Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 6135–6143
Bioorganic & Medicinal Chemistry

LNA nucleotides improve cleavage efficiency of singular and binary hammerhead ribozymes

Janne K. Christiansen,[a] Sune Lobedanz,[a,†] Khalil Arar,[b] Jesper Wengel[c] and Birte Vester[a,*]

[a]*Department of Biochemistry and Molecular Biology, The Nucleic Acid Center, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark*
[b]*Sigma-Proligo, Genopole Campus 1, 5, rue Henri Desbrueres, 91030 Evry Cedex, France*
[c]*Departments of Physics and Chemistry, The Nucleic Acid Center, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark*

Received 15 March 2007; accepted 13 June 2007
Available online 28 June 2007

Abstract—Variants of trans-acting hammerhead ribozymes were modified with Locked Nucleic Acid (LNA) nucleotides to reduce their size, to improve access to their RNA target and to explore combinational properties of binary constructs. Using low Mg^{2+} concentrations and low substrate and ribozyme concentrations, it was found that insertion of LNA monomers into the substrate binding arms allowed these to be shortened and results in a very active enzyme under both single and multiple turnover conditions. Incorporation of a mix of LNA and DNA residues further increased the multiple turnover cleavage activity. At high Mg^{2+} concentrations or high substrate and ribozyme concentrations, the enhancing effect of LNA incorporation was even more prominent. Using LNA in the stem of Helix II diminished cleavage activity, but allowed deletion of the tetra-loop and thus separating the ribozyme into two molecules with each half binding to the substrate. Efficient, binary hammerhead ribozymes were pursued in a combinatorial approach using a 6-times 5 library, which was analysed concerning the best combinations, buffer conditions and fragment ratios.

1. Introduction

Hammerhead ribozymes are relatively small RNA structures of around 30 nucleotides that can cleave RNA targets in *cis* or *trans* in a sequence-specific way. The natural hammerhead ribozymes are *cis* acting RNA structures coded on viroid RNA.[1] The cleavage is highly dependent on the presence of divalent metal ions with magnesium as the natural choice. The potential for clinical use of ribozymes as RNA cleaving pharmaceuticals is being exploited but their actual application has so far been limited. There are several explanations for this. The catalytic power of the known ribozymes is relatively poor compared to efficient proteins. In addition, the stability of ribozymes is limited due to their susceptibility hydrolysis mediated by RNases. Also, the binding properties of ribozymes to their RNA target by Watson–Crick base pairing set some inherent limitations influenced by the accessibility to the target sequence. Thus some improvements of ribozymes are needed to make them more applicable.

Hammerhead ribozymes have been the subject of numerous studies (recently reviewed in Refs. 2–6). The idea of introducing nucleotide modifications in hammerhead ribozymes has been explored both to elucidate the RNA cleaving mechanism and to increase the nucleolytic stability of the ribozymes. Specific nucleotides have been replaced by, for example, fluorobenzene-*C*-nucleosides, 2′-aminothymidine, 2′-deoxyuridine, 2′-*O*-methyl-RNA nucleosides or 3-phosphorothioate derivatives (Refs. 7–9 and references therein). A major breakthrough in understanding the structural arrangement underlying the ribozyme-mediated phosphodiester cleavage came with the recent X-ray determination of a full-length *Schistosoma mansoni* hammerhead ribozyme.[10] The structure solved a long-standing puzzle to fit all biochemical data with previous Y-shaped hammerhead structures.

Locked Nucleic Acids (LNA) are modified nucleosides with a conformationally locked ribose moiety.[11] They are capable of targeting complementary RNA and

Keywords: Hammerhead ribozymes; LNA; Modified nucleotides; RNA cleavage.
* Corresponding author. Tel.: +45 6550 2377; fax: +45 6550 2467; e-mail: b.vester@bmb.sdu.dk
† Present address: Novozymes A/S, Krogshojvej 36, 2880 Bagsvaerd, Denmark.

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.06.045

DNA with high affinity and incorporation of LNA into oligonucleotides provides resistance against nuclease degradation, reviewed in Ref. 12. Therefore, LNA nucleotides are potentially interesting as components in ribozymes to increase target affinity and thereby substrate accessibility as well as provide nuclease resistance. LNA monomers in a helix induce a local A-form helix structure.[13] Recent reports have shown that inclusion of LNA nucleotides into the binding arms of DNAzymes enhance cleavage of structured RNA targets and even promotes cleavage of targets in cases where the targets are intractable to unmodified DNAzymes.[14–18] These studies specify that incorporation of LNA into hammerhead ribozymes might be beneficial with a view to enhance its applicability.

Here, we have incorporated LNA nucleotides into hammerhead ribozymes to explore the effect on ribozyme performance in various ways. First, LNA nucleotides were incorporated in the ribozyme sequences binding to the target RNA to improve affinity and thereby increase cleavage. The insertions were expected to permit shortening of the binding arms by stabilising substrate binding without inhibiting RNA cleavage. An LNA modified ribozyme performed superior to the unmodified ribozyme at various conditions. Finally, we explore dividing the ribozyme in two fragments that self-assemble by interactions stabilised by LNA nucleotides, a strategy that allowed a combinatorial approach for optimising ribozyme modifications. This is the first report on LNA modified ribozymes and it clearly shows that incorporation of LNA nucleotides can improve ribozyme performance considerably.

2. Results

2.1. Design of LNA-modified hammerhead ribozymes

The purpose is to characterise the overall effect of incorporation of LNA nucleotides into ribozymes in a qualitative way and at conditions similar to those normally useful in molecular biology and enzymatic studies. To assess the effects of incorporation of LNA nucleotides, we designed the two unmodified ribozymes, Rz1 at 37n and Rz2 at 34n. The presumed secondary structures are outlined in Figure 1 together with secondary structures for LNA modified ribozymes. The sequences of all the investigated ribozymes are shown in Table 1. The ribozymes contain the catalytic core with its essential nucleotides (Fig. 1) and a small helix II as previously selected by Persson et al.[19] Helix I and III bind via

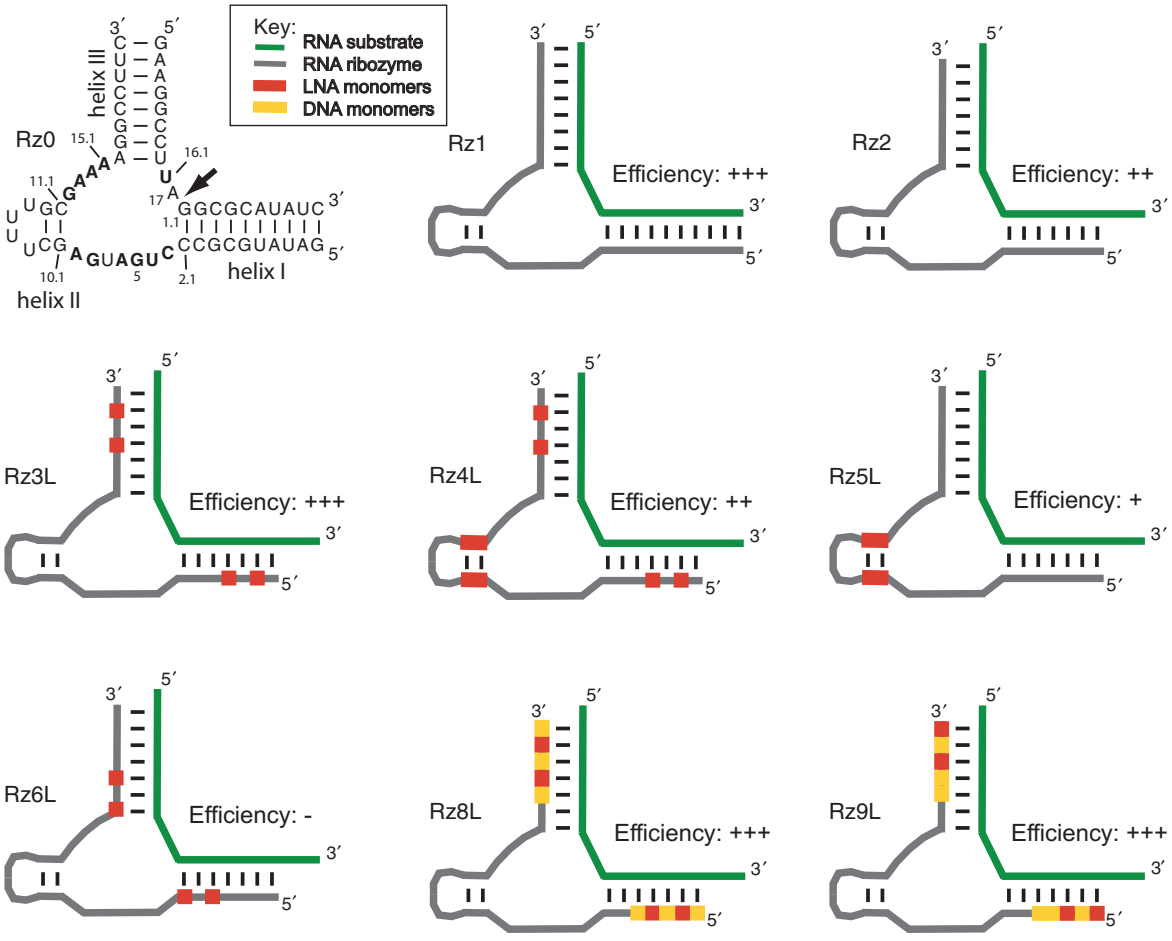

Figure 1. Secondary structures of the ribozymes and the substrate RNA. The sequence and general numbering for hammerhead ribozymes are indicated for Rz1 as well as the essential nucleotides that are marked in bold. The arrows show the sites of cleavage. The RNA parts of ribozymes are marked in grey, LNAs in red, and DNAs in yellow, and the RNA substrate in green.

Table 1. List of ribozymes and substrate

Ribozymes	Length	Sequence						
		HI	ss	HII	Loop	HII	ss	HIII
Rz1	37n	5′ GAUAUGCGCC	cugauga	GC	uuuu	GC	gaaa	AGGCCUUC 3′
Rz2	33n	5′ AUGCGCC	cugauga	GC	uuuu	GC	gaaa	AGGCCUU 3′
Rz3L	33n	5′ A**T**G**C**GCC	cugauga	GC	uuuu	GC	gaaa	AGG**C**C**T**U 3′
Rz4L	33n	5′ A**T**G**C**GCC	cugauga	**GC**	uuuu	**GC**	gaaa	AGG**C**C**T**U 3′
Rz5L	33n	5′ AUGCGCC	cugauga	**GC**	uuuu	**GC**	gaaa	AGGCCUU 3′
Rz6L	33n	5′ AUGC**G**C**C**	cugauga	GC	uuuu	GC	gaaa	**A**G**G**CCUU 3′
Rz7L	33n	5′ AUGCGCC	cugau**ga**	GC	uuuu	GC	**ga**aa	AGGCCUU 3′
Rz8L	33n	5′ dA**T**dG**C**dGCC	cugauga	GC	uuuu	GC	gaaa	AGdG**C**dC**T**dT 3′
Rz9L	33n	5′ **A**dT**G**dCdGCC	cugauga	GC	uuuu	GC	gaaa	AGdGdC**C**dT**T** 3′
R10L	20n	5′ GAUAUGCGCC	cugauga	**GCA**3′				
R11L	15n					5′ **TGC**	gaaa	AGGCCUUC 3′
R12L	20n	5′ GAUA**T**G**C**GCC	cugauga	**GCA** 3′				
R13L	15n					5′ **TGC**	gaaa	AGG**C**C**T**UC 3′
R14L	17n	5′ A**T**G**C**GCC	cugauga	**GCA** 3′				
R15L	14n					5′ **TGC**	gaaa	AGG**C**C**T**U 3′
R16L	22n	5′GAUAUGCGCC	cugauga	GC**AA**G 3′				
R17L	17n					5′ C**TT**GC	gaaa	AGGCCUUC 3′
R18L	19n	5′ A**T**G**C**GCC	cugauga	GC**AA**G 3′				
R19L	22n	5′ GAUA**T**G**C**GCC	cugauga	GC**AA**G 3′				
R20L	17n					5′ C**TT**GC	gaaa	AGG**C**C**T**U 3′
RNA substrate	20n	5′ GAAGGCCUUAGGCGCAUAUC 3′						

LNA monomers are in bold and underlined. DNA monomers are denoted by d. Other nucleotides are RNA. The helix regions (H) are indicated in upper case with the numbering in the top row. ss indicates single-stranded regions.The binary ribozymes are named Rz[..+..] with the numbers of both the half ribozymes in brackets.

Watson–Crick basepairing to the substrate RNA, a 20n substrate with an A–G cleavage site in the middle. LNA nucleotides were inserted into the middle of the binding arms of the ribozyme to maximise their effect on helix stability (Rz3L and Rz4L) and near the catalytic core to investigate a potential effect on the cleavage mechanism (Rz5L and Rz6L). One ribozyme with LNA nucleotides in the catalytic core was also constructed (Rz7L). Furthermore, DNA nucleotides were inserted together with LNA nucleotides to improve the nuclease stability of the ribozyme (Rz8L and Rz9L). Combinatorial possibilities were explored by deleting ribozyme loop II and dividing the ribozyme into two fragments (Fig. 2). The interaction of the fragments, and thus self-assembly

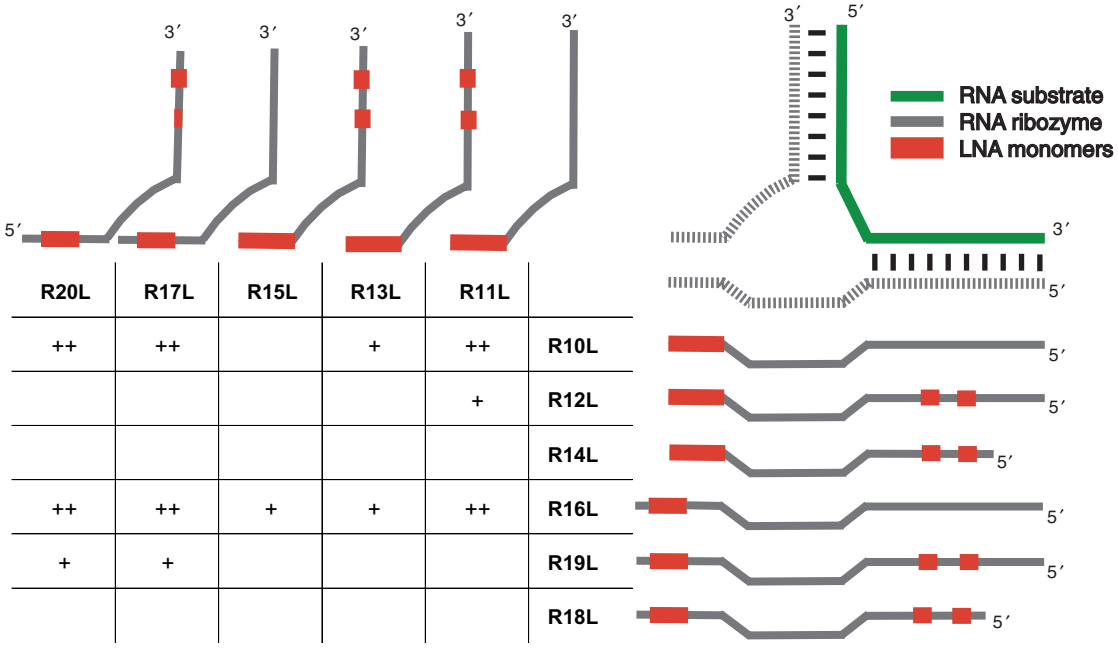

Figure 2. Secondary structure representation of the binary ribozymes together with a qualitative comparison of their cleavage efficiency. ++ means 30–50% of cleaved product, + means 15–30% and blank are <15%. The colouring is as described in Figure 1 legend. The data were obtained with 1 nM substrate RNA and 10 nM of each ribozyme fragment, and at 10 mM Mg^{2+} ions.

of the binary ribozymes, were stabilised by incorporation of LNA nucleotides in helix II. The length of the fragments and the positions and numbers of LNA nucleotides were varied to search for efficient bimolecular ribozymes.

2.2. The effect of LNA modifications on ribozyme performance

Ribozyme cleavage was assessed by incubating the ribozymes with 5′-end labelled substrate RNA followed by gel electrophoresis and phosphor image scanning. The exact conditions are described in Section 4. Reaction curves were obtained and k_{obs} and percentage of cleaved substrate were quantified whenever possible. The cleavage reactions were performed at various conditions as described below to quantify and compare the cleavage efficiencies and to characterise the effect of incorporation of LNA nucleotides. The common conditions for comparison of all the ribozymes included 3 mM $MgCl_2$ and 37 °C and with concentrations of substrate RNA and ribozyme at 1–10 nM. This setting is similar to what is often termed physiological conditions and what others have been using when judging the relevance for future application of ribozymes as therapeutics. Our rationale is that if the ribozymes perform well at these conditions they will only be even better at higher concentrations of Mg^{2+} ions, substrate and enzyme, as we also show below.

Comparative analyses of ribozymes were performed at selected conditions for both single turnover and multiple turnover reactions and are tabulated in Table 2. An example of PAGE analysis of cleavage and the curves for the reactions used for determining k_{obs} are shown in Figure 3. When fitting to a single exponential curve was not appropriate, either because of a low R^2 value or an obvious bad representation by visual inspection as seen with the Rz9L curve in Figure 3b, a double exponential function was used. Such biphasic curves represent the combination of two reactions with different k_{obs}. This complicates a direct comparison with reactions represented by a single k_{obs} and also influences the reproductivity of data as small changes can highly influence the k_{obs} values. Some of the reactions seemed to be at the borderline between a monophasic and a biphasic reaction and we have no obvious explanation as to why we observe the biphasic reactions.

Ribozyme Rz3L with LNA nucleotides in the arms was more efficient than the unmodified Rz2 ribozyme under single turnover conditions in presence of 3 mM Mg^{2+} ions and also better than the unmodified Rz1 that has longer binding arms than Rz2 (Table 2). Rz8L and Rz9L with both LNA and DNA nucleotides in the binding arms were also better than Rz2 but difficult to compare directly because of the biphasic reaction. The effect of LNA is much more prominent in the case of multiple turnover, where only LNA modified ribozymes show the ability to cleave more than one RNA substrate. The ribozymes with LNAs in both the arms and helix II showed decreased cleavage ability compared to Rz3L. In general incorporation of LNA nucleotides close to or in the catalytic loop results in very poor cleavage ability and for Rz7L no cleavage was obtained.

For most in vitro applications the concentration of Mg^{2+} ions can be higher than 3 mM and therefore we checked whether the Mg^{2+} dependence would also be influenced by the incorporation of LNA nucleotides. The Rz1, Rz2 and Rz3L ribozymes were assayed at 10 mM Mg^{2+} ions and the reaction rates and percentage of cleaved product are shown in Table 3. The k_{obs} was increased up to a factor of 100 for Rz3L relative to the one obtained at 3 mM Mg^{2+}, with about 20-fold increase for Rz1 and only about 7-fold for Rz2. To see if changes in the concentrations of reactants affect LNA modified ribozymes and unmodified ribozymes in similar ways the Rz1, Rz2 and Rz3L ribozymes were assayed at higher substrate and enzyme concentrations. As seen in Table 3, k_{obs} increase about 20-fold for Rz3L and Rz1, and about 100-fold for Rz3L resulting in very similar k_{obs} when using high concentrations of reactants.

Table 2. Ribozyme cleavage at 3 mM Mg^{2+} concentration

Name	Single turnover			Multiple turnover	
	Eff.	k_{obs} ± SD (min^{-1})	% P ± SD	Eff.	% P[a]
Rz1	+++	0.0123 ± 0.0027	84 ± 5	—	10
Rz2	++	0.0026 ± 0.0008	60 ± 10	—	13
Rz3L	+++	0.0170 ± 0.0006	77 ± 3	++	47
Rz4L	++	0.0032 ± 0.0006	47 ± 9	+	23
Rz5L	+	0.0015 ± 0.0001	57 ± 6	—	12
Rz6L	+	0.0009 ± 0.0002	34 ± 4	nd	nd
Rz7L	—	—	<2	nd	nd
Rz8L	+++	0.314^1 ± 0.026	19^1 ± 3	++	39
		0.009^2 ± 0.001	58^2 ± 4		
Rz9L	+++	0.231^1 ± 0.019	24^1 ± 6	+++	58
		0.010^2 ± 0.002	52^2 ± 6		

The overall efficiency is indicated with a number of plus signs for easy comparison. Single turnover at 1 nM substrate and 10 nM ribozyme: $k_{1,2\ obs}$ are defined as in Figure 3 and % P are the end point of cleaved product from regression analysis of cleavage curves. Data are averages of two to five independent experiments and the standard deviations are from this averaging. All curves had R^2 of $\geqslant 0.99$. For Rz8L and Rz9L a double exponential plot was needed to fit the data appropriately, thus indicating a biphasic reaction kinetic. Multiple turnover at 10 nM substrate and 1 nM ribozyme: % P are the value after an overnight incubation.

[a] Ten percent P can be from single turnover and therefore only P > 10% can be taken as evidence of multiple turnover. 1 and 2 refer to the rate constants k_{1obs} and k_{2obs} for biphasic reactions.

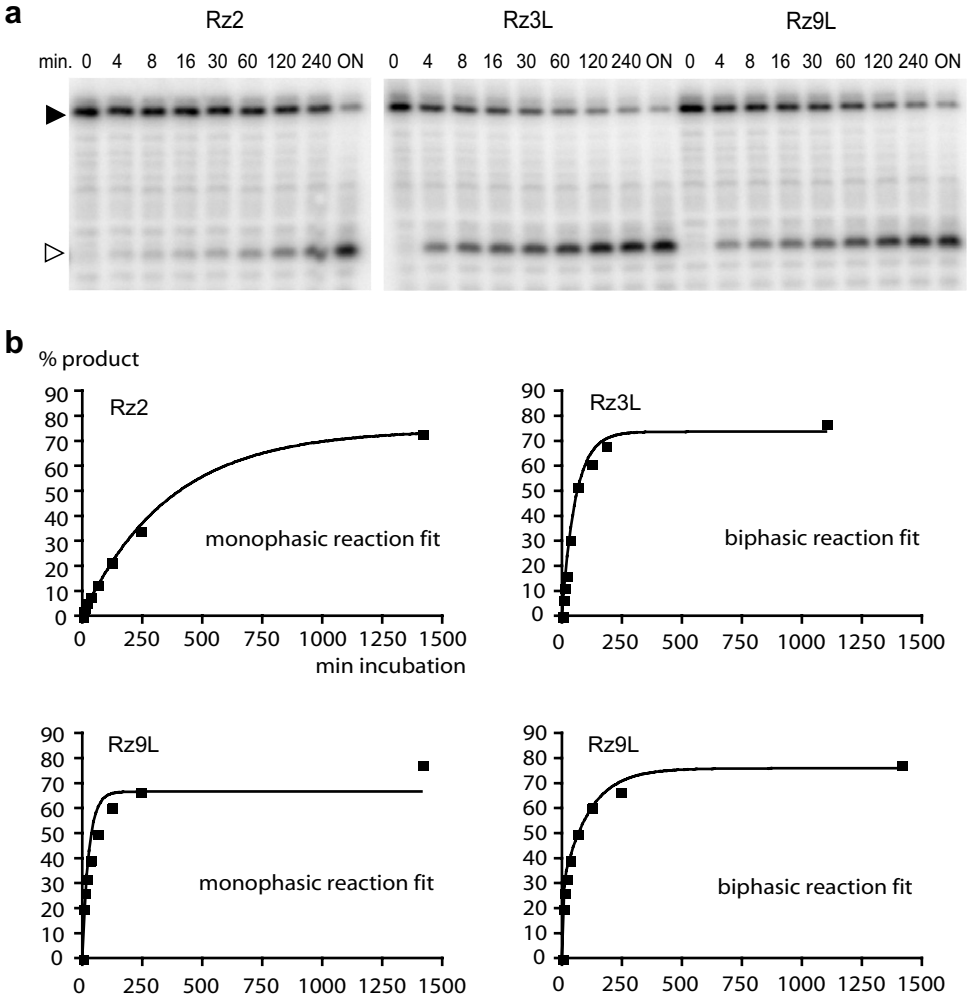

Figure 3. (a) Gel analysis of cleavage by Rz2, Rz3L and Rz9L at single turnover conditions with 1 nM substrate, 10 nM enzyme and 3 mM Mg^{2+}. (b) Examples of curves showing cleavage data used for determining k_{obs} by regression analysis. The two curves for Rz9L illustrate the differences between a monophasic reaction curve fit and a biphasic curve fit.

Table 3. Single turnover cleavage at 10 mM Mg^{2+} or with higher concentrations of reactants at 3 mM Mg^{2+} compared to the cleavage at the conditions used for Table 2

	1 nM sub: 10 nM Rz, 10 mM Mg^2		15 nM sub: 300 nM nM Rz, 3 mM Mg^{2+}		1 nM sub: 10 nM nM Rz, 3 mM Mg^{2+} (Table 2 data)	
	k_{obs} ± SD (min^{-1})	% P ± SD	k_{obs} ± SD (min^{-1})	% P ± SD	k_{obs} (min^{-1})	% P
Rz1	0.262^1 ± 0.029	35^1 ± 10	0.234 ± 0.039	82 ± 5	0.012	84
	0.021^2 ± 0.004	45^2 ± 12				
Rz2	0.022^1 ± 0.005	14^1 ± 2	0.371^1 ± 0.032	63^1 ± 4	0.003	60
	0.004^2 ± 0.001	49^2 ± 9	0.005^2 ± 0.001	7^2 ± 1		
Rz3L	1.663^1 ± 0.420	37^1 ± 6	0.373^1 ± 0.039	47^1 ± 2	0.017	77
	0.0336^2 ± 0.0002	44^2 ± 7	0.006^2 ± 0.003	33^2 ± 3		

Abbreviations: k_{obs} and % P are defined as in Table 2 and Figure 3. Data are from the average of at least two independent experiment. All curves had R^2 of >0.99. Double exponential plots were needed to fit some of the data appropriately and are thus indicating biphasic reaction kinetics where 1 and 2 are k_{1obs} and k_{2obs} respectively.

2.3. A combinatorial approach with binary ribozymes

The hypothesis behind this strategy is that incorporation of LNA nucleotides in helix II will allow deletion of loop II, and even though such a design may not be optimal with regard to catalytic activity we are presently interested in relative differences. The initial experiments were performed to determine if the divided ribozyme fragments would combine correctly to form a functional ribozyme with RNA cleaving activity. The starting point was the binary ribozyme Rz10L + 11L (Table 1 and Fig. 2) with a 3n long helix II composed entirely of LNA nucleotides in order to stabilise the hybridisation of the two oligonucleotides into a fully functional ribozyme. The ability of the binary R10L and R11L ribozyme fragments to complex with the substrate RNA

and to make a ribozyme complex was investigated by non-denaturing gel electrophoresis after incubation with the substrate RNA as shown in Figure 4a. From this we infer that the low cleavage efficiency (see below) is not due to lack of hybridisation between the ribozyme fragments and substrate but to a low fraction of complexes containing all three components. The Rz10L + 11L binary ribozyme cleaved with a k_{obs} at approximately 0.002 min^{-1}. As our assay is not suitable for accurately comparing the cleavage rates of the binary ribozymes at the used conditions, the binary ribozymes were compared according to the total amount of cleavage product obtained after 16 h incubation. The results for the combinations are displayed schematically in Figure 2.

The first step after confirming that the binary ribozyme Rz10L + 11L had the ability to cleave its target was an attempt to improve it by additional incorporation of LNA nucleotides into the binding arms (Rz12L + 13L; Table 1 and Fig. 2). Surprisingly, this change decreased the overall percentage of cleavage from 40% to <15% (Fig. 4b). A shortening of the binding arms (RzL14L + 15L), thus limiting the ability to make alternative structures, also did not improve cleavage. Another attempt to improve the cleavage efficiency was Rz16L + 17L with the helix II ends extended by two additional RNA nucleotides. This resulted in 35% cleavage (Fig. 4b), that was almost as good as the 40% seen with Rz10L + 11L. Using the same approach as before, this combination was modified with LNAs in the binding arms, as well as shortened, without success (R18L, R19L and R20L). When all possible combinations were assayed for cleavage only the RzL10L + 17L combination turned out to be better than the initial Rz10L + 11L combination with 47% cleavage compared to 40% (Fig. 4b).

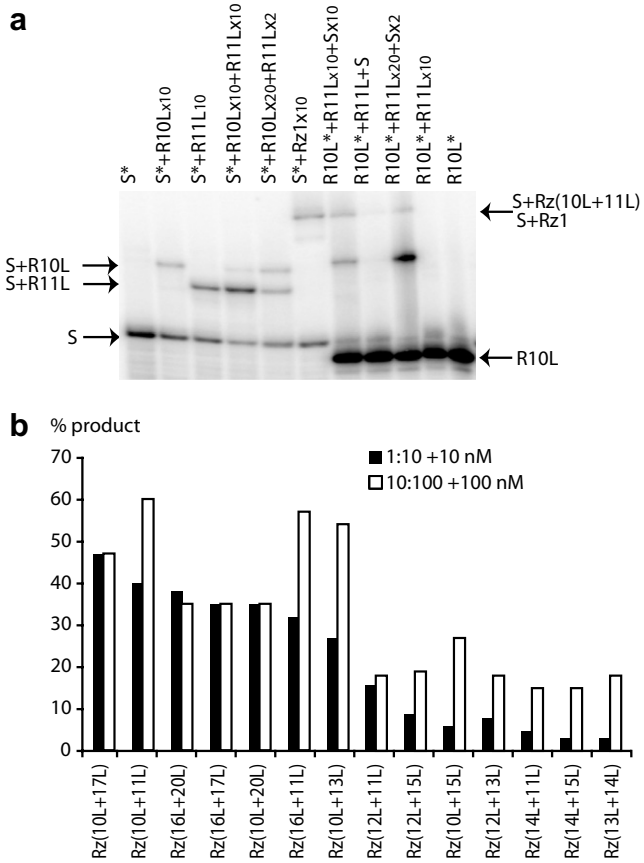

Figure 4. (a) Non-denaturing gel analysis of complexing of substrate and the binary ribozyme Rz10L + 11L. Substrate RNA or R10L was radioactive labelled and complexed in absence of Mg^{2+} and analysed by electrophoresis in a 15% non-denaturing acrylamide gel followed by phosphor imaging analysis. *Indicates the radioactive component. The small numbers; '2', '10' and '20' after the components are the molecular excess of the component at incubation. (b) Comparison of cleavage efficiency of the binary ribozymes and the influence of increased concentration of the reactants. Cleavage was performed overnight (16 h) with 10 mM Mg^{2+}.

The assays were performed with very low concentrations of the oligonucleotides to compare with the data for undivided ribozymes. As the concentration of the reactants highly influences the cleavage rate, we increased the concentration of both binary ribozymes and RNA substrate 10-fold for selected binary ribozymes and the resulting percentages of cleavage are shown in the white columns in Figure 4b. This concentration change improved the overall cleavage for some of the combinations but not for all. The highest level obtained was 60% for Rz10L + 11L followed by 57% for Rz16L + 11L, 54% for Rz 10L + 13L and 47% for Rz10L + 17L. Finally, the effect of using non-equivalent concentrations of the ribozyme fragments was tested with the binary ribozyme Rz10L + 11L and Rz10L+17L as shown in Table 4. The results show that the ribozyme fragments influence the complex formation in different ways with an excess of fragment R10L being more favourable than excess of fragments R11L or R17L.

3. Discussion

3.1. The hammerhead ribozyme designs

Our goal in this study is to assess the effect of incorporation of LNA nucleotides rendering ribozymes more applicable. Such ribozymes should be able to cleave at low concentrations and in low excess relative to the substrate and, ideally, perform multiple turnover. It is evident from other studies that incorporation of LNA into oligonucleotides improves nuclease stability.[12] It is also clear that LNA modifications of the binding arms will increase the ability to access structured substrate

Table 4. Cleavage effects of variations in ratios of the binary ribozyme reaction

S:Rz[x+y] conc in nM	% P	
	Rz[10L + 11L]	RzL[10L+17L]
1:5 + 5	33	49
1:5 + 1	42	31
5:1 + 5	17	22
5:1 + 5	30	20
5:5 + 1	16	19
5:1 + 1	5	12

Cleavage was performed overnight (16 h) with 10 mM Mg^{2+}, 37 °C. S are substrate and x and y refer to the binary ribozyme fragments.

RNA.[14,17,18] In contrast, it is not known how LNA modification will affect ribozyme catalysis and overall cleavage efficiency and at what positions they will be allowed or even beneficial. It is expected that the modifications will enable a reduction of the size of the ribozymes. Also, we envisioned that LNA incorporation would allow a division of the ribozyme and thereby provide the opportunity of applying a combinatorial approach for investigation of ribozyme cleavage. We did not aim at creating a ribozyme with an optimal k_{obs} and therefore we did not relate to loop–loop interactions in our constructs.[10,20] Nor did we choose an optimal cleavage sequence according to NUH and NHH rule investigations.[21,22]

Rz2 (Fig. 1) with 7n-long substrate binding arms was chosen as the reference construct. To establish how sensitive this is to changes in the number of nucleotides in the substrate binding arms we also made Rz1 with 10n- and 8n-long substrate binding arms. Substitution of RNA with LNA nucleotides in the distal ends of the arms improves the cleavage efficiency (Rz3L > Rz1 ≫ Rz2) in single turnover experiments in presence of 3 mM Mg^{2+} ions (Table 2). LNA also facilitates multiple turnover where the ribozyme has to dissociate from the cleaved substrate and attack a new uncleaved substrate. In fact no significant multiple turnover was observed for Rz2 or Rz1 in contrast to Rz3L that shows 46% cleavage (Table 2).

An auxiliary addition of LNA nucleotides into the internal helix II creating Rz4L (Fig. 1) decreases both k_{obs}, the total amount of cleaved substrate and the efficiency of multiple turnover (relative to Rz3L, Table 2). The performance of Rz4L is inferior to Rz1 but similar to Rz2 at the single turnover conditions but in contrast to Rz1 and Rz2, Rz3L can carry out multiple turnover (Table 2). Rz5L encompass LNAs only in the internal helix II (Fig. 1) and show decreased k_{obs} relative to Rz2 and no significant multiple turnover. The LNA nucleotides were expected to stabilise this helix and thereby facilitate folding of the ribozyme, but surprisingly it resulted in lesser cleavage of the RNA substrate. As the locked conformation of LNA causes an A-form helical structure as for RNA, our results indicate that the ribozyme at some stage adopts a structure with an opened or a distorted helix II. A similar argument can be provided for helix I and III. Rz6L (Fig. 1) has LNAs in the proximal ends of the bindings arms and shows a strongly reduced cleavage efficiency (Table 2). Again it indicates that incorporation of LNA nucleotides close to the catalytic core region somehow interfere with a needed flexibility of the helix regions. It is well known that ribozyme activity is dependent on formation of a precise, three-dimensional folded structure of the complex between the ribozyme and the RNA, and that structural rearrangements take place during the reaction.[5]

There have been various reports[23–25] of enhanced rate of cleavage upon incorporation of DNA nucleotides in the part of the ribozyme that hybridise to the substrate RNA but with no consensus in what step in the cleavage process this modification influences. The Rz3L ribozyme was additionally modified by also introducing DNAs in the arms (Rz8L, Fig. 1), and with the LNAs moved to the ends of the binding arms (Rz9L, Fig. 1). As seen in Table 2, Rz8L and Rz9L showed a biphasic reaction kinetics that makes a direct comparison with the other ribozymes difficult but it is evident that they perform much better than Rz1. With regard to multiple turnover they appear even better than RzL2 (Table 2).

The data in Table 3 show that k_{obs} are strongly influenced by assaying conditions. We cannot from our analysis tell how this effect is mediated and also we cannot explain why some reactions apparently are biphasic. We have seen similar biphasic reactions with LNAzymes (our unpublished data) showing that it is not an effect specific for the structures investigated here. Although k_{obs} are very similar for Rz1, Rz2 and Rz3L at 3 mM Mg^{2+} and high concentrations of reactants (15 and 300 nM), the Rz3L ribozyme is clearly much better than Rz1 at 3 mM Mg^{2+} ions and low concentrations of reactants (1 and 10 nM). This is probably because the complex formation is the rate-limiting step when the concentration of reactants is low, and reveals that LNA nucleotides stabilise the substrate complex thereby lowering the off rate. When raising the Mg^{2+} to 10 mM all three ribozymes show increased k_{obs} but the superiority of Rz3L becomes very prominent with k_{1obs} being more than 80 times and six times higher than k_{1obs} for Rz2 and Rz1, respectively.

3.2. The combinatorial approach with binary ribozymes

The overall idea of the combinatorial method is that a splitting of the ribozyme into two fragments can conveniently be explored to optimise the design with regard to incorporation of modifications in general (not only LNA modifications), as the number of variant ribozymes is increased from n to n^2. Additionally, a binary ribozyme provides the ability to use smaller oligonucleotides, which is highly advantageous from the chemical synthesis viewpoint. Finally, one can imagine applications where a binary ribozyme can be used to control the order of events by addition of the second fragment.

Splitting of hammerhead ribozymes has previously been studied by Vlassov and co-workers[26] and references therein as a means to improve function. As incorporation of LNA nucleotides increases helix stability, we expected that LNAs in helix II could compensate for the effect of deleting loop II and thereby allowing division of our ribozymes. Thus, a library of relatively small oligoes containing the ribozyme fragments could be investigated to optimise ribozyme design. The first combination we looked at, Rz10L + 11L, could cleave, albeit with a low k_{obs}. According to the in vitro gel mobility shift the ribozyme fragments each hybridise with the RNA substrate but have problems forming the complete tri-molecular ribozyme/substrate complex (Fig. 4a). Attempts of improvement by additional incorporation of LNAs in the binding arms (Rz12L + 13L and Rz14L + 15L) induced only negligible cleavage. Also extension of the helix II arms did not improve cleavage. The relative low ability to cleave the RNA

substrate is probably due to restriction of structural rearrangements during the cleavage reaction as also suggested by some of the full-length LNA modified ribozymes described above. Ribozyme fragments with the LNA monomers placed further apart from the catalytic core did not significantly improve RNA cleavage as seen in Figure 2. The ribozyme fragments with the best performance are those with few LNA nucleotides placed away from the catalytic core. The overall cleavage could be improved for some of the splitted ribozymes by increasing the concentrations of the ribozyme fragments and RNA substrate by 10-fold (Fig. 4b). Additionally, the use of 10 mM Mg^{2+} instead of 3 mM Mg^{2+} increased the cleavage efficiency (data not shown). As seen in Table 4 there is some effect of changing the relative ratios of the two ribozyme fragments and the substrate that could be considered for optimising the performance of binary ribozymes. The relatively simple assay used here allows a quick screening of the relative efficiency of various combinations and can be used for selecting promising candidates for further developments.

3.3. Perspectives and conclusions

This study presents a starting point for incorporation of LNA nucleotides to improve ribozyme function. A combinatorial approach is introduced that can be used to optimise incorporation of various modifications or other structural aspects of hammerhead ribozymes. An optimal design will likely encompass various modifications and structural characteristics each providing certain advantages. We have shown that LNA nucleotides can be incorporated in the binding arms at the distal ends allowing shortening of the arms. Such modified ribozyme performs even better than the parent ribozyme. Furthermore, LNA incorporations strongly facilitate multiple turnover reactions, which is greatly advantageous for most applications. We also know from previous work that LNA incorporation improves the accessibility to structured targets so LNA modified ribozymes might alleviate the need for a pre-screening of accessibility. LNA modified ribozymes therefore hold promise for future diagnostic or therapeutic applications.

4. Materials and methods

4.1. Oligonucleotides

The modified and unmodified ribozymes are listed in Table 1 together with the RNA substrate and the deoxyribozyme. The modified ribozyme oligonucleotides with LNA nucleotides as well as the unmodified Rz2 were synthesized by Sigma-Proligo or synthesized at the Nucleic Acid Center, University of Southern Denmark. The unmodified ribozyme Rz1 and the 20n RNA substrate were made by T7 RNA polymerase transcription from a T7 promoter in front of a DNA sequence complementary to the RNA substrate. The oligonucleotides for transcription were as follows: 5′-G<u>A</u>AGGCCTTTTCG CAAAAGCTCATGAGGGCGCATATC*<u>TATAGTGA GTCGTATTA</u>*-3′ (Rz1),5′-G<u>A</u>TATGCGCCTAAGGC CTTC*<u>TATAGTGAGTCGTATTA</u>*-3′ (substrate RNA), 5′-TAATACGACTCACTATAG-3′ (second promoter strand). Underlined letters are modified with 2′-O–CH_3 and letters in italic are T7 promoter sequence. DNA oligonucleotides were purchased at MWG Biotech AG.

4.2. Transcription of Rz1 and the RNA substrate with T7 RNA polymerase

The RNAs were made by transcription from oligodeoxynucleotide templates encoding the T7 RNA polymerase promoter in front of a DNA sequence complementary to the RNA substrate.[27,28] The in vitro transcriptions were performed with modifications according to Kao et al. [29]. Briefly, 40 μmol of the second promoter strand was annealed to 40 μmol of the template oligonucleotide in the hybridisation buffer [Hepes, pH 7.0 (250 mM), KCl (500 mM)] in a total volume of 20 μl. The annealing reaction was used for the transcription reaction and added to final concentrations in 100 μl: 2× TSC (80 mM Tris–HCl, pH 8.0, 10 mM DTT, 0.02% Triton X-100), NTP (8 mM each), PEG8000 (0.1 mg/ml), $MgCl_2$ (28 nM), sodium glutamate, pH 8.1 (50 μM) and T7 RNA polymerase (Promega) (100 U). After transcription, the DNA templates were degraded by adding DNase I (Roche) (40 U) and the RNAs were extracted with phenol/chloroform and precipitated with glycogen (0.04 μg/μl). The full-length RNAs were isolated on a 13% denaturing polyacrylamide–7 M urea gel and the appropriate gel bands were visualized by UV shadowing and excised from the gel. The RNAs were extracted by crush soaking with H_2O followed by further extraction with phenol/chloroform. The RNAs were recovered from the aqueous phase by ethanol precipitation with glycogen as before and were redissolved in H_2O.

4.3. RNA cleavage conditions: single and multiple turnover analyses

RNA transcripts were 5′-radiolabelled by dephosphorylating using Shrimp phosphatase (USB) followed by incubation with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. The RNA substrate was cleaved by mixing ribozyme and RNA substrate in reaction buffer containing NaCl (150 mM) and Tris–HCl, pH 7.5 (50 mM). The ribozymes were pre-folded at 50 °C for 5 min. The samples were subsequently shifted to 37 °C for cleavage. The cleavage reactions were started by addition of $MgCl_2$ at concentrations as specified. At timepoints between 0 min and 24 h, the reactions were stopped by adding ice-cold 90% formamide loading buffer containing 20 mM EDTA and stored at −20 °C. The cleavage efficiencies were assayed by quantifying the amount of cleaved ^{32}P-labelled RNA substrate by PAGE using 13% denaturing polyacrylamide–7 M gels. Immediately prior to loading, the samples were heated to 80 °C for 2 min. All gels were autoradiographed and scanned on a Typhoon TRIO Variable Mode Imager (Amersham Biosciences) and quantified by ImageQuant.

For standard single turnover conditions, the concentration of the substrate RNA was held fixed at 1 nM and the ribozyme was in an excess of 10 nM. For multiple turnover experiments the RNA substrate concentration was 10 nM and the ribozyme concentration 1 nM. Deviations from these concentrations are described in the Table legends.

4.4. Determination of k_{obs} using regression-analyses

Reaction curves were determined from the RNA cleavage experiments. Each reaction was performed at least two times. The numbers of time points were in all cases at least six and were adjusted according to the efficiency of the ribozyme in question. Cleavage ability was calculated as percentage cleaved of total RNA substrate. Regression-analyses were performed by using the formula $y = a(1 - e^{-Kx})$ if this provided an appropriate fit. If not the data were fitted to $y = a1(1 - e^{-K1x}) + a2(1 - e^{-K2x})$.

4.5. In vitro gel mobility shift assay

The RNA substrate was radioactive labelled as described above. The binding assays were performed in KCl (100 mM), Hepes (20 mM), DTT (1 mM), binary ribozymes (10 nM) and RNA substrate (1 nM) in a final volume of 10 μl, incubated at 37 °C for 60 min and then placed in ice. The samples were analysed on a 15% nondenaturing polyacrylamide gel with 1× running buffer (Tris-base, pH 8.3 (90 mM), boric acid (90 mM)) at 15 °C. The gel was dried, autoradiographed, and the bands quantified by a Typhoon TRIO Variable Mode Imager (Amersham Biosciences).

Acknowledgments

Lykke H. Hansen is thanked for excellent technical assistance. We thank The Danish National Research Foundation and The Lundbeck Foundation for financial support.

References and notes

1. Symons, R. H. *Annu. Rev. Biochem.* **1992**, *61*, 641.
2. Hammann, C.; Lilley, D. M. *Chembiochem* **2002**, *3*, 690.
3. Puerta-Fernandez, E.; Romero-Lopez, C.; Barroso-delJesus, A.; Berzal-Herranz, A. *FEMS Microbiol. Rev.* **2003**, *27*, 75.
4. Peracchi, A. *Rev. Med. Virol.* **2004**, *14*, 47.
5. Blount, K. F.; Uhlenbeck, O. C. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 415.
6. Citti, L.; Rainaldi, G. *Curr. Gene Ther.* **2005**, *5*, 11.
7. Beigelman, L.; Karpeisky, A.; Matulic-Adamic, J.; Haeberli, P.; Sweedler, D.; Usman, N. *Nucleic Acids Res.* **1995**, *23*, 4434.
8. Klopffer, A. E.; Engels, J. W. *Nucleosides Nucleotides Nucleic Acids* **2003**, *22*, 1347.
9. Hendry, P.; McCall, M. J.; Stewart, T. S.; Lockett, T. J. *BMC Chem. Biol.* **2004**, *4*, 1.
10. Martick, M.; Scott, W. G. *Cell* **2006**, *126*, 309.
11. Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301.
12. Vester, B.; Wengel, J. *Biochemistry* **2004**, *43*, 13233.
13. Petersen, M.; Nielsen, C. B.; Nielsen, K. E.; Jensen, G. A.; Bondensgaard, K.; Singh, S. K.; Rajwanshi, V. K.; Koshkin, A. A.; Dahl, B. M.; Wengel, J.; Jacobsen, J. P. *J. Mol. Recognit.* **2000**, *13*, 44.
14. Vester, B.; Lundberg, L. B.; Sorensen, M. D.; Babu, B. R.; Douthwaite, S.; Wengel, J. *J. Am. Chem. Soc.* **2002**, *124*, 13682.
15. Schubert, S.; Gul, D. C.; Grunert, H. P.; Zeichhardt, H.; Erdmann, V. A.; Kurreck, J. *Nucleic Acids Res.* **2003**, *31*, 5982.
16. Fahmy, R. G.; Khachigian, L. M. *Nucleic Acids Res.* **2004**, *32*, 2281.
17. Schubert, S.; Kurreck, J. *Curr. Drug Targets* **2004**, *5*, 667.
18. Vester, B.; Hansen, L. H.; Lundberg, L. B.; Babu, B. R.; Sorensen, M. D.; Wengel, J.; Douthwaite, S. *BMC Mol. Biol.* **2006**, *7*, 19.
19. Persson, T.; Hartmann, R. K.; Eckstein, F. *Chembiochem* **2002**, *3*, 1066.
20. Khvorova, A.; Lescoute, A.; Westhof, E.; Jayasena, S. D. *Nat. Struct. Biol.* **2003**, *10*, 708.
21. Shimayama, T.; Nishikawa, S.; Taira, K. *Biochemistry* **1995**, *34*, 3649.
22. Kore, A. R.; Vaish, N. K.; Kutzke, U.; Eckstein, F. *Nucleic Acids Res.* **1998**, *26*, 4116.
23. Hendry, P.; McCall, M. J.; Santiago, F. S.; Jennings, P. A. *Nucleic Acids Res.* **1992**, *20*, 5737.
24. McCall, M. J.; Hendry, P.; Jennings, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5710.
25. Taylor, N. R.; Kaplan, B. E.; Swiderski, P.; Li, H.; Rossi, J. J. *Nucleic Acids Res.* **1992**, *20*, 4559.
26. Vorobjeva, M.; Zenkova, M.; Venyaminova, A.; Vlassov, V. *Oligonucleotides* **2006**, *16*, 239.
27. Krupp, G.; Soll, D. *FEBS Lett.* **1987**, *212*, 271.
28. Milligan, J. F.; Uhlenbeck, O. C. *Methods Enzymol.* **1989**, *180*, 51.
29. Kao, C.; Rüdisser, S.; Zheng, M. *Methods.* **2001**, *23*, 201.