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Brief Communication

Hairpin Ribozyme-Catalyzed Ligation in Water-Alcohol Solutions

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ABSTRACT

The hairpin ribozyme (HPR) is a naturally existing RNA that catalyzes site-specific RNA cleavage and ligation. At 37°C and in the presence of divalent metal ions (M^{2+}), the HPR efficiently cleaves RNA substrates in *trans*. Here, we show that the HPR can catalyze efficient M^{2+}-independent ligation in *trans* in aqueous solutions containing any of several alcohols, including methanol, ethanol, and isopropanol, and millimolar concentrations of monovalent cations. Ligation proceeds most efficiently in 60% isopropanol at 37°C, whereas the reverse (cleavage) reaction is negligible under these conditions. We suggest that dehydration of the RNA is the key factor promoting HPR activity in water-alcohol solutions. Alcohol-induced ribozyme ligation may have practical applications.

INTRODUCTION

THE HAIRPIN RIBOZYME (HPR) is a small catalytic RNA derived from the minus strand of the tobacco ringspot virus satellite RNA, which also harbors a hammerhead ribozyme in its plus strand (Buzayan et al., 1986a,b, 1988; van Tol et al., 1990). The presumed biological function of its catalytic activity is to generate unit-length genomes from products of rolling circle replication through site-specific self-cleavage and circularization (Buzayan et al., 1995; Chay et al., 1997). The catalytic core of the ribozyme catalyzes both cleavage and ligation of RNA substrates, with cleavage yielding 5′-hydroxyl and 2′,3′-cyclic phosphate termini. The equilibrium between cleavage and ligation depends on salt concentration, temperature, and whether the reaction is *cis* or *trans*. Under physiological conditions (37°C and the presence of Mg^{2+}), the HPR efficiently cleaves substrates in *trans*, and the resulting products readily dissociate from the ribozyme. In contrast, when RNA-RNA interactions are stabilized by reduced temperature or increased salt concentration, release of cleavage products is slowed, and the equilibrium shifts toward ligation (Nesbitt et al., 1999; Fedor, 2000).

The hairpin ribozyme consist of two structural domains, each containing an internal loop and two short helices. The active site comprises multiple conserved functional groups located at and around the interface of the two domains in the docked, active enzyme-substrate complex (Rupert and Ferre-D'Amare, 2001; Hampel, 1998; Burke, 2001; Strobel and Ryder, 2001). Detailed investigations of the HPR reaction have shown that divalent metal ions (M^{2+}) have a structural but not catalytic role, as they can be substituted by cobalt (III) hexammine, spermidine, or high concentrations of monovalent cations, such as Na^{+} (Nesbitt et al., 1997; Nesbitt et al., 1999; Chowrira et al., 1993; Young et al., 1997; Hampel and Cowan, 1997; Earnshaw and Gait, 1998; Murray et al., 1998). Crystallographic studies have provided no ev-

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idence for specific binding sites for divalent metal ions in the proximity of the HPR catalytic center (Rupert and Ferre-D'Amare, 2001).

Several authors have noted slow spontaneous cleavage by the HPR under certain conditions in the absence of added M^{2+}, including ethanol precipitation (Prody et al., 1986), repeated freezing and thawing (Donahue and Fedor, 1997), and in partially hydrated dried films (Seyhan and Burke, 2000). We have described surprisingly efficient M^{2+}-independent ligation by the HPR both in *cis* and in *trans* under freezing conditions (Kazakov et al., 1998; Vlassov et al., 2004).

Here, we report characterization of HPR *trans*-ligation in water-alcohol solutions, including methanol, ethanol, and isopropanol. We have found that under optimal conditions, all these alcohols promote assembly of ribozyme-substrate complexes and ligation in the absence of divalent metal ions. This ligation requires millimolar concentrations of monovalent cations and is temperature dependent. It proceeds most efficiently in 60% isopropanol at 37°C, and the cleavage reaction is negligible under these conditions. We suggest that partial dehydration of the RNA is the key factor promoting the ribozyme ligation activity in water-alcohol solutions. Also, we briefly discuss potential biotechnological applications of alcohol-induced ribozyme ligation.

MATERIALS AND METHODS

Hairpin ribozyme

A 57-nt HPR (Fig. 1) was generated by *in vitro* transcription of a DNA fragment containing a T7 promoter. The sequence of the template strand was 5′-TACCAGGTAATGTACCACGACTTACGTCGTGTGTTTCTCTGGTTGACTTCTCTGCCCTATAGTGAGTCGTATTA-3′ (T7 promoter underlined) (IDT, Coralville, IA). RNA was mixed with 3× denaturing loading solution (7 M urea, 10 mM EDTA, and 0.02% xylene cyanol and bromphenol blue) and purified by electrophoresis on 15% polyacrylamide gels containing 7 M urea. RNA bands were localized by UV shadowing and isolated by excising bands from the gel, crushing/soaking, and extracting the RNA in 0.3 M sodium acetate, pH 5.0. After ethanol precipitation, the samples were dissolved in 5 mM Tris-HCl, pH 7.5, and stored at −20°C.

Ligation substrates

Ligation substrates 3′-LS and 5′-LS (Fig. 1) were obtained by HPR-catalyzed cleavage of an internally ^{32}P-labeled oligonucleotide (LP). LP was generated by T7 promoter-driven *in vitro* transcription from a DNA whose template strand was 5′-CACAGTCTAGTCGTCAGC-

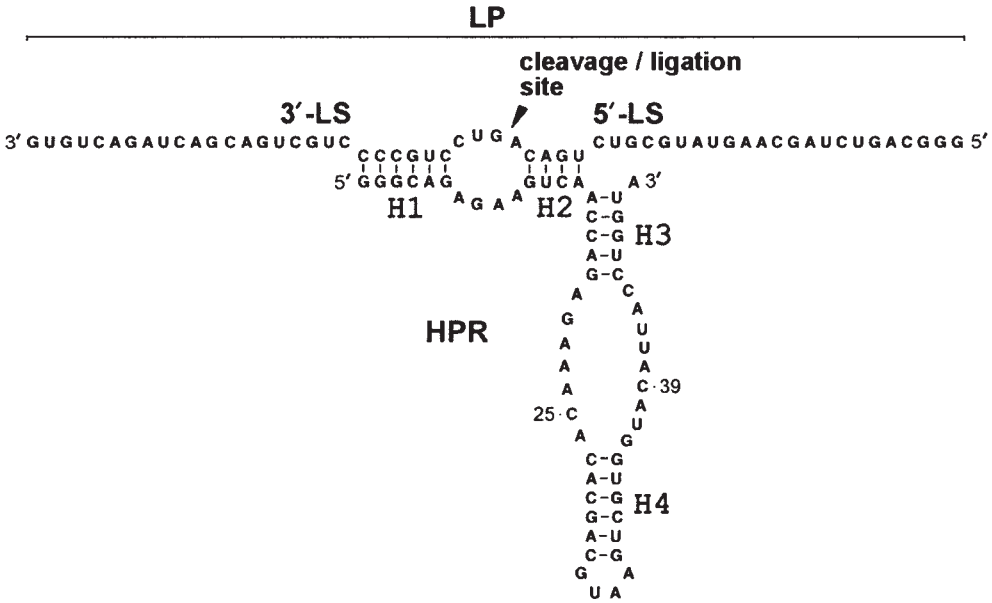

FIG. 1. Structure of the *trans*-acting HPR with ligation substrates used. Helical segments H1–H4 as well as ligation substrates 3′-LS and 5′-LS and the cleavage/ligation site are as indicated. This HPR incorporated modifications known to improve activity over the canonical sequence (Yu and Burke, 1997): additional stabilizing GC base pairs in helix 4 capped with a tetraloop structure and a U39 → C substitution. Substrate fragments were obtained by HPR-catalyzed cleavage of an internally ^{32}P-labeled 57-nt oligonucleotide (LP) consisting of the HPR substrate sequence (14 nt, in bold) extended with flanking sequences on both sides: 5′-gggCAGUCUAGCAAGUAUGCGUC**UGACA/GUCCUG*CCC***CUGCUGACG ACUAGACUGUG-3′. Cleavage was performed in 10 mM $MgCl_2$, 1 mM Tris-HCl, pH 7.5, at 37°C and was followed by gel purification of the 3′-LS and 5′-LS fragments (see Materials and Methods).

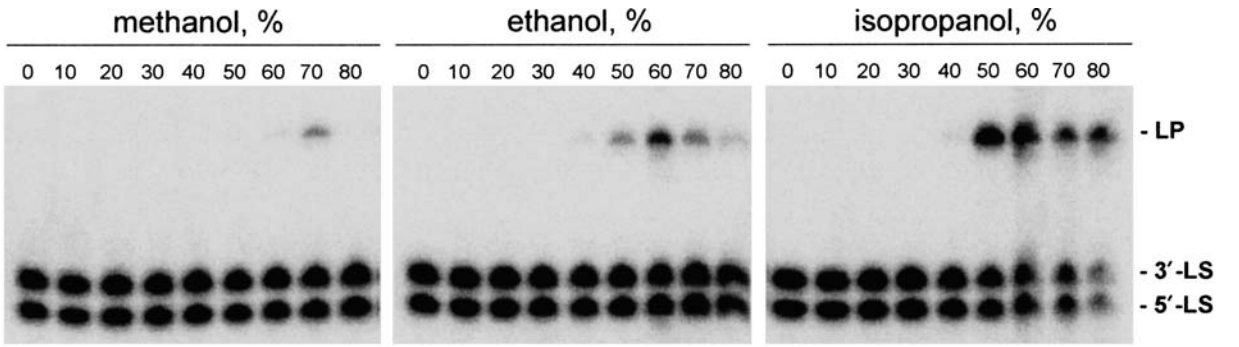

FIG. 2. Alcohol-induced *trans*-ligation catalyzed by HPR; autoradiography of a denaturing 15% polyacrylamide gel. HPR was incubated with ligation substrates (3′-LS and 5′-LS at a 1:1 ratio) (Fig. 1) at 25°C for 14 hours in 25 mM NaCl, 1 mM Tris-HCl, pH 7.5, and alcohol at the indicated concentrations.

AGGGGCAGGACTGTCAGACGCATACTTGCTAG-ACTGCCCTATAGTGAGTCGTATTA-3′ (T7 promoter underlined). LP consisted of the normal HPR substrate sequence plus flanking sequences that are not complementary to the HPR. After cleavage was performed in 10 mM $MgCl_2$, 1 mM Tris-HCl, pH 7.5, at 37°C, the two products were gel purified as described above.

Reaction procedures and analysis of products

For reactions in water-alcohol solutions, typical 10-μl reactions contained 0.3 μM internally ^{32}P-labeled ligation substrates 5′-LS and 3′-LS and 0.3 μM unlabeled HPR in 25 mM NaCl, 1 mM Tris-HCl, pH 7.5, and alcohol (methanol, ethanol, or isopropanol) at concentrations ranging from 10% to 80%. Low concentrations of Tris (1 mM) were used because HPR activity is reduced in Tris buffer at the commonly used concentrations of 20–50 mM (S.A. Kazakov, unpublished observations). It was confirmed that the addition of up to 80% alcohol does not change the pH by more than 0.2 units, and the reaction proceeds with the same efficiency over the pH range 7.0–9.0. EDTA (0.5–5 mM) was used in preliminary studies to prove that the HPR-catalyzed ligation is M^{2+} independent. Because the presence of EDTA made no difference in ligation yield, it was omitted in later experiments.

The freezing reactions were performed essentially as previously described (Vlassov et al., 2004). Typical 10-μl reactions contained 0.3 μM internally ^{32}P-labeled 5′-LS and 3′-LS and unlabeled ribozyme core (0.3–3 μM) in 25 mM NaCl, 1 mM Tris-HCl, pH 7.5. To prevent variation in the rate of freezing, we used a quick-freezing techniques; 10-μl samples in 1.5 ml Eppendorf microcentrifuge tubes were frozen by immersing in liquid nitrogen (−196°C) for 30 seconds, followed by transfer to a circulating ethanol-water bath and incubation at −10°C.

The reaction was stopped by removing tubes at the desired times, mixing with 3× loading buffer, and analyzing on denaturing 15% polyacrylamide gels. ^{32}P-Labeled reaction products were quantified using a Molecular Imager FX and Quantity One-4.2.0 software (Bio-Rad, Hercules, CA). Apparent initial rates for the ligation reaction were determined from the initial slope of the plots. The fraction of substrate ligated per minute was calculated using the three initial time points (5, 10, and 15 minutes).

RESULTS AND DISCUSSION

HPR catalyzes trans*-ligation in water-alcohol solutions*

We examined the enzymatic activity in water-alcohol solutions of a *trans*-acting version of the HPR previously used for studies under freezing conditions (Vlassov et al., 2004) (Fig. 1). Unlabeled HPR in M^{2+}-free buffer was mixed with equimolar amounts of ^{32}P-labeled ligation substrates 5′-LS and 3′-LS at a ribozyme/substrates ratio of 1:1. Alcohols were added to a final concentration of 10%–80%, and after 14 hours' incubation at 25°C, the products were analyzed by denaturing polyacrylamide gel electrophoresis. In the case of ethanol (Fig. 2, middle panel), ligation in *trans* proceeded efficiently, with a clearly defined optimum (15% ligation) at 60% ethanol. No reaction was observed in solutions containing ethanol at concentrations $<$40% or $>$80%. Variation of Tris concentration (1–20 mM), pH (7.0–9.0), and NaCl concentration (25–1000 mM) did not significantly affect the reaction yield (data not shown). The addition of EDTA (0–5 mM) also did not affect reaction yields, indicating that the reaction is truly M^{2+} independent.

HPR ligation in water-alcohol solutions is much more efficient than cleavage. For example, in ethanol solutions, the maximum ligation yield of 10% was achieved after only 2 hours, whereas cleavage of the full-length substrate was $<$0.5% under the same conditions. Similar results were seen with freezing-induced ligation (Vlassov et al., 2004). Longer incubation (up to 24 hours) did not

provide substantially higher yields for either reaction; hence, 2 hours at 25°C was taken as approximating equilibrium conditions in our observations. At equilibrium, however, HPR cleavage and ligation percentages should add up to 100%. The fact that this sum is much less than 100% suggests that a significant fraction of the ribozyme population is in an inactive conformation under these conditions. Similar conformational trapping (although to a lesser extent) has also been observed with the HPR in normal solution reactions in the presence of M^{2+} (Feldstein and Bruening, 1993; Esteban et al., 1997). An obvious source of conformational trapping in alcohol-water solutions is that alcohols can induce the rapid formation of potentially inactive RNA aggregates or precipitates, limiting the time available for productive intermolecular contacts. Indeed, when samples were incubated for 2 hours in 60% ethanol and then centrifuged at 20,000 G for 1 hour, the RNA was quantitatively pelleted.

To determine if ethanol was unique in inducing HPR ligation, we investigated the ability of other alcohols to promote assembly of ribozyme-substrate complexes and catalyze *trans*-ligation at 25°C (Fig. 2). We found that isopropanol (C_3H_7OH) promotes ligation more efficiently than ethanol (C_2H_5OH), providing a maximum yield of ligated product of about 40% over a broad range of isopropanol concentrations (50%–80%). Methanol (CH_3OH) was much less efficient, with a ligation yield of only 5% at the optimal methanol concentration of 70%. Next, n-Butyl (C_4H_9OH) and isoamyl ($C_4H_9CH_2OH$) alcohols were tested for their abilities to stimulate HPR ligation. These alcohols are not completely miscible with water, but there was still a possibility that reactions could be promoted in the water phase saturated with the alcohol. However, no ligation was observed (data not shown).

Effects of temperature on reaction kinetics

Ethanol and isopropanol were chosen as the most potent inducers of HPR ligation for further investigation of the effects of temperature on the reaction. The kinetics of the ligation reaction were studied in 60% ethanol and 60% isopropanol solutions at 4°C, 25°C, 37°C, and 45°C (Fig. 3). The reaction yields were calculated by phosphorimager-based quantitation of polyacrylamide gel separations. Apparent initial rates were determined from the initial slope of the plots. For both ethanol and isopropanol, the initial rate and maximum yield were higher

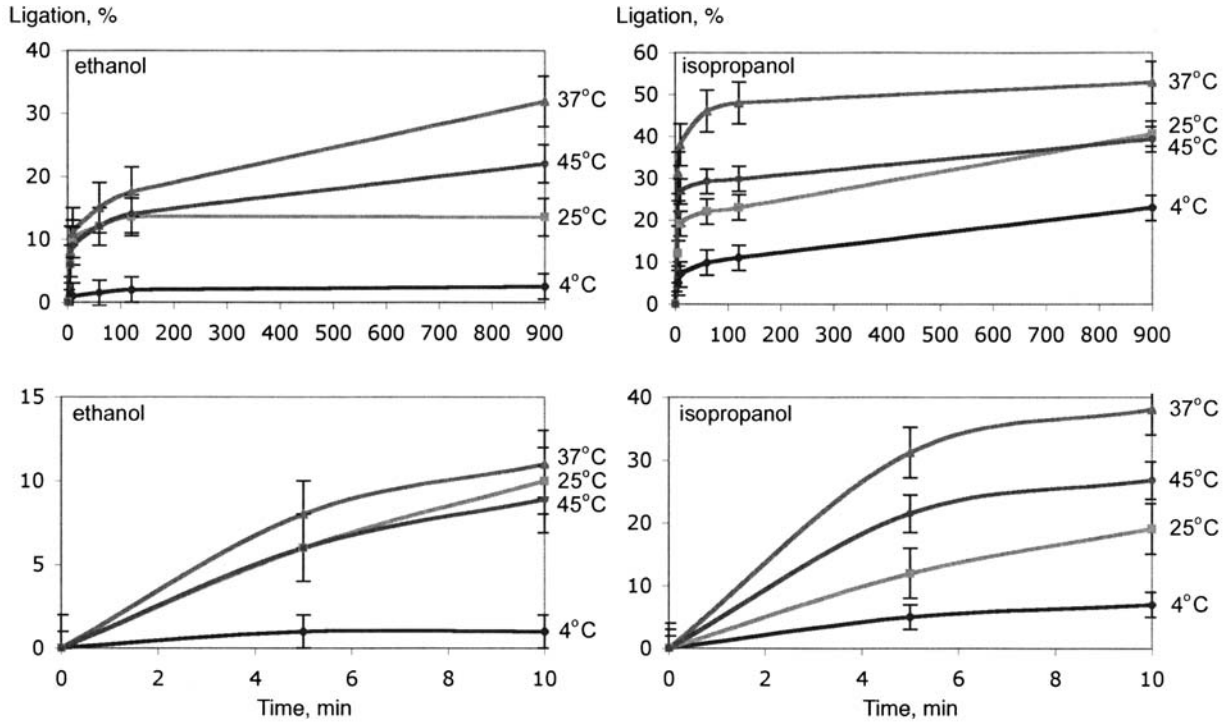

FIG. 3. Kinetics of the alcohol-induced *trans*-ligation catalyzed by HPR at various temperatures. Reactions were performed at 4°C, 25°C, 37°C, and 45°C for up to 15 hours in 25 mM NaCl, 1 mM Tris-HCl, pH 7.5, and 60% alcohol. (**Left**) 60% ethanol: *top*, kinetics of the ligation, 5 minutes–15 hours; *bottom*, enlarged initial 5–10-minute interval. (**Right**) 60% isopropanol: *top*, kinetics of the ligation, 5 minutes–15 hours; *bottom*, enlarged initial 5–10-minute interval. Average values from four experiments are shown.

at 37°C than at the other temperatures tested (Fig. 3). The most efficient ligation was achieved in 60% isopropanol at 37°C, where >30% of substrate was ligated in 5 minutes. After 14 hours' incubation, the ligation yield reached a maximum of 50%.

It is not possible to quantitatively characterize HPR-catalyzed ligation in alcohol solutions by the usual approach (Esteban et al., 1997; Hegg and Fedor, 1995), where kinetic parameters for ligation are determined from single-turnover reactions in which a small amount of radioactive 5′-LS is combined with a saturating excess of a complex containing HPR and 3′-LS. In water-alcohol solutions and also under freezing conditions, if 3′-LS is present in large excess, it competes with 5′-LS for binding to the 5′ binding site on the ribozyme as a result of relaxed sequence requirements compared with normal solution conditions (Vlassov et al., 2004) (data not shown). Moreover, it is not possible to determine the final concentration of all components because of RNA precipitation after the addition of alcohol. For this reason, a 1:1 ratio of the ligation substrates 3′-LS and 5′-LS was used for all the reactions, and the reactions were characterized by initial rate and maximal ligation yield. The optimal HPR/substrates ratio for ligation in alcohol was found to be 1:1, whereas for freezing-induced catalysis, it was approximately 20:1 (Vlassov et al., 2004). Apparently, the reaction proceeds in rapidly forming aggregates, and there is no free substrate exchange between ribozymes.

Given that HPR-catalyzed ligation is stimulated in water-alcohol solutions at above-zero temperatures as well as under freezing conditions (−10°C) in the absence of alcohol, the question of whether the combination of alcohol and low temperature could further increase the ligation yields was of interest. However, it was found that the presence of as little as 0.1% ethanol suppressed the freezing-induced reaction at −10°C, with complete inhibition achieved at 10% (data not shown). This happens presumably because of the inability of ethanol to promote ligation at subzero temperatures, as well as lowering of the freezing point, which prevents the possibility of freezing-induced ligation.

Dehydration and RNA aggregation are key factors promoting ligation in water-alcohol solutions

There are several effects caused by alcohol that could stimulate HPR-catalyzed ligation. These include (1) generation of a high local concentration of monovalent cations, (2) RNA precipitation (aggregation, concentration), and (3) dehydration. The first possibility was excluded because, in contrast to the freezing reaction, the salt concentration around RNA does not increase on addition of 40%–60% ethanol at 25°C (Fink and Geeves, 1979). Also, increasing the NaCl concentration up to 1 M did not affect the ligation yield.

It is hard to draw solid conclusions at the moment about the other two possibilities. RNA precipitation indeed takes place in 60% alcohol, under conditions optimal for ligation, and an increase in RNA concentration causes elevation of the ligation yield. On the other hand, ligation is more efficient at higher (25–37°C) than at lower (4°C) temperature (Fig. 3), whereas precipitation is faster at lower temperatures. Moreover, freezing-induced ligation involves no obvious aggregation, whereas both freezing and the presence of alcohols cause dehydration of RNA (Vlassov et al., 2004). These considerations indicate that aggregation of RNA is not the only factor stimulating the reaction and that dehydration may play an important role.

Reinforcing this view is the fact that interactions of polynucleotides require that interacting surfaces be partially dehydrated so that specific contacts can form (Rau et al., 1984; Lundback and Hard, 1996); that is, the formation of RNA-RNA contacts depends on competition between RNA-RNA and RNA-water interactions. Ethanol is known to affect the conformation and stability of nucleic acids, presumably through its effect on their hydration (Vollenweider et al., 1978; Beneventi and Onori, 1986); it displaces water and partially immobilizes H_2O molecules. For example, it was reported previously that ethanol at concentrations ~20% can significantly increase the activity of ribozymes in Mg^{2+}-containing solutions (Feig et al., 1998; Hanna and Szostak, 1994; Gardiner et al., 1985). Higher ethanol concentrations diminished their activity, apparently due to rapid aggregation of RNA promoted by the combined effects of ethanol and Mg^{2+} ions.

In the present system, HPR ligation in *trans* is significant only at alcohol concentrations of at least 40% (for isopropanol and ethanol) and reaches a maximum yield at 60% alcohol. At alcohol concentrations >70%, the ligation efficacy decreases, presumably because of excessive dehydration of RNA molecules and their aggregation and precipitation. The fact that the degree of ligation stimulation by three tested alcohols follows their hydrophobicity (isopropanol > ethanol > methanol) suggests that they may work by lowering water activity. We propose that dehydration, perhaps combined with aggregation, induces the same catalytically active conformation that in the alcohol-free reaction is induced by divalent metal ions or high concentrations of salts. However, the exact mechanism is not yet clear.

Possible utility of alcohol-induced ligation

The cleavage efficiency of HPR under normal solution conditions and the ease with which its sequence specificity can be manipulated inspired its early use as a se-

quence-specific ribonuclease. Although not so intensively studied as the hammerhead ribozyme, many applications for this ribozyme have been explored, including the analysis of gene function and the development of therapeutics for genetic and viral diseases (Hampel, 1998; Kruger et al., 1999).

The discovery of HPR-catalyzed ligation in water-alcohol solutions and upon freezing is, to our knowledge, the first example of RNA catalysis that is more efficient at extreme, rather than normal, solution conditions. Freezing and alcohol-induced HPR ligation is highly efficient compared with typical solution conditions under which cleavage prevails for reaction in *trans*. The RNA is protected by low water activity, low temperature, and lack of divalent metal ions. Moreover, the system is protein free, reducing the potential for contamination by ribonucleases, and there is no need for subsequent protein removal. These attractive features suggest possible applications in diagnostic, biosensor, and other biotechnology settings. Further study of RNA catalysis under freezing and dehydrating conditions may provide new insights into the mechanisms of RNA catalysis, its possible roles in the RNA world (Vlassov et al., 2005), and ways in which these reactions can be of practical use.

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