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Attempts to Obtain More Efficient GAC-Cleaving Hammerhead Ribozymes by In Vitro Selection

Anilkumar R. Kore, Christophe Carola and Fritz Eckstein*

Max-Planck-Institut für Experimentelle Medizin, Hermann-Rein-Str. 3, D-37075 Göttingen, Germany

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Abstract—An in vitro selection was carried out to identify hammerhead ribozymes cleaving 3′ to GAC triplets more efficiently than the wild type ribozyme. A double-stranded DNA containing the sequence for the hammerhead ribozyme with 10 randomizations in the catalytic core, designed for in *cis* cleavage, was transcribed and the cleavage product amplified by reverse transcription and PCR. After seven selection cycles, the DNA was cloned and 50 colonies sequenced. One sequence, appearing six times, was active for in *cis* cleavage of GAC. It was identical to the consensus sequence except for a mutation at position 7. Another cleaved GUC and two more, cleaved GUA. The cleavage rates of these ribozymes for in *trans* cleavage were slower than the rate of the consensus ribozyme. Interestingly, the consensus sequence was not found in the selection. This strongly suggests that the consensus hammerhead ribozyme has evolved to an optimal sequence.

Introduction

The hammerhead ribozyme is an RNA motif which is capable of sustaining either in *trans* or in *cis* cleavage of a phosphodiester bond (Fig. 1).[1–4] The core region contains invariant nucleotides, i.e. change of any nucleotide results in a considerable reduction in cleavage activity. Sequence specificity of cleavage is controlled by the hybridizing arms of the ribozyme which anneal with the substrate RNA by formation of Watson-Crick base pairs to form helices I and III. Cleavage is limited to sequences of the form 5′-NUH-3′ where N is any nucleotide and H is A, U or C. However, the central U of the substrate (position 16.1) can be changed to C if the complementary nucleotide of the ribozyme at position 15.1 is changed to inosine resulting in a very efficient ribozyme.[5] When the conventional base pair A15.1-U16.1 is inverted to A16.1-U15.1 cleavage occurs but at approximately 5% of that of the conventional sequence.[6] We were interested in having in hand a hammerhead ribozyme which cleaves triplets of an 5′-NAH-3′ sequence more efficiently than the wild type, not only to get some insight into what might determine the cleavage specificity but also to expand the use of the hammerhead ribozyme for the inhibition of gene expression.[7] We have previously used in vitro selection to obtain hammerhead ribozymes with altered core sequences and also with altered triplet specificities, particularly hammerhead-like ribozymes to very efficiently cleave NUG triplets which are not cleavable by the conventional hammerhead ribozyme.[8–10] Thus in vitro selection seemed to be a reasonable approach to obtain a ribozyme with better cleavage activity 3′ to NAH than the consensus ribozyme. Here we describe our attempts to select for GAC-cleaving ribozymes, using the same selection procedure.

Results and Discussion

The selection procedure to obtain GAC-cleaving hammerhead ribozymes was based on the previously described method successfully used for the selection of mutant hammerhead ribozymes.[8–10] Double-stranded DNA was synthesized to contain the sequence for the hammerhead ribozyme with the 10 central core nucleotides randomized. This corresponds to a library of 4^{10} (1.05×10^6) ribozyme sequences. The hammerhead sequence was flanked by restriction sites for cloning, and the sequence for the T7 promoter for transcription (Fig. 2). Transcription of this DNA results in full length transcripts except for those sequences which contain an active ribozyme resulting in *cis* cleavage. The cleavage product which does not contain terminal GTPγS is isolated by mercury-containing polyacrylamide gel electrophoresis where any products with a 5′-phosphorothioate remain in the well. The cleavage product is subsequently reverse transcribed and amplified by PCR for a new round of transcription. With each successive selection cycle, the

*Corresponding author. Tel.: +49-551-3899-274; fax: +49-551-3899-399; e-mail: eckstein@mail.mpiem.gwdg.de

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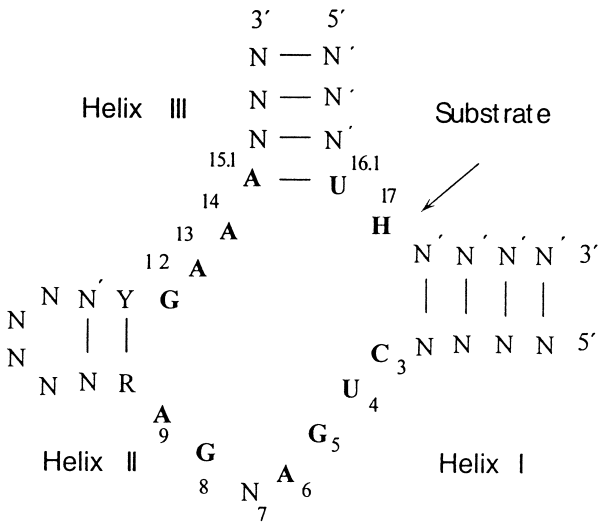

Figure 1. Generalized representation of the hammerhead ribozyme–substrate complex. Nucleotides in boldface indicate the conserved catalytic core nucleotides; arrow, cleavage site. Ribozyme numbering is according to Hertel et al.[16]

time allowed for cleavage was reduced progressively to enrich the dsDNA pool with the sequences of more efficiently cleaving ribozymes.

After seven such selection cycles the DNA was restricted with *Hind* III and *EcoR* I for ligation into pUC19 and transformation of TG1 cells. White colonies were isolated randomly for isolation of DNA and run-off transcription after linearization with *EcoR* I. Of the 98 clones picked, only 18 showed convincing in *cis* cleavage during transcription. Of these, six had the same sequence as clone 24 containing the original sequence in the randomized region, including the GAC triplet for which selection had been targeted. Another clone, No. 14, contained an altered triplet GUC as well as mutations in helix III and deletions in helices I and II. Yet another set, clone 2, appeared five times with the same changes as clone 14 but with yet another triplet, GUA. The sequence of clone 8 was present six times and was identical with that of clone 2 except for a mutation at position 7. An additional 26 clones were sequenced even though they did not show any convincing in *cis* cleavage (Fig. 3). One of these, clone 50, had only one deviation from the consensus sequence whereas the rest showed multiple differences.

The sequence of clone 24 was synthesized for an in *trans* cleaving ribozyme to determine its cleavage parameters by single turn over kinetics (Fig. 4) (Table 1). It had half the activity with $k_{cat'}$ 0.15 min^{-1} as the wild type sequence with 0.30 min^{-1}. Clone 2 was also tested for in *trans* cleavage and had $k_{cat'}$ 1.0 min^{-1} for GUA cleavage as compared with 1.9 min^{-1} for the consensus sequence. This sequence was also examined for GAC cleavage but had only $k_{cat'}$ 0.08 min^{-1}. Clone 14 showed $k_{cat'}$ 0.88 min^{-1} thus being considerably less active than the consensus sequence with 4.63 min^{-1}.

These results are remarkable for several reasons. Although a single sequence, that of clone 24, supporting cleavage of GAC has been selected for, it is not more active than the consensus sequence from which it differs only in position 7. Surprisingly the wild type sequence has not been found. Thus there must be some selection pressure to prevent the wild type sequence to evolve. This has also been observed in the previous selection using the same protocol to look for an improved GUC-cleaving ribozyme.[5] This is presumably due to certain sequence or structure specificities of the enzymes used in the selection process. Apparently the wild type sequence is favoured for GAC cleavage within the hammerhead sequence context. Very surprising is the detection of sequences, represented by clones 14, 2 and 8, which show considerable changes in the part of the DNA sequence which was kept constant in the selection protocol. This also includes the triplet sequence which was mutated from GAC to either GUC or GUA. The cause for these mutations is not clear but they have probably arisen because of the limited fidelity of the Taq polymerase.

Thus this selection method did not identify a ribozyme that was more active than the GAC-cleaving consensus hammerhead ribozyme although it yielded variants of the consensus sequence. The sequence space for this selection was deliberately kept small to preserve the classical hammerhead structure. This was done with the idea that the consensus sequence and the selected sequences, could be easily compared to identify the nucleotides responsible for improved cleavage activity. Additionally the hammerhead ribozyme is a small molecule which limits the number of ways a given motif can be present.[11,12] These restrictions might have prevented the selection of a more active ribozyme. It could well be that by increasing the number of randomized nucleotides ribozymes with better GAC-cleaving potential might be found. Indeed, expanding the number of randomizations to 22 had resulted in a hammerhead ribozyme capable of cleaving AUG, a triplet not cleavable by the consensus sequence.[10] However, the selected sequence no longer fully conformed to that of the consensus ribozyme.

The result of our selection suggests that the evolution of the hammerhead ribozyme is close to optimal and that the identification of a more active sequence than the consensus central core seems unlikely. This view is supported by our previous failures to obtain more efficient hammerhead ribozymes for the cleavage of AUA and GUC.[8,9]

Experimental

Materials

Oligodeoxyribonucleotides and oligoribonucleotides were synthesized and purified as previously described.[8,13] Sequenase 2.0, MMLV reverse transcriptase and Taq polymerase were from Amersham Buchler (Germany), QIAquick Nucleotide Removal kit and plasmid DNA purification columns (P-20) from Qiagen (Germany). The sequences of the primers and templates used are as follows:

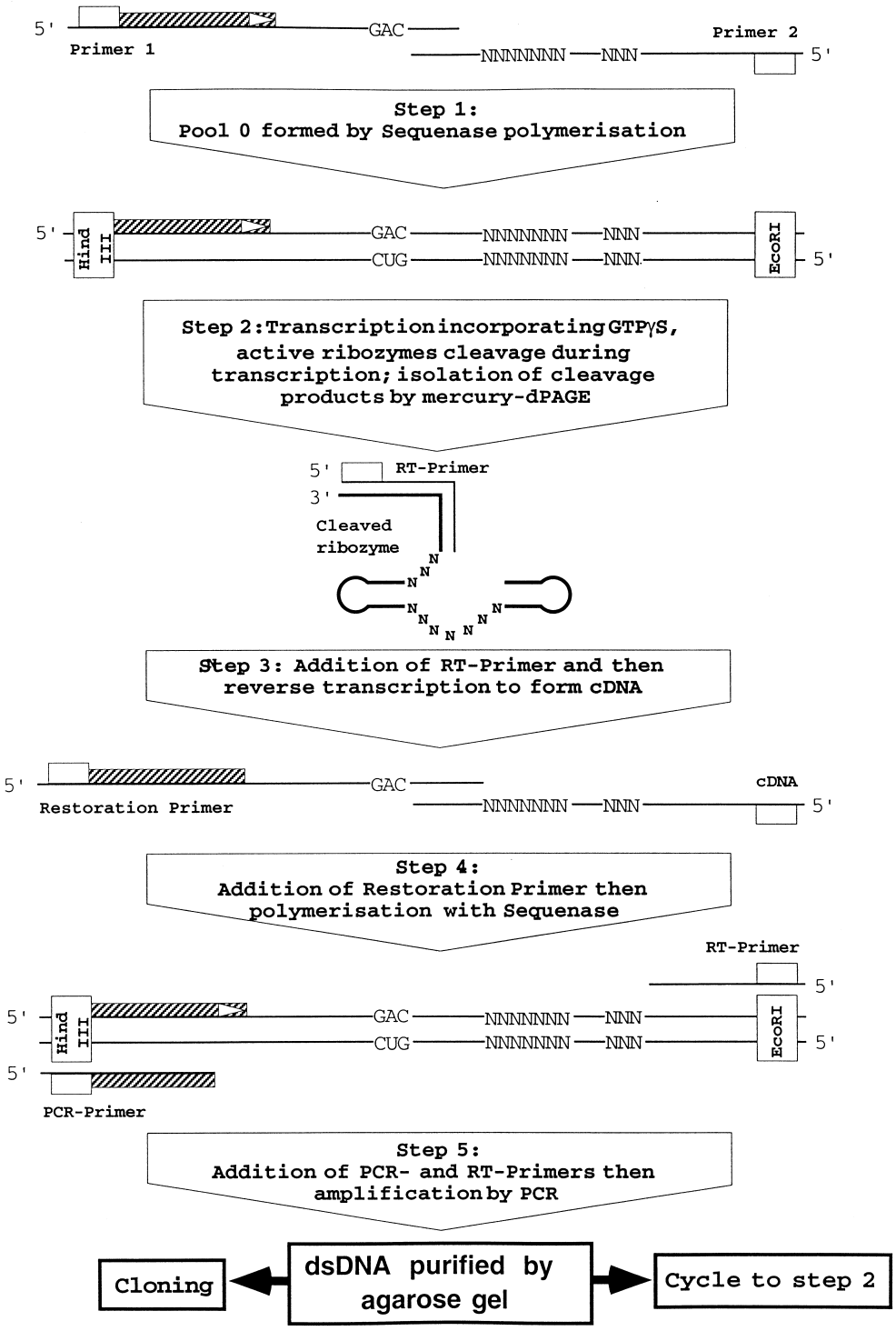

Figure 2. Outline of the selection procedure.

Primer 1 (restoration)
5′CTGATGC**AAGCTT**AATACGACTCACTATAGG-GAGACTGTAACTTTTC*GAC*TCAGCCAGCTATG3′

Primer 2 (random)
5′TCAT**GGAATT**CTAGTTTCGANNNGTCCTTGC-GGACNNNNNNNTCAGCCATAGCTGGCTGA 3′

These two primers were used for the initial Sequenase reaction, and primer 1 also functioned as a restoration primer for the re-introduction of the lost sequence after cleavage.

Primer 3 (RT and PCR)
5′TCATGGAATTCTAGTTTCG 3′ and

Primer 4 (PCR) PCR-Primer:
5′ CTGATGCAAGCTTAATACGACTCACTATAGG 3′. Where the *Hind* III (5′-end) and *EcoR* I (3′-end) restriction sites are indicated in boldface; the T7 promoter

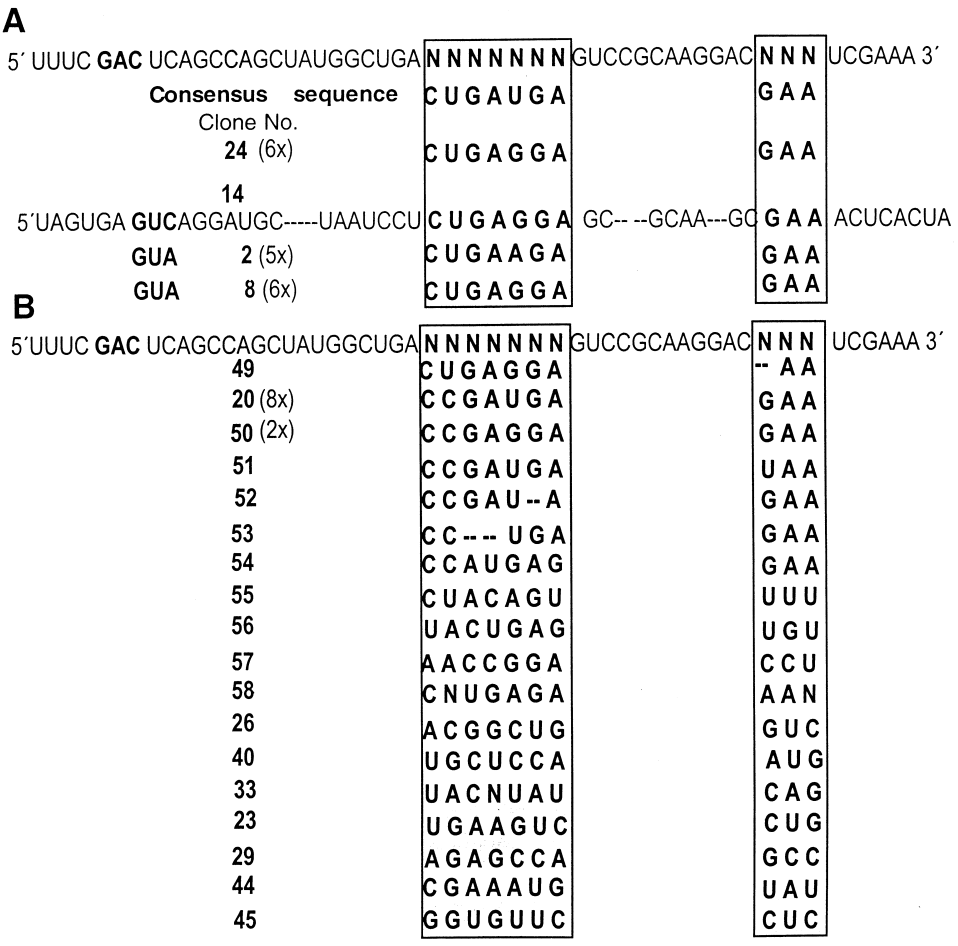

Figure 3. RNA sequences of selected colonies. A, sequences active in *cis* cleavage; B, inactive sequences. N, randomized positions of the core; triplets in bold, cleavage triplets; bold numbers, number of clones; numbers in brackets, number of identical sequences.

is underlined; the ribozyme cleavage triplet is in italics; and N indicates the randomized nucleotides.

RNA selection

RNA selection was performed as previously described.[9,10] Transcriptions from pool 0 were performed on a large scale, 6 times in 500 μL. Successive transcriptions were performed at 250 μL volume. Transcription reactions were for decreasing periods of time from 7 h for the first cycle, 3 h for the second, 1 h for the third, 30 min for the fourth, 15 min for the fifth, 2 min for the sixth cycle and 1 min for the seventh cycle, at 37 °C. Reactions for short periods were quenched by addition of EDTA (75 mM final concentration). All transcriptions were performed with T7 RNA polymerase on 1 μM DNA and with [α-^{32}P]UTP and GTPγS. The full length and cleavage products were separated by mercury-containing polyacrylamide gel electrophoresis. The desired band was isolated by the crush and soak method and reverse transcribed with a minimum RNA template concentration of 1×10^{-8} M. This cDNA was annealed to the restoration primer and sequenase used for synthesis of the full length dsDNA which was amplified with Taq polymerase using a DNA concentration of at least 1×10^{-15} M. From pool 3 not only the expected full length DNA was observed but also deletion products which were separated on a 2.5% Metaphor intermediate melting agarose gel (Biozym, Oldendorf, Germany). The full length product was isolated by JETsorb gel extraction (Genomed, Bad Oeynhausen, Germany). This DNA was further amplified by PCR to obtain enough material for the next round of selection. The concentrations were determined assuming a molar extinction coefficient at 260 nM of 6600 per nucleotide.

Table 1. Kinetic parameters for consensus and selected ribozymes[a]

Ribozyme	Triplet $N^{16.2}Y^{16.1}X^{17}$	$k_{cat'}$ (min^{-1})	K_m (nM)	k_{cat}/K_m ($\mu M^{-1}\,min^{-1}$)
Consensus	GAC	0.3	31	9.67
Clone 24	GAC	0.15	107	1.40
Clone 2	GAC	0.082	11	7.45
Consensus	GUA	1.9	16	118.75
Clone 2	GUA	1.0	14	71.42
Consensus	GUC	4.63	65	71.23
Clone 14	GUC	0.88	16	55.00

[a]STO kinetics with 10 mM $MgCl_2$, Tris–HCl pH 8, at 37 °C.

Cloning and sequencing

These were performed as described with digestion of double-stranded DNA (dsDNA) with *Hind* III and *EcoR* I[9] and purification with the QIAquick nucleotide

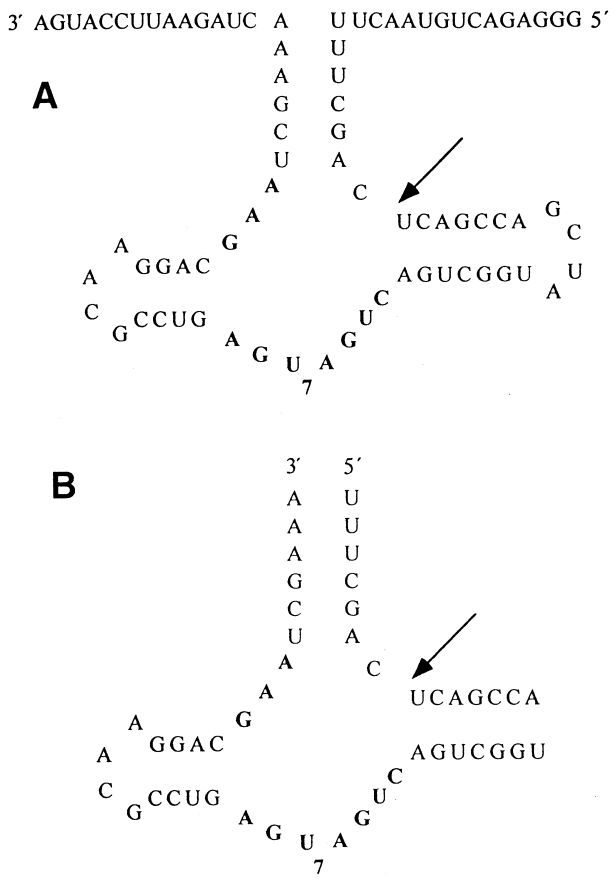

Figure 4. Structure of ribozymes. A, for in *cis* cleavage; B, for in *trans* cleavage.

removal kit for ligation. Plasmid DNA was isolated by the plasmid DNA purification columns from Qiagen. Colonies were tested for transcript self-cleavage from linearized plasmids. Colonies which showed cleavage activity, during run-off transcriptions were sequenced.

Cleavage kinetics

Cleavage kinetics of intermolecularly cleaving ribozymes were performed with chemically synthesized ribozyme and substrate in 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$ with a 25–500 nM ribozyme and 25 nM substrate for single turnover kinetics.[13–15] Data were fitted to the Michaelis–Menten and Eadie–Hofstee equations by KaleidaGraph (Synergy Software, Reading, PA).

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