Nucleic Acids Research, 1993, Vol. 21, No. 2 185–190

# Cross-ligation and exchange reactions catalyzed by hairpin ribozymes

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Received November 12, 1992; Accepted December 10, 1992

## ABSTRACT

**The negative strand of the satellite RNA of tobacco ringspot virus (sTobRV(−)) contains a hairpin catalytic domain that shows self-cleavage and self-ligation activities in the presence of magnesium ions. We describe here that the minimal catalytic domain can catalyze a cross-ligation reaction between two kinds of substrates *in trans*. The cross-ligated product increased when the reaction temperature was decreased during the reaction from 37°C to 4°C. A two-stranded hairpin ribozyme, divided into two fragments between G45 and U46 in a hairpin loop, showed higher ligation activity than the nondivided ribozyme. The two stranded ribozyme also catalyzed an exchange reaction of the 3′-portion of the cleavage site.**

## INTRODUCTION

The existence of several types of RNA that have self-cleavage or self-ligation activities, has been reported (1−4). These RNAs are classified into groups based on their primary or secondary structures and their catalytic activities.

The satellite RNA of tobacco ringspot virus (sTobRV RNA) is encapsidated within the coat protein of tobacco ringspot virus and its replication is dependent on the virus. This satellite RNA consists of plus and minus strands and each strand shows self-cleavage activity in vitro (5,6). The minimal domains for the self-cleavage activities of the plus and minus strands were identified, and their secondary structures were assumed to be hammerhead (5,7,8,9) and hairpin (6,10,11,12), respectively. Both ribozymes cleave an excess of substrate RNAs and produce RNA fragments containing a 5′-hydroxyl group and a 2,′ 3′-cyclic phosphate at their termini (5,6). This *trans* esterification occurs by an in-line mechanism (13,14).

The catalytic domain of the hairpin ribozyme consists of 50 bases, and has four stems in the ribozyme-substrate complex (12). Recently, some mutagenesis experiments that targeted the self-cleavage activity of this domain were reported(12,15,16,17). We have previously described the minimum catalytic domain that catalyzes the reverse reaction like a whole strand (6,10) and that this ribozyme can be divided into two strands (17).

The plus and minus strands of sTobRV are thought to be replicated by the rolling circle mechanism (18). In this replication, the linear multimeric RNA undergoes self-cleavage and subsequent cyclization. This may be assumed to be a cross-ligation between the cleaved fragment and the neighboring cleavage site. We have previously found that the 50-base hairpin ribozyme catalyzed a cross-ligation with a low efficiency (17). In this report, we describe the influence of the reaction temperature on the yields of cross-ligated products. We also report that the cross-ligation can be catalyzed more efficiently by a two stranded hairpin ribozyme, and that this has the activity of the RNA exchange reaction, in which the 3′-side fragment of the substrate RNA was replaced with another RNA fragment. In these reactions, RNA fragments cleave and join simultaneously *in trans*. The number of base pairs between the ribozyme and either the substrate or introduced RNA fragments was an important factor in this exchange reaction. This novel *trans* exchange reaction, which has not been previously found in ribozyme reactions, will provide a useful tool for RNA modification techniques.

## MATERIAL AND METHOD

### Enzymes

T4 polynucleotide kinase (*E.coli* A19) and T4 RNA ligase were purchased from Takara Shuzo Co. Ltd.

### Oligoribonucleotides

Oligoribonucleotides were synthesized by the phosphoramidite method using 2′-*O*-*tert*-butyldimethylsilyl-5′-*O*-dimethoxytrityl 3′-*O*-phosphoroamidite which was purchased from American Bionetics, Inc. These oligonucleotides were synthesized with a DNA synthesizer, Applied Biosystems model 381A. The oligonucleotides were cleaved from the support at room temperature in 3 : 1 $cNH_4OH$ : EtOH (2 ml) for 2hr. When deprotection is done in $cNH_4OH$ : EtOH (3 : 1), the silyl groups are removed and the neighboring phosphodiester linkage is cleaved (19). The solvent was evaporated, 3 ml of ethanolic ammonia was added, and the oligoribonucleotides were deprotected at 55°C for 16hr (19). After removal of the solvent, the reaction mixture was treated with 1.0 M tetrabutyl ammonium fluoride (TBAF, Aldrich) in THF (50 equivalents per TBDMS), at room temperature for 18hr. The reaction was quenched by addition of five volumes 0.1 M triethylammonium acetate

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(TEAA) per volume TBAF solution (20). The reaction mixture was loaded onto a C-18 open column pre-equilibrated with 50 mM triethylammounium bicarbonate (TEAB), and was eluted with a linear gradient of 5−40% $CH_3CN$ in 50 mM TEAB. After removal of the dimethoxytrityl group by treatment with HCl (pH2) for 1hr at room temperature, the oligoribonucleotides were purified by reverse-phase and anion-exchange high performance liquid chromatographies (HPLC).

A fifty base ribozyme (E50) was prepared by ligation of a 30 mer RNA (E30) and a 21 mer RNA by RNA ligase as described previously (17).

### 5′ or 3′-Labeled oligoribonucleotides

For 5′-end labeling, the purified substrate RNA was incubated in 50 mM Tris−HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, with [$\gamma$-$^{32}$P]ATP, and T4 polynucleotide kinase (*E.coli* A19) for 1 hr at 37°C. The labeled oligonucleotides were isolated and desalted with NENSORB 20 (Du Pont). To prepare 3′-end-labeled RNA, 500 pmol of the oligoribonucleotide was incubated in 10 $\mu$l of 50 mM HEPES-NaOH (pH 7.5), 20 mM $MgCl_2$, 3.3 mM DTT, 0.001% bovine serum albumin, 10% dimethyl sulfoxide, 0.1 mM ATP, and [5′-$^{32}$P]pCp ($^{32}$PCp) with 4.6 U/$\mu$l of T4 RNA ligase at 6°C for 12 hr. The 3′-end-labeled RNA was purified and desalted by the same procedure used for 5′-end-labeling.

### Cross-ligation reaction

The substrates, S1-$^{32}$PCp (10 pmol) and S2 (10 pmol), were dissolved in 5 $\mu$l of ligation buffer (40 mM Tris−HCl (pH7.5), 100 mM $MgCl_2$, and 2 mM spermidine-$^3$HCl) and heated at 65°C for 2min, then immediately transferred to an ice bath. E30,21 (100pmol) was dissolved in 25$\mu$l of ligation buffer, heated 65°C for 2min, and then annealed to 32°C. To start the reaction, 5 $\mu$l of E30,21 in buffer was added to the solution containing the substrates. The reaction was stopped by the addition of 50 mM $Na_2EDTA$ and the mixture was analyzed by electrophoresis on 20% PAGE containing 8 M urea. Cleavage rates were estimated from the gel radioactivity as measured by a Bioimaging analyzer (FUJIX BAS 2000).

### Exchange reaction

For the cross-ligation reaction, the substrates, 5′-$^{32}$P labeled S5 (10 pmol) and P2 (10 pmol), were dissolved in 5 $\mu$l of ligation buffer. E30,21 (100 pmol) was dissolved in 25 $\mu$l of ligation buffer. After E30,21 was annealed, 5 $\mu$l of E30,21 was added to the substrates and the reaction was started. The reaction was stopped by the addition of 50 mM $Na_2EDTA$. The percentages of exchanged substrates were estimated as above. The exchange reactions from S3 and P2 to S1 and from S3 and P3 to S5 were performed using similar conditions.

## RESULTS AND DISCUSSION

### Cross-ligation with E50 and E30,21

The minimal catalytic domain of sTobRV(−), E50, (Fig. 1) has been shown to catalyze a cross-ligation with low efficiency at 37°C (17). In order to improve the yield, the efficiency of the reaction at different temperature was analyzed. A two strand ribozyme, which is divided between G45 and U46 (Fig. 1), was also used as shown in Fig. 2. Fig. 3b indicates yields of the cross-ligation product (S2-$^{32}$PCp, 19 mer) from the S1-$^{32}$PCp (16 mer)

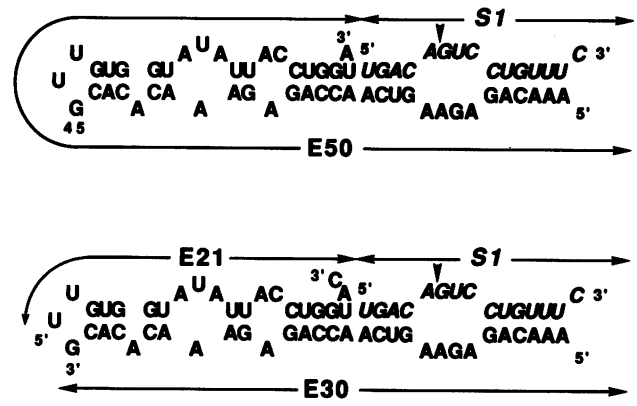


Figure 1. The hairpin ribozyme E50 and the two-stranded ribozyme E30,21.

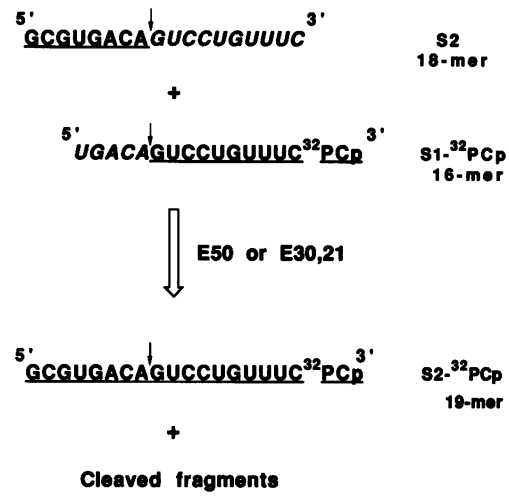


Figure 2. Cross-ligation between S1-$^{32}$PCp (16 mer) and S2 (18 mer). Radioactivities are detected by PAGE analysis after the reaction.

and the unlabeled donor (S2, 18 mer). With 2 $\mu$M of E50, which was the minimum concentration for the reaction at 37°C, the yield was 0.4%. When the temperature was decreased to 4°C after 15 min, the amount of product increased to 1.2%, probably because of suppressed cleavage and the facilitated binding of the substrate. The amount of starting material, shown in Fig. 3c, also increased with lowering of the temperature, indicating that the reverse-reaction to S1-$^{32}$PCp (16 mer) occurred. When the mixture was heated to 65°C and chilled in ice before incubation at 4°C, the yield increased slightly. The denaturation might stimulate the exchange of substrates for joining. Next, the cross-ligation with the two stranded ribozyme (E30,21) at 32°C, which is the optimal temperature of E29,21 for cleavage (17), was tried after annealing. As shown in figures 4a and 4b, 3−4% of the product was detected without lowering the temperature (lane 2−4). At 32°C, E50 did not give any detectable product (data not shown). This indicated that the high ligation activity of E30,21 was not driven by the reaction temperature. When the temperature was decreased to 4°C, the two stranded ribozyme gave 5% of the cross-ligation product (19 mer, Fig. 4b). The reverse reaction to the starting material, S1-$^{32}$PCp, was also increased from 5%

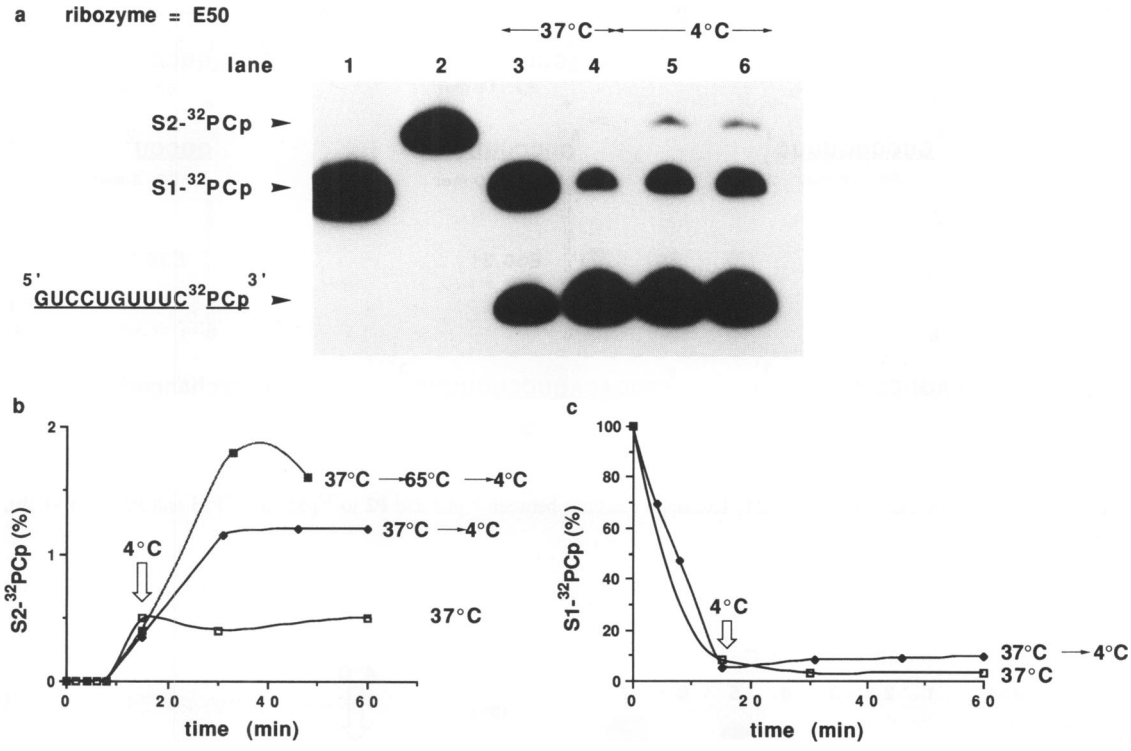


**Figure 3.** Cross-ligation reaction with E50. Autoradiogram of cross-ligation reaction with E50 (**a**). The reaction mixture was incubated for 15 min at 37°C, then transferred to 4°C. Lane 1, S1-$^{32}$PCp; lane 2, S2-$^{32}$PCp; lane 3, 4 min (37°C); lane 4, 15 min (37°C); lane 5, 31 min (4°C); lane 6, 60 min (4°C). Plots of the percentage of S2-$^{32}$PCp (**b**) and S1-$^{32}$PCp (**c**) in the cross-ligation reaction. Open arrows indicate the time when the reaction temperature was changed to 4°C. The reaction mixture was incubated at 37°C (open squares), incubated for 15 min at 37°C, then transferred to 4°C (solid diamonds), incubated at 37°C for 15 min, heated at 65°C, for 2 min, then chilled in ice and incubated at 4°C (solid squares).

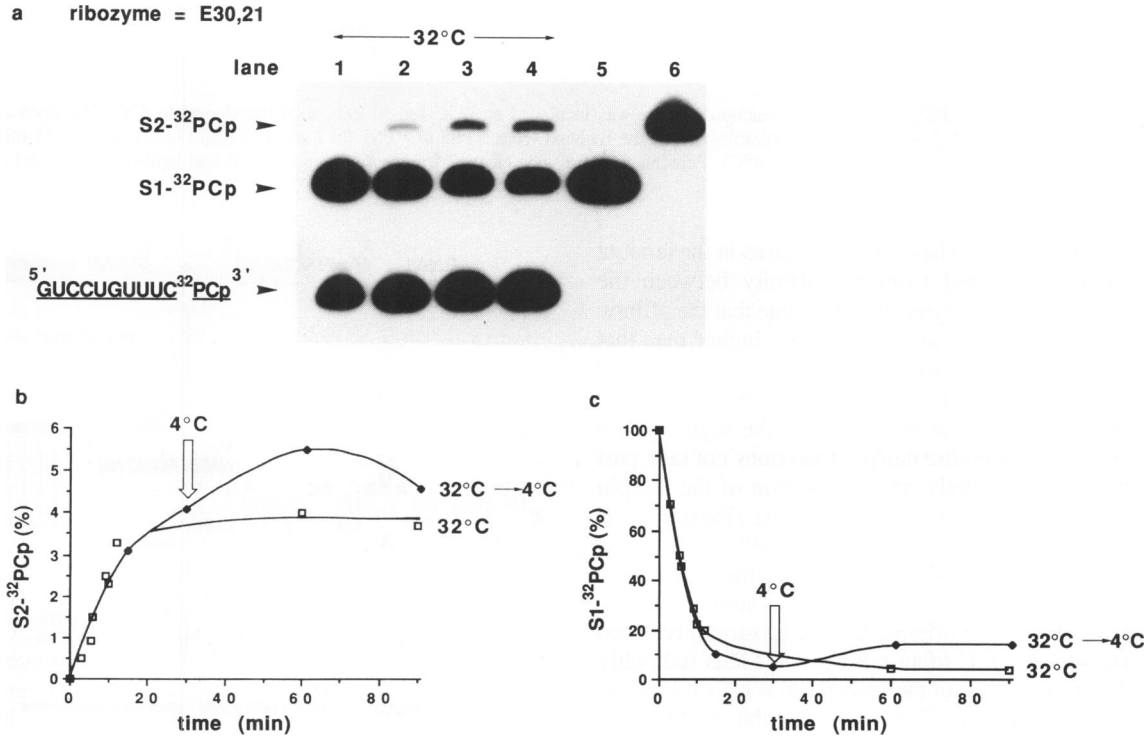


**Figure 4.** Cross-ligation reaction with E30,21 at 32°C. PAGE analysis of the cross-ligation (**a**). Lane 1, 3 min; lane 2, 6 min; lane 3, 9 min; lane 4, 12 min; lane 5, S1-$^{32}$PCp; lane 6, S2-$^{32}$PCp. Percentages of S2-$^{32}$PCp (**b**) and S1-$^{32}$PCp (**c**). Open arrows indicate the time when the reaction temperature was changed to 4°C. The reaction mixture was incubated at 32°C (open squares), incubated at 32°C for 30 min, then transferred to 4°C (solid diamonds).

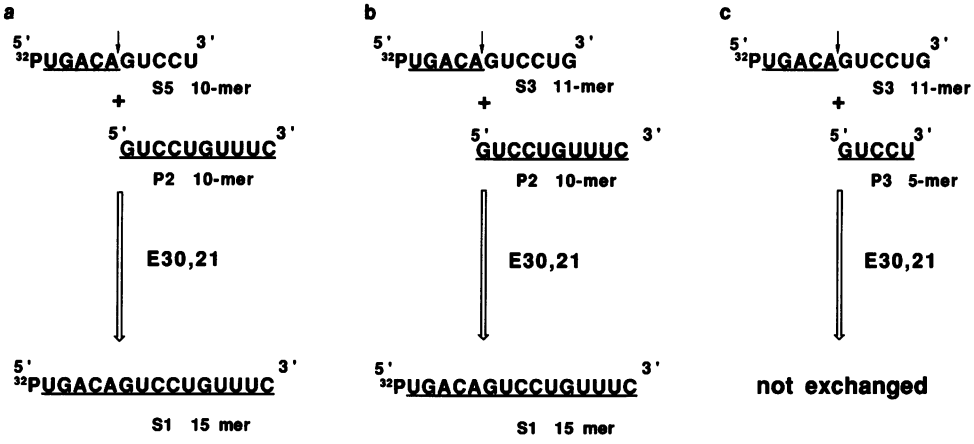


**Figure 5.** Exchange reactions of RNA catalyzed by E30,21. Exchange reactions between $^{32}$pS5 and P2 to $^{32}$pS1 (**a**), $^{32}$PS3 and P2 to $^{32}$PS1 (**b**), and $^{32}$PS3 and P3 to $^{32}$PS5 (**c**).

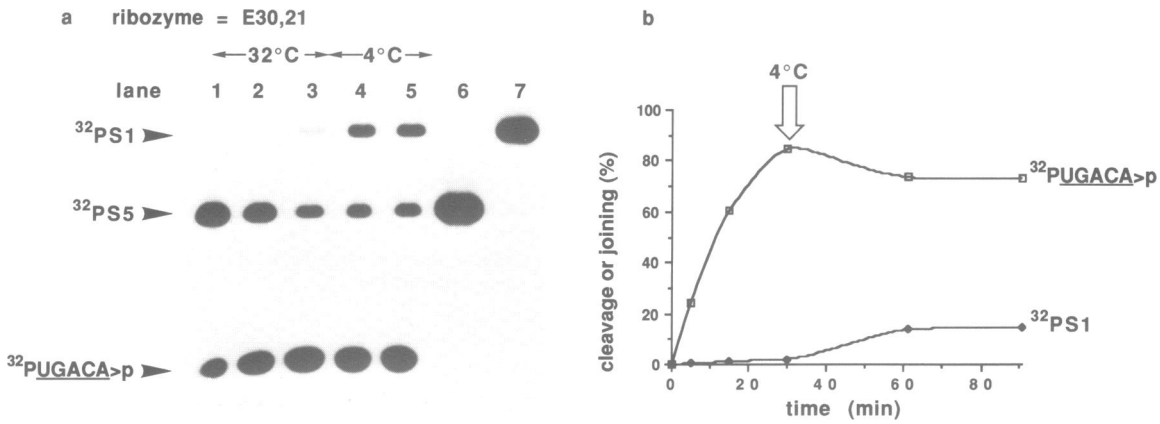


**Figure 6.** Exchange reaction from $^{32}$PS5 to $^{32}$PS1. The reaction mixture was incubated at 32°C for 30 min, then transferred to 4°C. The open arrow indicates the time when the temperature was decreased to 4°C. Autoradiogram of the reaction from $^{32}$PS5 to $^{32}$PS1 (**a**) Lane 1, 5 min (32°C); lane 2, 15 min (32°C); lane 3, 30 min (32°C); lane 4, 61 min (4°C); lane 5, 91 min (4°C). Percentages of 5′-side cleaved fragments (open squares) and ligated $^{32}$PS1 (solid diamonds) (**b**).

to 14%, as shown in Fig. 4c. These large increases in the amount of S1-$^{32}$PCp may be derived from the affinity between the cleaved fragments and the ribozyme. It is possible that the affinity between the cleaved fragments and E30,21 was higher than that of E50. The high affinity may prevent the release of the cleaved fragment from ribozyme and could lead to the increase of S1-[$^{32}$P]Cp. This affinity might be related to the high ligation activity of E30,21. Although the hairpin loop does not take part in the cleavage reaction directly, the destruction of the hairpin loop may influence the ternary structure of the ribozyme and suppress the release of the cleaved fragments. When the cross-ligation reaction was performed at 32°C for 30 min, incubated at 4°C for 29 min, and returned to 32°C, the amounts of the cross-ligated products and the original labeled substrates returned to the ratio obtained at 32°C (data not shown). This indirectly indicates that the linkage mode of the ligated site is a 3′-5′ linkage, because the substrate that had a 2′-5′ linkage at the cleavage site was not cleaved by the hairpin ribozyme (15).

## Exchange reaction with E30,21

A *cis*-acting ligation reaction with a hairpin ribozyme has been reported (21). We utilized the high ligation activity of E30,21

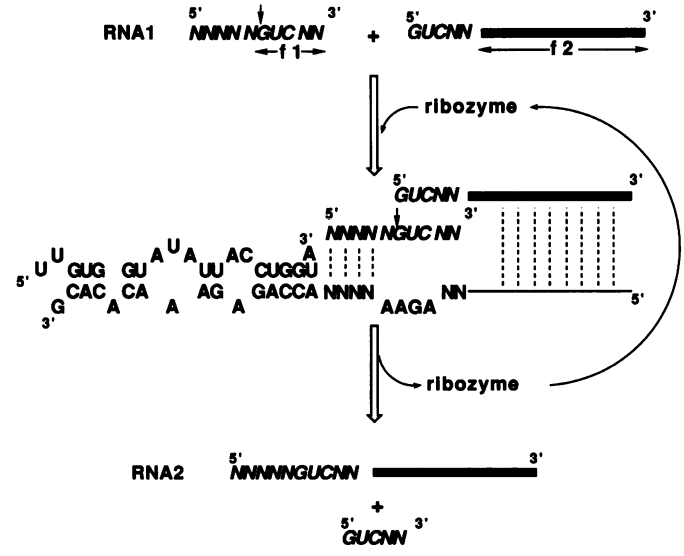


**Figure 7.** Design of a ribozyme for an efficient exchange reaction of RNAs. The 3′-Side fragment of RNA1 (f1) was substituted with f2, to produce RNA2.

to replace the 3'-side fragment of the substrate with the other RNA fragment *in trans* (Fig. 5). First, we investigated whether the 3'-side fragment of the substrate 10 mer (S5) could be exchanged with the other 3'-side fragment 10 mer (P2) (Fig. 5a). The substrate to be cleaved by the ribozyme needed to have 4 bases at the 5'-side and 6 bases at the 3'-side of the ApG junction (22). However, S5, which had only 5 bases at both sides of cleavage site, was able to be cleaved under the reaction conditions including 100 mM $MgCl_2$. The concentrations of $^{32}$PS5, P2, and E30,21 were the same as in the cross-ligation reaction (1 $\mu$M, 1 $\mu$M and 2 $\mu$M, respectively). As expected, the 15 mer RNA ($^{32}$PS1) from the exchange reaction between $^{32}$PS5 and P2 was detected with a yield of about 2% (30 min, Fig. 6a). However, the percentage of the ligated $^{32}$PS1 was smaller than that derived from the cross-ligation reaction. In the cross-ligation, the 3'-side fragment, which had more base pairs than the 5'-side, was labeled. In the exchange reaction, the 5'-side was labeled. The 5'-side fragment RNA was released more easily from the ribozyme and the yield of the ligated RNA appeared to be less in the exchange reaction. The amount of ligated $^{32}$PS1 increased from 2% to 14% by reducing the temperature to 4°C, as observed in the cross-ligation reaction. Both the cross-ligated products and the original labeled substrates increased in the cross-ligation reaction. However, in the exchange reaction, the longer ligated products increased with decreasing temperature. This indicates that the length of the 3'-side fragment is an important factor that affects the specific exchange of the 3'-side fragment of the RNA *in trans*. When 10 equivalents of P2 were used, the amount of S1 did not increase and conversely, the cleavage of the products ($^{32}$PS1) was inhibited (data not shown). The inhibition of the cleavage of S5 was derived from the binding of excess P2 to the ribozyme. The exchange reaction between the substrate RNA 11 mer (S3) and P2 was carried out in the same way (Fig. 5b) and the amount of the ligated $^{32}$PS1 was detected about the same as found in the reaction between S5 and P2. However, the cleavage rate of S3 was faster than that of S5, although S3 was longer than S5 by only one base on the 3'-side fragment. S3 might bind to the ribozyme more easily before the cleavage reaction.

An exchange reaction between S3 (11 mer) and P3 (5 mer) was carried out (Fig. 5c). This exchange reaction was a conversion to the shorter RNA (S5). P3 was added at either equal or 10 equivalents to S3, but S5 was not detected even though the reaction temperature was decreased from 32°C to 4°C (data not shown). By transferring the reaction mixture to 4°C, only the $^{32}$P labeled S3 increased. These results show that the ligation to the longer RNA occurred easily.

Although the ApG junction ligated by E50 was found to have a 3'-5' linkage (17), the mode of the linkage of the exchanged product by E30,21 was investigated. Substrates labeled with $^{32}$P at the phosphate of the ApG junction were synthesized with RNA ligase. After the exchange reaction was carried out with these substrates, the products ligated by E30,21 were purified from gel slices of PAGE. The purified products were digested by RNase $T_2$ and were analyzed by two-dimensional thin layer chromatography. If the ApG junction has 2'-5' linkage, A$^{32}$P(2'-5')Gp should be detected, but only a spot of A$^{32}$p was detected (data not shown). From this result, the linkage of the products ligated in the exchange reaction was proven to be the 3'-5' linkage, indicating that the ligation reactions catalyzed by E30,21, as well as E50, were not template dependent (23).

From the above results, one-tube cleavage and ligation reaction with a ribozyme and the substrate RNA can be designed, as shown in Fig. 7. For an efficient ligation reaction, it is required that 3'-side fragment of RNA1 (f1) must be shorter than that of RNA2 (f2), and f2 must have the sequences forming more base pairs with the ribozyme. It has been reported that 14, 16, 18, and 20 base RNAs, which were different in the lengths of their 3'-side fragments, could be cleaved by a hairpin ribozyme, even though these substrate RNAs showed different optimal temperatures (12). The maximum length of the introduced RNA to be ligated has yet to be determined. The exchange reaction of the 5'-side fragments of substrates RNA would be limited, because the newly introduced RNA fragments must have 2', 3'-cyclic phosphates at the 3'-terminus. It is not known whether the ribozyme will turn over by lowering the temperature and whether the concentration of the ribozyme affects the yield of the exchanged product.

For specific cleavage or reconstruction of RNA, ribozymes or RNases are used. For example, the 5'-fragment of the acceptor stem of a tRNA has been cleaved with RNaseH in the presence of 2'-*O*-methyl oligoribonucleotides, and using RNA ligase, was replaced with an oligoribonucleotide containing inosine residue (24). Purification of the cleaved RNA was required in such a two-step exchange reaction. The exchange reaction described in this report involves simultaneous cleavage and ligation of RNA, and purification of intermediates was not required. This exchange reaction which utilizes the reversible catalytic activities of the hairpin ribozyme, can be applied to the introduction of a functional oligoribonucleotide into an RNA *in trans*, although there are several limitations in the sequence. This technique should be useful in RNA engineering.

## ACKNOWLEDGEMENT

This research was supported in part by a grant pioneering research project in biotechnology from Ministry of Agriculture, Forestry and Fisheries Japan.

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