to peptide. However, In these assays, I can know whether binding affinity of $(Apt)_3$ to Tat peptide is higher or not in comparison to affinity of $(Apt)_1$ or $(Apt)_2$ to Tat peptide. In Figure 4, K_{rel} of $(Apt)_2$ is 0.8 and K_{rel} of $(Apt)_3$ is 0.5 against $(Apt)_1$. This result show apparent K_d value of $(Apt)_3$ is 2 fold lower than $(Apt)_1$ at least, and is lower than $(Apt)_2$.

Construction and Purification of Dihydrofolate Reductase and RE Peptide Fusion Proteins; DHFR-$(RE)_n$

I constructed the 3 types of plasmid vectors expressing the 3 kinds of DHFR-RE peptide fusion proteins, that is , DHFR-$(RE)_1$, DHFR-$(RE)_2$ and DHFR- $(RE)_3$. RE peptide, and 2 and 3 RE peptides, which were linked tandemly, are connected to C terminal end of DHFR respectively. I predicted that binding activities of tandem Tat peptide domains to tandem RNA aptamer motifs increase dramatically. I adopted DHFR as the model protein of selection (Iwakura et al., 1995; Fujita et al., 1997; Fujita et al., 1998). In future, I may carry out *in vitro* selection of DHFR containing random sequences using our system. 6 histidine amino residues were introduced to N terminal end of each DHFR-RE proteins because of easy purification of protein by nickel affinity chromatography. Linkers between DHFR and RE peptide, and between Tat peptides

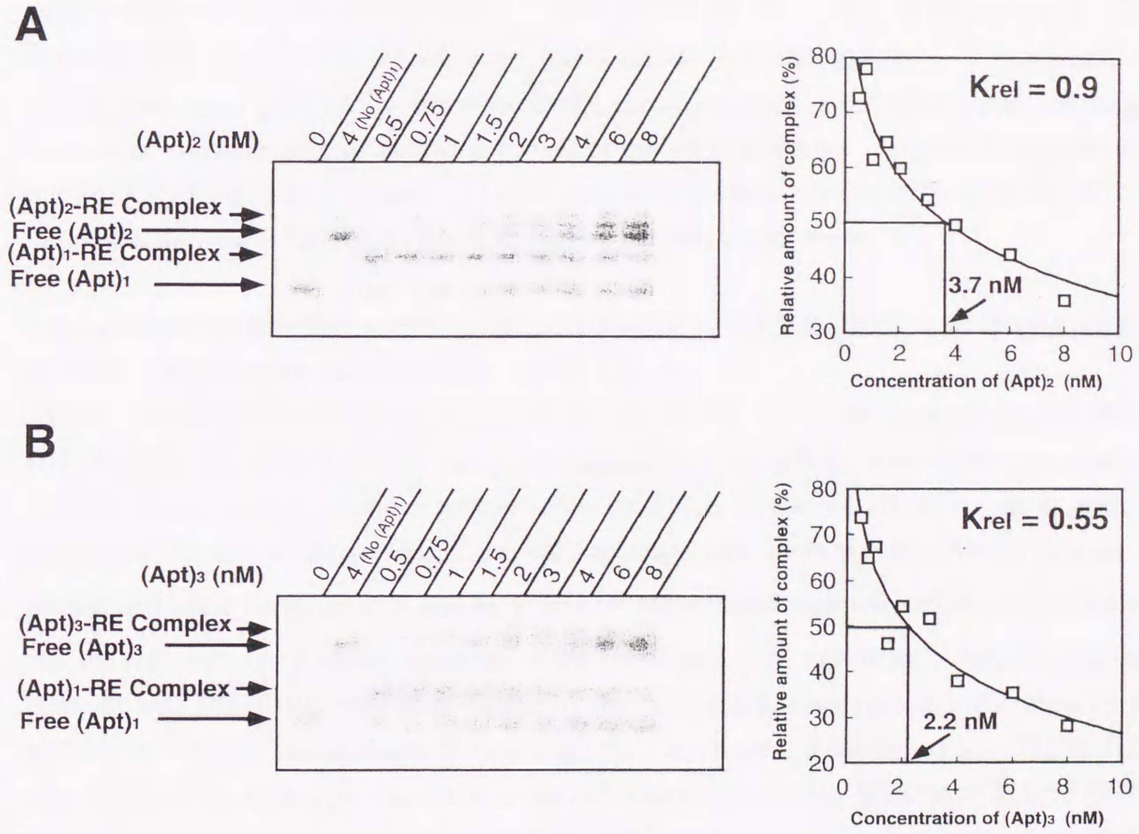


Figure 4. Competitive binding assay showing the formations of complexes between (A) $(Apt)_1$ or $(Apt)_2$ RNA and RE peptide, and (B) $(Apt)_1$ or $(Apt)_3$ RNA and RE peptide. labeled 4 nM aptamer $(Apt)_1$ and 4 nM RE peptide were pre-mixed with tRNA in Tat binding buffer after denature and restructure. After incubation for binding, 0.5 nM - 8 nM of tandem aptamer as a competitor ($(Apt)_2$ or $(Apt)_3$) were added to pre-mixture and these mixture were incubated in Tat binding buffer in a total volume of 10 μl at 30 °C for over 1 hours. Free and protein or peptide bound RNA were separated and were analyzed by 15 % non-denatureing gel.

were constructed by glycine and alanine, probably because glycine and alanine residues hardly affect the structure of DHFR and Tat peptides (Figure 2). I introduced the open reading frame of these 3 kinds of fusion proteins to pET30 plasmid vector. *E.coli* are transformed by pET vectors and each DHFR-RE proteins are induced by IPTG. *E. coli* was incubated at 30 °C to suppress the formation of inclusion body. After incubation for 4 hours, at first, crude proteins extracted from *E. coli* were purified by nickel affinity chromatography. In next, purified protein were more purified by cation exchange chromatography again after buffer exchange, because of contamination of degraded protein at the sites of linkers. Figure 5 showed each purified DHFR-RE fusion proteins. I confirmed each proteins have the activity of DHFR. It means that domain of DHFR in DHFR-RE fusion proteins folded correctly.

Comparison of Binding Activity of DHFR-$(RE)_1$, DHFR-$(RE)_2$ and DHFR-$(RE)_3$ to each Aptamers by Gel Mobility Shift Assay

At first, I carried out the filter binding assay of each DHFR-RE fusion proteins; DHFR-$(RE)_1$, DHFR-$(RE)_2$ and DHFR-$(RE)_3$, and each aptamers; $(Apt)_1$,$(Apt)_2$ and $(Apt)_3$, to confirm whether fusion proteins bind to aptamer with specificity (Data not shown). As a control, interaction between wild type DHFR and each aptamers were investigated. Mixed samples of protein and labeled aptamer at 5' end by γ^{32}P-ATP were passed over nitrocellulose membranes, and the radioactivity of trapped aptamer on the membrane were measured. Aptamers formed complex with DHFR-RE proteins is trapped by the membranes, since protein is absorbed by the membrane, whereas free aptamers flow through the membrane. I confirmed that DHFR-$(RE)_2$ and DHFR-$(RE)_3$ fusion proteins bind to each aptamers respectively, whereas wild type DHFR can not bind to each aptamers in spite of adding high concentration. In both cases, I can expect strong interaction under nM scale of K_d. However, DHFR-$(RE)_1$ hardly binds to $(Apt)_1$ despite of adding 30 nM of DHFR-$(RE)_1$.

I performed get mobility shift assay of DHFR-RE series; DHFR-$(RE)_1$, DHFR-$(RE)_2$ and DHFR-$(RE)_3$, and each aptamers; $(Apt)_1$, $(Apt)_2$ and $(Apt)_3$ to compare binding activity and to investigate whether interaction between DHFR-$(RE)_n$ and $(Apt)_n$ are strong enough to combine mRNA and protein for long time. To combine between RNA and protein for long time is important for a transport and recruitment of the protein. As a control, I investigated the binding of DHFR to each aptamers. Same as previous experiment of gel mobility shift assay, aptamers (30 pM) labeled γ^{32}P-ATP at 5' end and excess fusion proteins were mixed and loaded to gel after incubation. I measured the ratio of complex from radioactivities of free aptamers and aptamers forming complex. In figure 6, each aptamers form complex with each fusion proteins respectively as adding suitable concentration of proteins, whereas DHFR and each

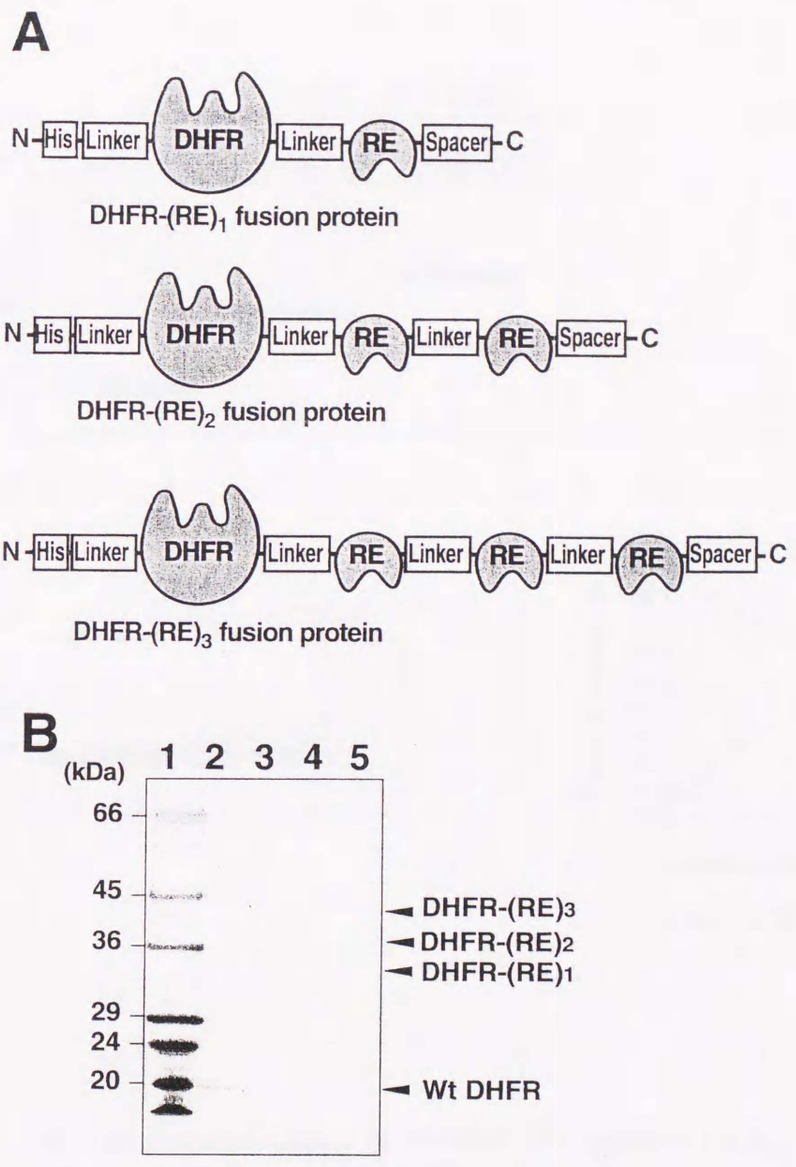


Figure 5. DHFR-$(RE)_1$, DHFR-$(RE)_2$ and DHFR-$(RE)_3$ fusion proteins and wild type DHFR protein. (A) Schematic drowing if the protien used in this study. (B) Purified, reombinant proteins (~100 μg) werre electrophoresed on a 10-20 % Tris-glysine polyacrylamide gradient gel in the presence of SDS and visualized by Coomassie blue staining. Lane 1, wild type DHFR; lane 2, DHFR-(RE)1; lane 3, DHFR-(RE)2; lane 4, DHFR-(RE)3. Molecular weight markers (kDa) are shown on the right side.

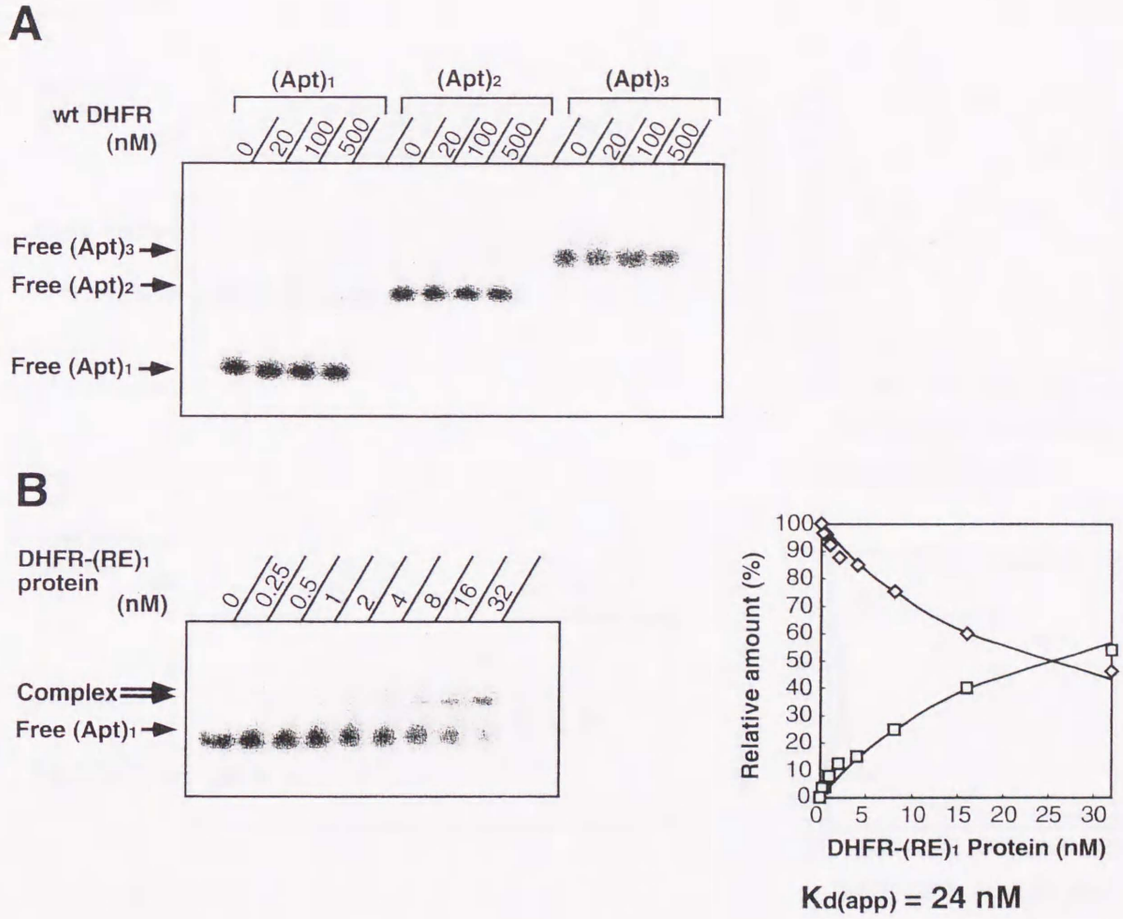


Fig. 6A, B. (A) Tat aptamer $(Apt)_1$ or tandem Tat aptamer $(Apt)_2$ or $(Apt)_3$ not binding to wild type DHFR protein. (B) Tat aptamer $(Apt)_1$ binding to DHFR-$(RE)_1$ fusion protein. In all cases, the mixtures of 5'-end labeled RNA and RE peptide were incubated at 30 °C for over 1 hour in Tat binding buffer. Free and peptide bound RNA were separated by non-denatureing gel electrophoreses through 15 % polyacrylamide. Binding reactions contained 5'-end labeled (A) RNA (1 nM, 1000 cpm) or (B) $(Apt)_1$ (30 pM, 100 cpm) and various concentration of RE peptides. ◇ shows the relative amount of free $(Apt)_1$ (%). □ shows the relative amount of total complex between $(Apt)_1$ and RE peptide (%).Apperent K_d value of $(Apt)_1$ are 24 nM.

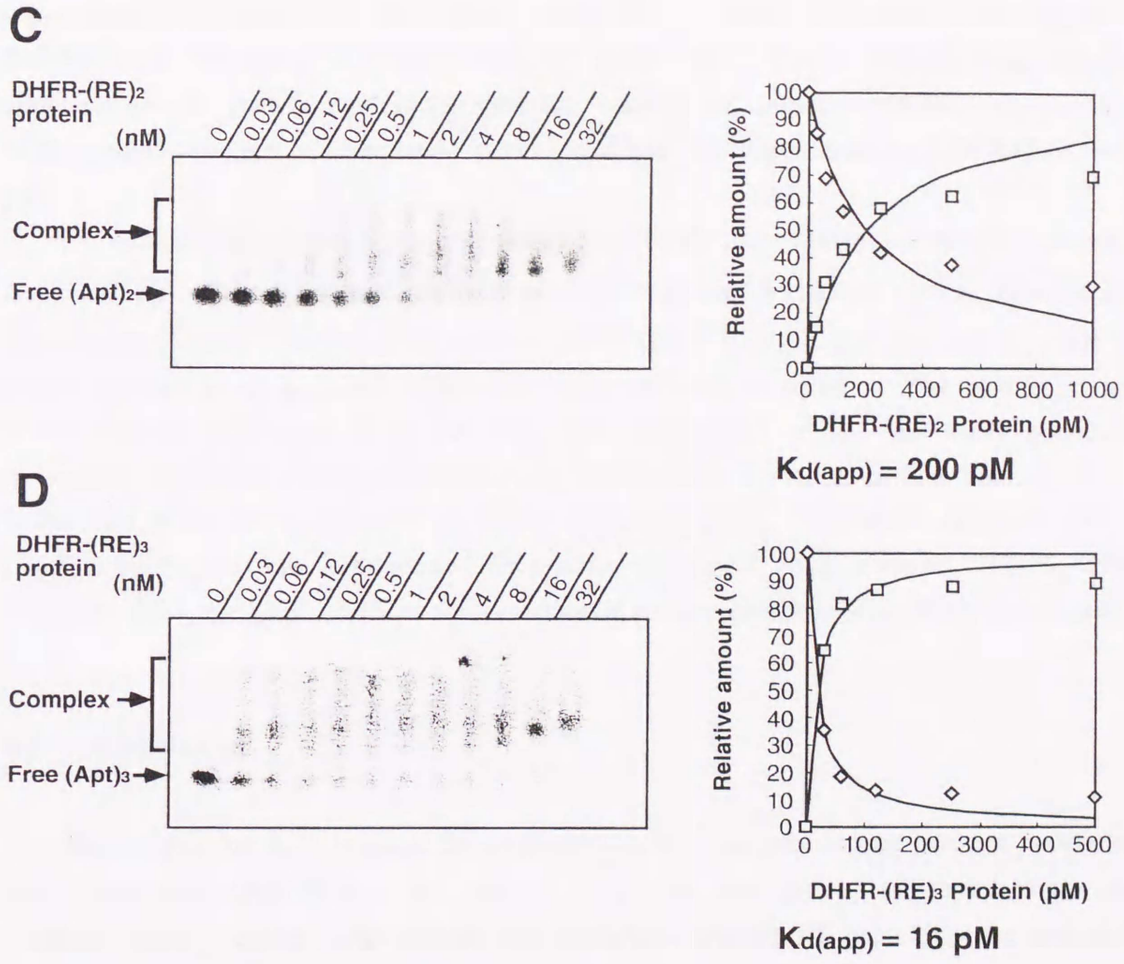


Fig. 6C, D. (C) Tat aptamer $(Apt)_2$ binding to DHFR-$(RE)_2$ fusion protein. (D) Tat aptamer $(Apt)_3$ binding to DHFR-$(RE)_3$ fusion protein. In all cases, the mixtures of 5'-end labeled RNA and RE peptide were incubated at 30 °C for over 1 hour in Tat binding buffer. Free and peptide bound RNA were separated by non-denatureing gel electrophoreses through 4% polyacrylamide. Binding reactions contained 5'-end labeled (C) $(Apt)_2$ (30 pM, 100 cpm) (D) $(Apt)_3$ (30 pM, 100 cpm) and various concentration of RE peptides. ◇ shows the relative amount of free $(Apt)_2$ (%) or $(Apt)_3$ (%). □ shows the relative amount of total complex between $(Apt)_2$ (%) or $(Apt)_3$ and RE peptide (%).Apperent K_d value of $(Apt)_2$, and $(Apt)_3$, are 200 pM and 16 pM respectively.

aptamers did not form complex despite of adding nM scale of DHFR. Binding activities of $(Apt)_2$ and DHFR-$(RE)_2$, and $(Apt)_3$ and DHFR-$(RE)_3$ are drastically higher than binding activity of $(Apt)_1$ and DHFR-$(RE)_1$. In DHFR-$(RE)_2$ and DHFR-$(RE)_3$, various complexes were formed. In the results of filter binding assay and gel mobility shift assay, it is suggested that RE peptide domains of DHFR-$(RE)_2$ and DHFR-$(RE)_3$ fusion proteins form suitable structures and are specific binding for aptamers, whereas RE domain of DHFR-$(RE)_1$ can not form suitable structure or some steric hindrance affects interaction between DHFR-$(RE)_1$ and $(Apt)_1$.

I calculated apparent K_d values of each cases by the most simple second order reaction : $A + B \rightleftarrows AB$. These reactions are likely to very complicated because theoretical curve did not fit to practical values. Apparent K_d value of DHFR-$(RE)_1$ to $(Apt)_1$ is higher than K_d value of normal aptamer to RE peptide. This result might reflected the result of filter binding assay. In the contrast, K_d values of DHFR-$(RE)_2$ and DHFR-$(RE)_3$ in gel shift assay decreased dramatically (200 pM and 30 pM respectively) according to the result of filter binding assay. In the case of the interaction between DHFR-$(RE)_3$ and $(Apt)_3$, because of adding 30 pM of $(Apt)_3$ to binding mixture, it is expected that practical apparent K_d value is lower. The K_d value of DHFR-$(RE)_3$ and $(Apt)_3$ suffices for formation of stable complex between RNA and protein.

4.5. Conclusion

Our ultimate purpose is to produce the mediators for the strongest linking between functional protein and functional RNA *in vivo* and *in vitro*. In this study, I suggested to use the strongest binding between RNA aptamer and Tat-derived peptide (RE peptide) as the mediators for linking between protein and RNA. RNA aptamer that was evolved by *in vitro* genetic selection recognized RE peptide with high affinity and specificity. Dissociation constant of Tat aptamer was over 20 folds lower than the dissociation constant of TAR RNA (Figure 3). To develop more superior binding RNA and peptide motifs with the strongest affinity based on the interaction between Tat aptamer and RE peptide is not only useful as the transport and delivery of the functional protein and RNA but also as the tools of various bio-technics. Furthermore, the analysis of strongest interaction between RNA and protein is meaningful at physical chemistry.

When protein (A) binds to the ligand (C), Gibbs free energy change of the protein A (ΔG_a) is the sum of the loss of the entropy (ΔG_s) to approach the ligand and intrinsic Gibbs free energy change of the protein A (ΔG^i_a) for binding to the ligand ($\Delta G_a = \Delta G_s + \Delta G^i_a$). When the protein having two domains (A-B) binds to the ligand having two sites (C-D) and after domain

A binds to site C, domain B binds to site D in turn, Gibbs free energy change of binding domain A to site C is $\Delta G_a = \Delta G_s + \Delta G^i_a$ and Gibbs free energy change of binding domain B to site C is $\Delta G_b = \Delta G^i_b$. Consequently, Gibbs free energy change of the protein A-B (ΔG_{ab}) is equal to the sum of ΔG_a and ΔG_b ($\Delta G_{ab} = \Delta G_s + \Delta G^i_a + \Delta G^i_b$). The dramatically reduction of Gibbs free energy is expected by the linking between protein having over 2 binding motif and ligand having over 2 binding site, because domain B approaches to site D after binding domain A to site C and there are no the loss of entropy for binding domain B to site D (Jencks, 1981).

At first, I designed the 3 kinds of tandem Tat aptamers; $(Apt)_1$,$(Apt)_2$ and $(Apt)_3$), based on these ideas. When I confirmed the dissociation constant of each Tat aptamers to RE peptide, the dissociation constant of tandem Tat aptamers and RE peptide decreased in compare to that of normal aptamer and RE peptide (Figure 3). I guessed that even if the complex is released, in the case of tandem aptamer, the possibility that the same complex is formed again is higher than the possibility in the case of normal aptamer (that is, vibration of molecules). These effect might be affect the suppression of the transcription of HIV-1 mRNA by the tandem TAR decoy that was reported. In the next, I constructed the 3 kinds of tandem RE peptide fusion proteins that were linked to DHFR as the model of functional protein; DHFR-$(RE)_1$, DHFR-$(RE)_2$ and DHFR-$(RE)_3$, and investigated the interaction between tandem aptamers; $(Apt)_1$, $(Apt)_2$ and $(Apt)_3$, and DHFR-tandem RE peptide fusion proteins. The affinity of normal aptamer, $(Apt)_1$, to RE peptide linked DHFR, DHFR-$(RE)_1$, is lower than the affinity of $(Apt)_1$ to free RE peptide, probably because of the steric hindrance of DHFR. Otherwise, the affinity of tandem peptide motifs (DHFR-$(RE)_2$ and DHFR-$(RE)_3$) and tandem RNA motifs ($(Apt)_2$ and $(Apt)_3$) is dramatically higher than the affinity of each normal motifs (DHFR-$(RE)_1$ and $(Apt)_1$) according to the theory of Jencks (Jencks, 1981). These experimental result was reported by the case that two ligands recognizing the different sites of same protein respectively were combined (Shuker et al., 1996; Hajduk et al., 1997). Some reports relate to the artificial strong binding between RNA and protein have been nothing. Especially, the dissociation constant of $(Apt)_3$ and DHFR-$(RE)_3$ is lower than 16 pM (Figure 6). However, the dramatic decrease of the dissociation constant according to the theory of Jencks was not detected same as the previous report in Nature. The change of intrinsic free energy by second or third binding might decreased by the restriction of motion of second or third domain. And the binding between aptamer and RE peptide might be insufficient for the steric hindrance of each domains. The broadening of the bands of complexes in gel mobility shift assay of DHFR-$(RE)_2$ and DHFR-$(RE)_3$ is because the complexes might formed various tertiary structure or two complexes might formed dimer. The formation of dimer might improve the stability of RNA-protein complex. The dimerization of complexes hardly affect the transport and delivery of functional molecules. On the other hand, the dimerization of complexes is undesirable for

protein selection system *in vitro*, because it is important that interaction between protein and RNA having protein information at 1 to 1 ratio is formed. Unfortunately, the optimization of linkers to combining aptamers or RE peptides might be insufficiently. However, I showed the strongest interaction between tandem aptamer and tandem RE peptide is useful as adapter to linking functional protein and RNA.

Because these adapter motifs consist of RNA or peptide, I can construct these motifs without the reaction of chemical synthesis. Hence, it is possible that linking between protein and RNA expressing *in vivo* without *in vitro* approach. And the strongest interaction is efficient for novel selection system of the protein *in vitro*, if interaction between RNA and protein is formed at 1 to 1 ratio.

Chapter 5

Approach for *in vitro* Rational Protein Selection Using Dihydrofolate Reductase (DHFR) as Model Protein

5.1. Summary

The rational screening system for protein *in vitro* (in cell-free) were developed. The binding protein can be enriched by the cycles of transcription, translation, affinity selection and PCR. Model protein and its encoding mRNA were linked by the strongest mediators; RNA aptamer and RNA binding peptide, in order to obtain genotype of the selected protein. Intact dihydrofolate reductase (DHFR), as the model, was enriched 9 folds over from a pool of intact and mutant DHFRs using the system. I enriched the mRNA encoding intact protein 2.5 folds by 1 cycle of the method.

5.2. Introduction

Protein and peptide are expected as the excellent functional molecules that avail to medical drugs and industrial usage, because they consisting of the combination of 20 kinds of amino acids have more complexity and diversity than another molecules, such as ribonucleic acid is compose of only 4 kinds of nucleotides. Indeed, almost cellular functional molecules, including enzyme, antibody, hormone, and regulation factor, are constructed by them. Therefore, a discovery of novel functional proteins and peptides have the advantage of industrial and medical developments.

The various screening systems to select the specific functional proteins from the pool of them having random sequences of amino acids have been developed. It is the common problems in all of these systems to amplify the information of selected proteins, to return them to the pool in next cycles and to read their information by peptide sequencer. To resolve these problems, it is important to couple genotype, namely ribonucleic acid, and phenotype, namely protein, for selecting proteins. However, the most of systems are not able to retain the diversity of pool, because they have the process based on living cells in order to link phenotype to genotype (Schatz et al., 1996; Moore and Arnord, 1996; Harada et al., 1996; Boder and Wittrup, 1997; Zhang et al., 1997). In the case of phage display, diversity of pool of proteins is about only population of 9 orders (Smith, 1985; Harrison et al., 1996; Smith and Petrenko,1997).

Recently, two types of selection techniques of special protein (peptides or antibody) in cell-free system, so-called ribosome display (polysome display) system and protein-RNA covalent binding system, have been reported (Mattheakis et al., 1994; Mattheakis et al., 1996; Hanes and Pluckthun, 1997; He and Taussig, 1997; Nemoto et al., 1997; Roberts and Szostak, 1997; Hanes et al., 1998; Makeyev et al., 1999; Hanes et al., 1999). In these approaches based on the concept of *in vitro* molecular evolution, multiple rounds of selection cycles, that DNA library is transcibed, and the RNA is translated, protein with RNA having the information of amino acid sequences is selected, selected RNA is reverse transcribed and amplified, are repeated to enrich functional proteins. These approaches can follow amino acid sequences of population of 12 orders in theory, because of cell-free. In the former case, ribosome forms the complex along with protein and ribonucleic acid, and complex is retained by eliminating stop codon. In the latter case, protein binds covalently to ribonucleic acid which is ligated to DNA labeled by puromysin at 3' end. In both cases, protein-RNA complex must be formed stably and largely *in vitro* to combine genotype and phenotype, thereby selecting functional proteins efficiently.

In this study, I developed the rational screening system for protein *in vitro* (in cell-

free) (Figure 1). In our system for protein, novel peptide and RNA motifs, described in Chapter 4, are used as mediators to form the strongest protein-RNA linkage without chemical synthesis. Novel peptide motif; $(RE)_n$ consisting of tandemly multiple Tat derived peptides binds to novel RNA motif consisting of tandemly multiple Tat aptamer with high affinity and specificity. Especially, binding affinity of DHFR fused 3 tandemly repeated RE peptides, namely DHFR-$(RE)_3$, and 3 tandem repeated aptamers, namely $(Apt)_3$, is stronger than that of almost antibody and antigen (K_d value is less than 30 pM) (refer in Chapter 4). Therefore, in our system, selection of protein will be performed stably and easily in comparison to the selection of protein in the other systems in cell-free. Namely, because, in ribosome display, the retaining of the RNA-ribosome-protein complex is difficult and, in protein-RNA covalent binding systems, synthesis for linking between puromycin and mRNA is difficult.

5.3. Materials and Methods

Construction of 3 Kinds of Plasmids; pDHFR-$(RE)_3$-$(Apt)_3$, pMDHFR-$(RE)_3$-$(Apt)_3$ and pDHFR-$(RE)_3$

Construction of pDHFR-$(RE)_3$ (Control) (Figure 2), that encodes DHFR-RE_3 fusion protein, was described in Chapter 4. For mutation of core region of DHFR, 4 kinds of oligonucleotides are prepared by DNA synthesizer (model 392A; Applied Biosystems); complimentary primer with *Xba* I site against upstream of DHFR gene 5'- ACA ATT CCC CTC TAG AAA TA -3', complimentary primer against downstream of gene of RE peptide 5'- GTG GTG GTG CTC GAG AAT TC -3', sense oligonucleotide for mutation 5'- CCT GCC GCC CTC GCC TGG GCC AAA CGC AAC ACC TTA AAT AAA -3', anti-sense oligonucleotide for mutation 5'- GCG TTT GGC CCA GGC GAG GGC GGC AGG CAG GTT CCA CGG -3'. 2 parts of mutations, were introduced to DHFR gene using the primers and oligonucleotides by mega primer method, lead to 2 sites of mutations of amino acid sequences in DHFR; Asp 27 -> Ala 27 and Phe 31 -> Ala 31, namely the plasmids encoding mutant DHFR fused 3 tandemly repeated RE peptides; pMDHFR-$(RE)_3$-$(Apt)_3$, was constructed. Next, 3 tandem aptamer sequences $(Apt)_3$ described by Chapter 4 were introduced to *Eco* RI site in the downstream of RE peptides in pDHFR-$(RE)_3$ and pMDHFR-$(RE)_3$, that is pDHFR-$(RE)_3$-$(Apt)_3$ (Intact), pMDHFR-$(RE)_3$-$(Apt)_3$ (Mutant) (Figure 2) (Taira et al., 1987; Taira and Benkovic, 1988); hereafter, each plasmids expressing mutant DHFR-$(RE)_3$ fusion protein and expressing intact DHFR-$(RE)_3$ fusion protein are abbreviated to pMDHFR-$(RE)_3$-$(Apt)_3$ and pDHFR-$(RE)_3$-$(Apt)_3$.

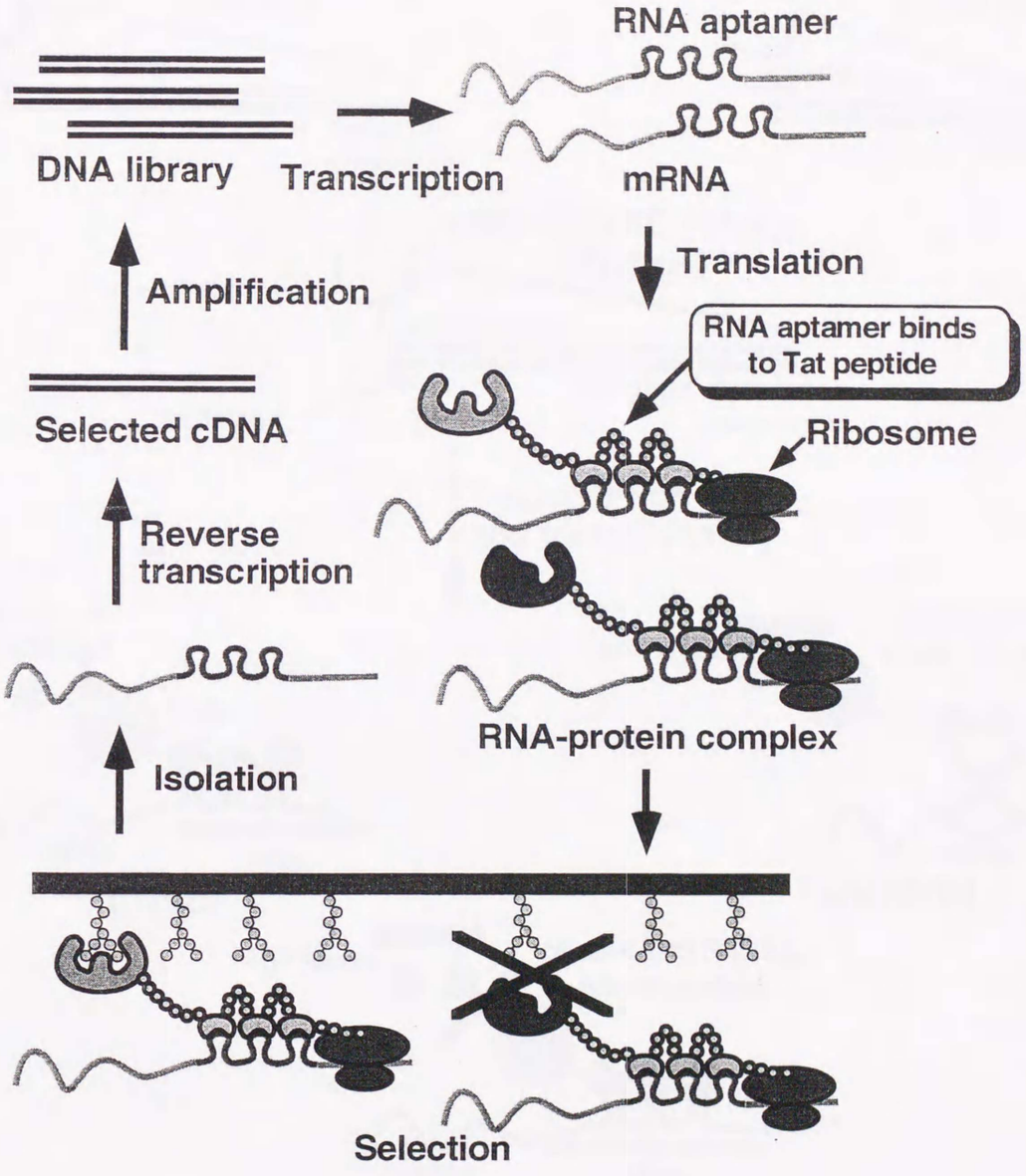


Figure 1. The schematic diagram of rational protien selection *in vitro.* The binding protein are enriched by the multiple cyclces of transcription, translation, affinity selection and PCR. In translation step, tandemly repeated RNA aptamer binds to Tat peptide with high affinity. The binding associate the coupling between the binding protein and its encoding mRNA. In this model study, we used DHFR as the taget of seletion.

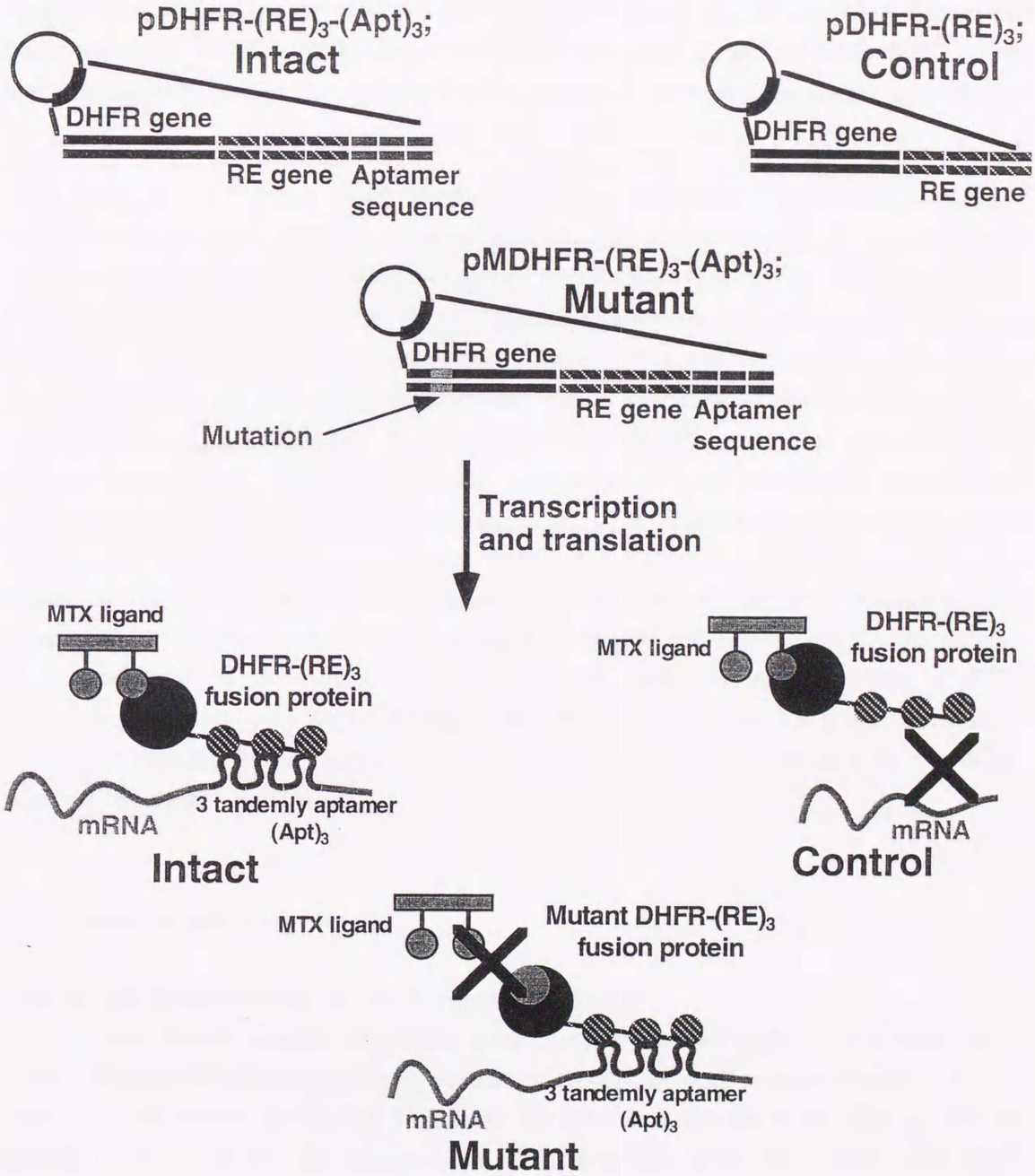


Figure 2. Model of rational selection for protein *in vitro*. I prepared 2 types of plasmids (pDHFR-$(RE)_3$-$(Apt)_3$; Intact, pDHFR-$(RE)_3$-$(Apt)_3$; Mutant) expressing 2 kinds of proteins (DHFR-$(RE)_3$ fusion protein, mutant DHFR-$(RE)_3$ fusion protein). mRNA is selected, only when DHFR bind to MTX ligand. As the control, plasmid except aptamer sequences (pDHFR-$(RE)_3$) was prepared. In control, mRNA is not selected regardless of binding activity of DHFR.

Preparation of 3 Kinds of mRNAs and Concentration of Intact mRNA and Protein

Each plasmids, pDHFR-$(RE)_3$-$(Apt)_3$, pMDHFR-$(RE)_3$-$(Apt)_3$ and pDHFR-$(RE)_3$, were linearized by restriction enzyme, namely *Xho* I. Linear DNAs were transcribed to mRNA with RNA-cap-structure-analog (New England Bio Labs Inc.) and α^{32}P-CTP by T7 polymerase *in vitro* at 37 °C for 2 hours using T7 Ampliscribe kit (Epicentre Technologies). Labeled mRNAs were purified by NICK™ Columns (Amersham Pharmacia Biotech). 1 μg of capped-mRNAs coding DHFR-$(RE)_3$ (Intact) and mutant DHFR-$(RE)_3$ (Mutant) are translated in 20 μl of rabbit reticulcyte lysate for 20 min at 30 °C (by Rabbit Reticulocyte Lysate System; Promega) (Figure 2). As the control, mRNA in absence of aptamer was translated too (Control) (Figure 2). Each proteins are labeled by ^{35}S-methionine. After translation, 180 μl of binding buffer; 10 mM Na·phosphate buffer (pH 7.5), 70 mM NaCl, 10 μM tRNA, and 20 μl of MTX-agarose (Sigma), were treated by succinic anhydride, were added to lysate and binding reaction was performed for 30 min at 4 °C. 200 μl of supernatants of the binding solutions containing non-binding mRNA and protein were removed from binding solution, and MTX-agaroses were washed by 180 μl of binding buffer at 2 times. 1 μl of lysate including mRNA and translated protein, 20 μl of supernatants of binding solution containing non-binding mRNA and protein, 20 μl of supernatants of washing solution and 10 μl of mRNA and protein binding to MTX-agarose were isolated by 15 % SDS-PAGE. The efficiency of the selection was determined by quantity of radioactivity in bands of the mRNA and protein of SDS-PAGE with Bio-Image Analyzer (BAS2000, Fuji Film).

5.4. Results and Discussion

Design and Construction of the Screening System

Our suggested rational selection for protein in cell-free is shown in Figure 1. It is important to couple genotype and phenotype, namely protein and ribonucleic acid, to select proteins. I used 3 tandemly RE peptide and tandem 3 tandemly Tat aptamer motifs for strong linking between genotype and phenotype. In our approach, multiple rounds of selection cycles, that DNA library is transcibed, the RNA is translated, protein with RNA encoding the information of amino acid sequences is selected, and selected RNA is reverse transcribed and amplified, are repeated to enrich functional proteins.

As the model to construct our rational screening system for protein, DHFR derived from *E. coli* is employed as the target of selection (Figure 1) DHFR that reduces dihydrofolate to tetrahydroforate with the assistance of coenzyme; nicotinamide adenine dinucleotide phoshate, reduced form (NADPH), is essential for the synthesis of thymidylic acid. I prepared 2 types of

plasmids expressing 2 kinds of DHFR-$(RE)_3$ fusion-proteins [Intact; pDHFR-$(RE)_3$-$(Apt)_3$, Mutant; pMDHFR-$(RE)_3$-$(Apt)_3$] (Figure 2). Each plasmids have the sequences encoding DHFR and 3 tandemly linked RE peptides; $(RE)_3$. DHFR proteins fused $(RE)_3$ peptides by linker at C terminal end are translated from these plasmids. Linkers between DHFR and RE peptide, and between each RE peptides are formed by poly-glycine, because of keeping distances and flexibility. Furthermore, 3 tandemly linked Tat-aptamer sequences $(Apt)_3$ are connected in downstream of open reading frame of DHFR-$(RE)_3$ fusion protein (Figure 2). Appropriate linkers formed by poly-adenine are inserted between aptamers for keeping distances. One of the 2 types of plasmids differ from the other one by only two nucleotide mutations encoding the part of binding core of DHFR. The mutations abolish the binding activity of DHFR to MTX (Figure 2) (Taira et al., 1997; Taira ad Benkovic, 1988). Moreover, as control, I prepared the intact DHFR encoding plasmid without aptamer sequences, namely, pDHFR-$(RE)_3$ (Figure 2).

Because T7 promoter sequences are inserted to upstream of DHFR gene in 3 kinds of plasmids, mRNA can be transcribed by T7 polymerase from linearized plasmids *in vitro*. Next, when transcribed 2 types of mRNAs containing 3 tandemly Tat-aptamers have been translated, translated 3 tandemly RE peptides will bind to 3 tandemly Tat-aptamers. Because cis-reactions takes precedence over trans-reactions in this binding reactions, I expect that the translated DHFR-$(RE)_3$ fusion-protein binds to template-mRNA, namely the protein-RNA complexes contain both genotype and phenotype. When 2 kinds of complexes including intact DHFR and mutant DHFR are selected by the binding affinity against MTX, I expect that mRNA containing intact DHFR sequences is obtained (Figure 1).

Optimization of Translation and Selection Condition of the Rational Screening System for Protein

To perform the selection for protein in cell-free efficiently, I investigated the applied conditions of the translation and the selection. In translation, I did not accept *E. coli* extract but rabbit reticulcyte lysate, because folding of model proteins in *E. coli* is post-translational, and leads to intramolecular misfolding of concurrently folding domains. In contrast, during eukaryotic translation same proteins fold efficiently by sequential and co-translational folding of their domains (Netzer and Hartl, 1997). Purified capped-mRNAs, which are transcribed from 3 kinds of plasmids, were translated in rabbit reticulcyte lysate and the production of each proteins (Figure 3) and the activity of DHFR (data not shown) were detected. Efficiency of translation of each proteins was lower than that of wild type DHFR. It might be because of formation of strong RNA-protein complex inhibiting translation. I thought it is important to shorten the time of translation and selection for retaining complex. I confirmed that translation for 10-20

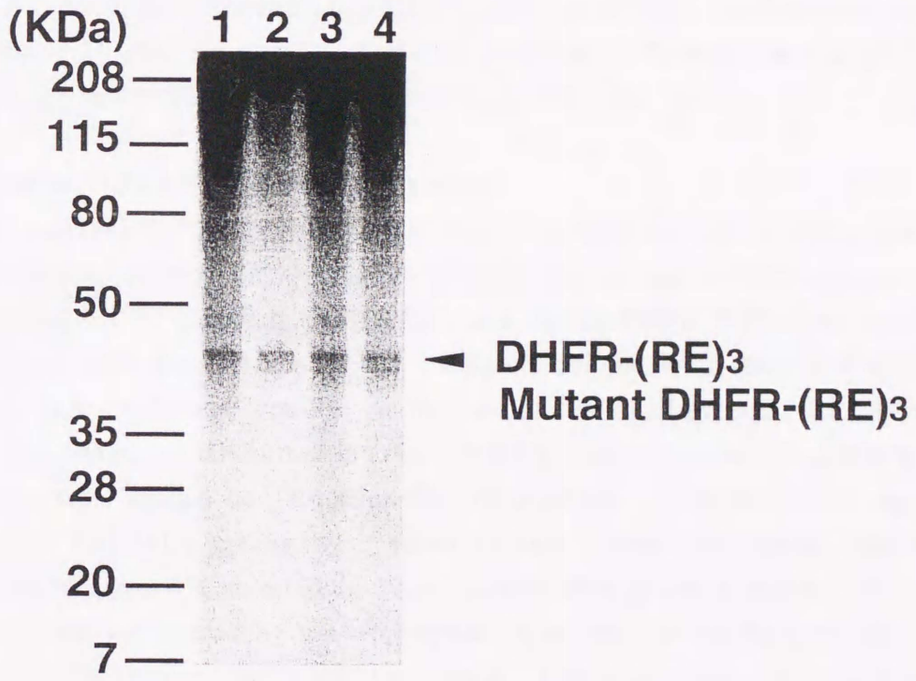


Figure 3. Translational efficiency of each DHFR-$(RE)_3$ fusion proteins. 1 μg of capped-mRNAs are translated in 20 μl of rabit reticulcyte lysate for 20 min at 30 °C in the presence of ^{35}S-Methionine. 1 μl of lysates were subjected to electrophoresis on an 15 % SDS-polyacrylamide gel. Lane 1, mutant DHFR-$(RE)_3$ fusion proteien derived from pMDHFR-$(RE)_3$-$(Apt)_3$ (Mutant). Lane 2, DHFR-$(RE)_3$ fusion protein derived from pDHFR-$(RE)_3$-$(Apt)_3$ (Intact). Lane 3, Mixture of DHFR-$(RE)_3$ and mutant DHFR-$(RE)_3$. 1:1 mixture of Intact and Mutant mRNA was translated. Lane 4, DHFR-$(RE)_3$ fusion protein derived from pDHFR-$(RE)_3$ (Control). Translational efficiency of each DHFR-$(RE)_3$ fusion proteins were almost same.

min produced proteins sufficiently. In our model of selection for DHFR, I adopted MTX-agarose, which is treated by succinic anhydride for neutralization of positive charge in agarose. I confirmed that non-specific binding of mRNA to non-treated agarose with positive charge is extremely high (data not shown). MTX is analog of substrate of DHFR, and it is known that wild type DHFR binds to MTX with high affinity and mutant DHFR with two substitutions; Asp 27 -> Ala 27 and Phe 31 -> Ala 31. Next, I checked the applied buffer-condition of selection of DHFR and confirmed DHFR-$(RE)_3$ fusion protein can bind to treated MTX-agarose in 10 mM Na·phosphate buffer at pH 5.5-pH 8.0 with high affinity. And aptamer can bind to RE peptides in 10 mM Na phoshate buffer in presence of 70 mM NaCl at pH 7.0-8.0. Aptamer can not bind to RE peptide in the absence of NaCl at all.

Enrichment of DHFR-$(RE)_3$ Fusion Protein

To confirm that DHFR-$(RE)_3$ protein-mRNA complex (Intact) can bind to MTX-agarose and mutant DHFR-$(RE)_3$ protein-mRNA complex (Mutant) can not bind to MTX-agarose, capped-mRNAs labeled by ^{32}P encoding DHFR-$(RE)_3$ and mutant DHFR-$(RE)_3$ were translated in rabbit reticulcyte lysate for 20 min at 30 °C. As the control, mRNA in absence of aptamer was translated at same condition (Control). In the case of Control, I expected that DHFR-$(RE)_3$ protein-mRNA complex is not formed in order that mRNA do not contain 3 tandemly aptamers. Each proteins were labeled by ^{35}S-methionine for detection. After translation, appropriate binding buffer and MTX-agarose were added to each lysates, and binding reaction was performed for 30 min at 4 °C to avoid the dissociation of RNA-protein complex. Supernatants of the binding solutions containing non-binding mRNA and protein were removed from binding solution, and MTX-agaroses were washed at 2 times. Lysate containing mRNA and translated protein, supernatants of binding solution containing non-binding mRNA and protein, supernatants of washing solution and mRNA and protein binding to MTX-agarose, were isolated by SDS-PAGE (Figure 4A). Figure 4 shows that amounts of translated proteins and input mRNAs were same in each cases; intact, mutant and control (lanes 1, 2, 3). Many protein and mRNA can not bind to MTX-agarose after binding reaction (lanes 4, 5, 6), because, probably, there is not enough the amount of MTX-agarose. Washing at 2 times were sufficient (lanes 7, 8, 9) in order not to detect bands. Proteins were concentrated in Intact and Control, while hardly Mutant protein can be detected (lanes 10, 11, 12). mRNA was only detected strongly in Intact (lane 10). Intact protein was selected by MTX-agarose 9 fold over Mutant protein, and Intact mRNA was selected 2.5 fold over Mutant mRNA too (Figure 4B). In Control in the absence of aptamer, only protein was enriched 16 fold over Mutant protein, while concentration of mRNA was same as Mutant mRNA (Figure 4B). Moreover, in denaturing PAGE, I confirmed that mRNA of Intact was only concentrated in comparison to

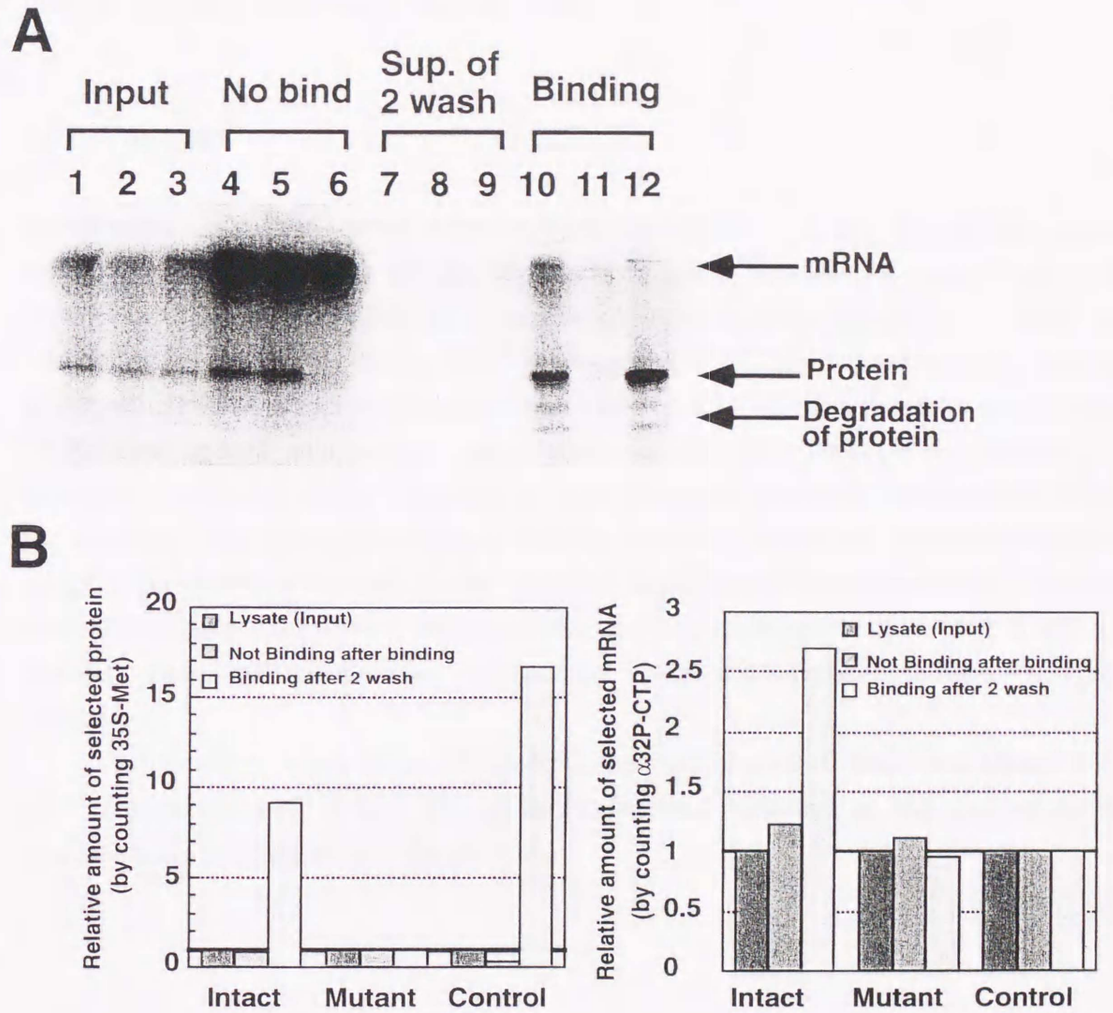


Figure 4. (A) Enrichment of DHFR-(RE)3 fusion protein and mRNA derived from pDHFR-(RE)3-(Apt)3. After translation of 3 kinds of mRNA, 180 μl of binding buffer and 20 μl of MTX-agarose were added to 20 μl of lysate for the binding of protein and mRNA against MTX. After incubation for 30 min at 4 °C, supernatants containing non-binding mRNA and protein were removed. MTX-agaroses were washed at 2 times. Finally, binding mRNA and protein were isolated from MTX-agarose in boiling water. Each samples were subjected to electrophoresis on an 15 % SDS-polyacrylamid gel. mRNAs and proteins were labeled by radioisotope. Lane 1-3; lysate after the reaction of translation. Lane 4-6; supernatants containing non-binding mRNA and protein. Lane 7-9; supernatants after washing. Lane 10-12; binding mRNA and protein against MTX. Lane 1,4,7,10 are Intact samples derived from pDHFR-(RE)3-(Apt)3. Lane 2,5,8,11 are Mutant samples derived from pMDHFR-(RE)3-(Apt)3. Lane 3,6,9,12 are Control samples derived from pDHFR-(RE)3. (B) Efficiency of enrichment of mRNA (left) and protein (right).

mRNAs of Mutant and Control (data not shown).

5.5 Conclusion

I constructed the rational protein screening system in cell-free. In this system, the originality deserving special mention is that the strongest interaction between Tat derived peptide (RE peptide) and Tat aptamer is useful for linking between genotype and phenotype. At first, in the selection using several non-treated MTX-agaroses and MTX linked magnet beads, binding of non-specific mRNA is extremely high, because mRNA with negative charge is easy to bind to MTX-linked ligands with positive charge (data not shown). To solve this problem, it is important to neutralize charge of ligands. I used 3 tandem repeated RE peptides and aptamers for selection. The strongest binding of tandem structures is important for the stabilization of complex. Intact mRNA containing only 1 aptamer, that binds to DHFR fused only 1 RE peptide, was enriched only 1.5 fold over mutant mRNA with 1 aptamer, that binds to DHFR with 1 RE peptide. Binding affinity between 1 aptamer and 1 peptide is insufficient to form RNA-protein complex.

Attempts to select Intact mRNA form the mixture pool of Intact and Mutant mRNA were unsuccessful yet. I think that applied linkers and regulation of time and condition on translation and selection resolve the problem.

Chapter 6

General Conclusion

In this study, at first, I developed *in vivo* screening system for ribozyme and succeeded the selection of active ribozyme using our system, that is introduced in Chapter 2 and 3. Natural ribozymes have RNA-cleavage activity and exhibit high substrate specificity. Therefore, they are expected to be useful for some diseases that are caused by the expression of abnormal mRNA. Because *in vivo* selection of ribozyme reflect intracellular environment, high active ribozymes that are found by our system would retain high activity in cell. If the background "noise" could be removed completely in the future, it might useful complement existing selection system.

Protein are more expected as the excellent functional molecules, because protein consisting of the combination of 20 kinds of amino acids have more complexity and diversity than another molecules. Thus, in next, I developed rational protein selection system *in vitro* and succeeded the enrichment of model binding protein (DHFR), described in Chapter 4 and 5. In the selection of protein, *in vitro* method is desirable, because of ensuring the diversity of structure. Because in the construction of *in vitro* system, the coupling of protein and RNA is important, I developed the strongest binding motifs for linking between RNA and protein.

Functional RNA and protein can be applied various field of industry. Especially, I expects that these molecules are useful as the powerful tools in gene therapy. Development of rational screening system for selection of functional RNA and protein would provides novel functional molecules and would contribute to the gene therapy of incurable disease including acquired immune deficiency syndrome (AIDS) and cancer.

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Journal Articles & Letters

1) Koguma, T., Ohkawa, J., Mori, K., **Fujita, S.**, Khoda, T., Kise, H., Nishikawa. S., Iwakura, M. and Taira, K. (**1995**)
A novel screening system for construction and selection of active ribozyme using DHFR(dihydrofolate reductase) gene as selection marker.
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2) **Fujita, S.**, Koguma, T., Ohkawa, J., Mori, K., Khoda, 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.*
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3) **Fujita, S .**, Hamada, M., Jigami, Y., Kise, H. and Taira, K. (**1998**)
A novel system for selection of intracellularly active ribozyme using the gene for dihydrofolate reductase (DHFR) as a selective marker in *Escherichia coli.*
Gene Ther. Mol. Biol. 1, 419-434.

4) Hamada, M., **Fujita, S .**, Kise, H., Jigami, Y. and Taira, K. (**1998**)
Comparison of *in vivo* activities between 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.*
J Biochem. 123, 684-692.

Reviews

5) Warashina, M., **Fujita, S .**, Utsunomiya, T., Zhou, D-M. and Taira, K. (**1997**)
Mechanism of action of a hammerhead ribozyme, a metalloenzyme.
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7) Hamada, M., Kawasaki, H., **Fujita, S .**, and Taira, K. (**1999**)
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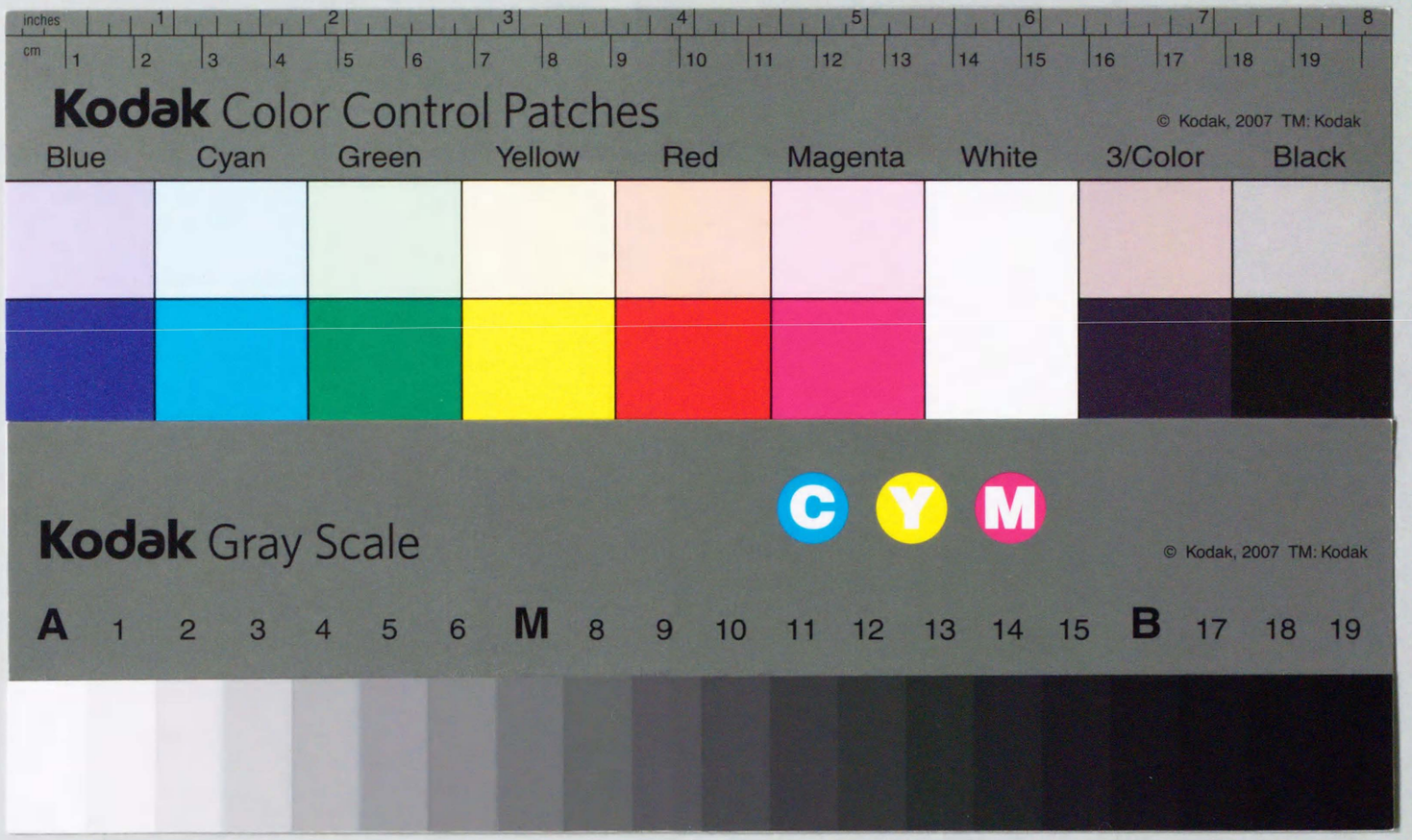
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