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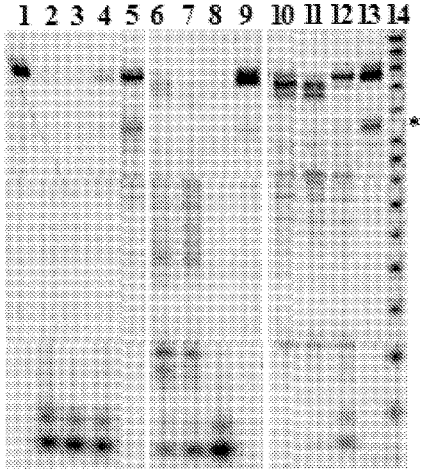
(54) Title: SEQUENCE-SPECIFIC ENGINEERED RIBONUCLEASE H AND THE METHOD FOR DETERMINING THE SEQUENCE PREFERENCE OF DNA-RNA HYBRID BINDING PROTEINS

Fig. 3



(57) Abstract: The subject of the invention is the ribonuclease which cleaves RNA strand in DNA-RNA hybrids, wherein ribonuclease comprises fusion protein comprising catalytic domain of RNase HI (RNase HI) or derivative thereof with a zinc finger DNA-RNA hybrid binding domain, and wherein the zinc finger binding domain has the ability to bind to specific sequences in the DNA-RNA hybrid. The invention also relates to new method for determination of the sequence preference of DNA-RNA hybrid binding protein(s) or its domain(s) and allows determining the sequence recognized by sequence specific binding protein in the DNA-RNA hybrid.

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Sequence-specific engineered ribonuclease H and the method for determining the sequence preference of DNA-RNA hybrid binding proteins

DESCRIPTION

TECHNICAL FIELD

The invention concerns a sequence-specific ribonuclease H, which comprises a fusion of an engineered ribonuclease HI (RNase HI) domain with a zinc finger domain. The present invention is applicable in genetics. The sequence-specific ribonuclease that acts on DNA-RNA hybrids is useful in any application, where cleavage of the RNA strand in a DNA-RNA hybrid can be performed, for example in *in vitro* manipulation of nucleic acids, particularly DNA-RNA hybrids with a specified sequence. In addition to in vitro use, enzymes that cleave DNA-RNA hybrids in a sequence specific manner may also find use in the therapy of certain RNA viruses infections (eg. oncogenic viruses, retroviruses, hepatitis B, influenza), which replicate by transiently forming a DNA strand on a RNA template or to cleave other DNA-RNA hybrids that are formed in vivo.

The invention also relates to a method for determination of the sequence preference of DNA-RNA hybrid binding protein(s) or its domain(s) and by this determining the sequence recognized by a DNA-RNA hybrid binding protein. The present invention in this scope is applicable in genetics. The method for determination of the sequence preference of DNA-RNA hybrid binding protein is applicable in determining the sequence preference of any protein or its domain that binds to specific sequences in the DNA-RNA hybrid and by this determining the sequence recognized by a DNA-RNA hybrid binding protein(s) or its domain. The method can be applied complementarily to any specificity engineering technique of such proteins. Sequence-specific DNA-RNA hybrid binding proteins can be used in the diagnosis of certain RNA viruses (e.g. oncogenic viruses, retroviruses, hepatitis B, influenza), which replicate by the transition to the creation of a DNA strand RNA template. Such domains can also be applied in protein engineering to obtain enzymes with new specificity such as DNA-RNA binding protein fusions with enzymatic domains, such as nuclease, RNA modification or DNA modification enzymes and others.

BACKGROUND ART

Cleavage of nucleic acids in a specific location is frequently used in many genetic engineering techniques. There are many methods to fragment DNA molecules, including the widely used commercially available restriction enzymes. Not many RNA processing enzymes are known and most of them are characterized by a low sequential specificity or its total absence.

The WO 2010076939 A1 describes the compositions and methods for carrying out targeted genetic recombination or mutation using the chimeric zinc finger nuclease. The WO 03087341 A2 describes the use of a zinc finger nuclease for the targeted editing of the human cystic fibrosis transmembrane conductance regulator gene, thereby providing a potential therapy for cystic fibrosis. The WO 2009146179 A1 describes the development of a highly efficient and easy-to-practice modular-assembly method using publicly available zinc fingers to make zinc finger nucleases that are able to modify the DNA sequences of several genomic sites in human cells. None of WO 2010076939 A1, WO 03087341 A2 or WO 2009146179 A1 describes or suggests the use of the fusion of RNase HI or part thereof with a zinc finger, particular with ZfQQR nor in particular they disclose or suggest possibility for the targeted cleavage of DNA-RNA hybrids or any type of targeted cleavage of DNA-RNA hybrids by any protein. From descriptions WO2007014181A2 and WO2007014182A2 there are known fusions of many zinc finger domains and FokI nucleases that facilitate targeted genome editing. However, unlike in the former case, the fusion of native proteins alone, RNase HI and the zinc finger, does not lead to a sequence-specific enzyme.

The method for determination of the sequence preference of DNA-RNA hybrid binding protein(s) or its domain(s) and by this determining the sequence recognized by a DNA-RNA hybrid binding protein is a modification of the SELEX procedure. SELEX stands for systematic evolution of ligands by exponential enrichment. The principle of this method is based on iterative selection and enrichment of molecules from a large diverged library of nucleic acids sequences that exhibit a high affinity towards a ligand. The enrichment step is accomplished by binding of the nucleic acids to a ligand and removal of the unbound sequences. This method was so far used for obtaining RNA and DNA aptamers that bind ligands with high specificity (Ellington, A.D., Szostak, J.W., 1990. Nature 346, 818–822; Huizenga DE, Szostak JW. 1995. Biochemistry 34(2):656-65"), for determining a sequence preference of a DNA or RNA binding protein (Blackwell TK & Weintraub H. 1990. Science 250:1104-1110), but was never used for proteins that bind to DNA-RNA hybrids. The described oligonucleotide libraries used in known modifications of SELEX consisted of either single stranded oligonucleotides (RNA, ssDNA,

modified RNA or modified ssDNA, PNA), or double stranded DNA (dsDNA), but none characterize the use of DNA-RNA hybrid library.

The WO 2010076939 A1 describes the compositions and methods for carrying out the targeted genetic recombination or mutation using the chimeric zinc finger nuclease. The WO 03087341 A2 describes the use of a zinc finger nuclease for the targeted editing of the human cystic fibrosis transmembrane conductance regulator gene, thereby providing a potential therapy for cystic fibrosis. The WO2009146179 A1 describes the development of a highly efficient and easy-to-practice modular-assembly method using publicly available zinc fingers to make zinc finger nucleases that are able to modify the DNA sequences of several genomic sites in human cells. None of the aforementioned publications describe the use of SELEX method for determining the substrate preference of DNA-RNA hybrid binding proteins or use of zinc finger for binding of specific sequences in DNA-RNA hybrids or use of ZfQQR in particular for that purpose.

Herskovitz M.A. et al., Mol Microbiol. 2000 Dec:38(5):1027-33, " Endoribonuclease RNase III is essential in Bacillus subtilis." describes growth of a strain in which Bs-RNase III (rncS) expression was dependent upon transcription of rncS from a temperature-sensitive plasmid and at the non-permissive temperature resulted in 90-95% cell death, and virtually all the cells that survived retained the rncS-expressing plasmid. Thus, authors concluded that rncS is essential in *B. subtilis*. Dasgupta S. et al., Mol Microbiol. 1998; 28 (3): 629-40, "Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNase III--the effect of dsRNA binding on gene expression." describes the phenotypes of bacteria carrying point mutations in rnc, the gene encoding RNase III. Karen Shahbabian et al., The EMBO Journal (2009) 28, 3523-3533, " RNase Y, a novel endoribonuclease, initiates riboswitch turnover in *Bacillus subtilis*" describes an essential protein of earlier unknown function, YmdA, identified as a novel endoribonuclease (now called RNase Y) that was capable of preferential cleaving in vitro of the 5′-monophosphorylated yitJ riboswitch upstream of the SAM-binding aptamer domain. None of Herskovitz M.A. et al., Dasgupta S. et al. or Karen Shahbabian et al. mention or suggest the use of SELEX method for determining the substrate preference of DNA-RNA hybrid binding proteins or zinc fingers that bind to DNA-RNA hybrids or any method of obtaining DNA-RNA hybrids.

The US 2006057590 describes the generation of a double stranded RNA molecule that substantially covers the whole transcribed region of a gene and cleaving this molecule using an RNA endonuclease to generate small RNA molecules, which are already or may be subsequently labeled. The JP54059392 patent describes a novel nuclease B-1 which attacks the single chain scission area of one of the DNA chains of a double-stranded deoxyribonucleic acid, and

specifically splits the other DNA chain at its complementary area. The US 2006057590 and JP54059392 are silent of the use of SELEX method for determining the substrate preference of DNA-RNA hybrid binding proteins or use of zinc finger for binding of specific sequences in DNA-RNA hybrids or use of ZfQQR in particular for that purpose.

Therefore, there is a need for a ribonuclease that cleaves the RNA strand of the DNA-RNA hybrids in a specific location. There is also a need for a new improved method of determining the substrate preference of DNA-RNA hybrid binding proteins or use of zinc finger for binding of specific sequences in DNA-RNA hybrids or in particular of use ZfQQR for that purpose.

DISCLOSURE OF INVENTION

In the light of described state-of-the-art, the object of the presented invention is to overcome the indicated disadvantages and to deliver ribonuclease that cleaves the RNA strand of the DNA-RNA hybrids in a specific location. Therefore the object of the invention is to provide engineered enzyme based on combination of catalytic domain of RNase HI and the zinc finger domain that recognizes the sequence in DNA-RNA hybrids and to obtain a sequence-specific enzyme that cleaves only the RNA strand of the DNA-RNA hybrids.

The next object of the invention is to provide a new, improved method for obtaining a library of DNA-RNA hybrids with a random sequence and its use for determining the sequence preference of DNA-RNA hybrid binding protein, preferably sequence preference of DNA-RNA hybrid for binding by zinc finger, more preferably by ZfQQR. Such a method can be effectively used for screening DNA-RNA hybrid library. The next object of the invention is to provide a new and improved method of obtaining a library of DNA-RNA hybrids.

The inventors have unexpectedly found out that a sequence-specific engineered ribonuclease H that cleaves the RNA strand of the DNA-RNA hybrids in a specific location could be obtained by fusing a catalytic domain of RNase HI or a derivative thereof with a zinc finger DNA- RNA hybrid binding domain, in which the zinc finger binding domain has the ability to bind to specific sequences in the DNA-RNA hybrid.

In the first aspect, the invention provides a ribonuclease which cleaves the RNA strand in DNA-RNA hybrids, wherein the ribonuclease is a fusion protein comprising catalytic domain of RNase HI or a derivative thereof with a zinc finger DNA- RNA hybrid binding domain, and wherein the zinc finger binding domain has the ability to bind to specific sequences in the DNA-RNA hybrid. The preferred ribonuclease is a derivative of the catalytic domain of RNase HI

comprising a deletion of the RNase HI hybrid binding domain, preferably the catalytic domain of RNase HI is from *Bacillus halodurans*, more preferably comprises polypeptide encoded by nucleotides 175 to 588 of *rnhA* gene shown in SEQ ID No:1. In the ribonuclease, the preferred catalytic domain of RNase HI comprises at least one substitution of one amino acid in the substrate binding region selected from: K81A, K89E and K123A, and preferably contains all substitutions K81A, K89E and K123A.

In the preferred ribonuclease, the zinc finger domain is a derivative of the zinc finger ZfQQR, preferably a polypeptide encoded by nucleotides from 19 to 303 of the sequence ZfQQR shown in SEQ ID No.2. The ribonuclease preferably comprises fusion protein catAEA-ZfQQR as shown in SEQ ID No.4. The ribonuclease preferably comprises fusion protein GQ as shown in SEQ ID No.6. The ribonuclease also preferably comprises fusion protein GGKKQ as shown in SEQ ID No.8.

In the next aspect, the invention provides the composition comprising a ribonuclease according to the invention.

The invention also concerns the use of the ribonuclease according to the invention or a composition according to the invention for the cleavage of the RNA strand in DNA-RNA hybrids. In such use, the preferred cleavage of the RNA strand in DNA-RNA hybrids is located 2-16 nucleotides, preferably 5-7 nucleotides, away from the binding site for the zinc finger.

The invention also provides the method of obtaining engineered RNase HI, which cleaves RNA strand in DNA-RNA hybrids comprising the following steps:

a) obtaining a RNase HI catalytic domain, that does not bind the substarate but retains the catalytic activity, preferebly by removal of binding domains and/or substitution of amino acids involved in substrate binding;

b) obtaining an engineered RNase HI by making fusion protein comprising the RNase HI catalytic domain obtained in step a) with binding domain that has the ability to bind to specific sequences in the DNA-RNA hybrid, preferebly with zinc finger DNA- RNA hybrid binding domain. In preferred method of obtaining an engineered RNase HI, the zinc finger domain is a derivative of zinc finger ZfQQR, preferably a polypeptide encoded by nucleotides from 19 to 303 of the sequence ZfQQR shown in SEQ ID No.2. In such method the catalytic domain of RNase HI is preferably from *B. halodurans*, preferably comprising polypeptide encoded by nucleotides 175 to 588 of *rnhA* gene shown in SEQ ID No:1. The catalytic domain of RNase HI preferably comprises changes in the substrate binding region, preferably selected from substitution, deletion and/or insretion of at least on amino acid.

The ribonuclease that cleaves the RNA strand of the DNA-RNA hybrids, of the invention contains a fusion of RNase HI or derivative thereof and a DNA-RNA hybrid binding zinc finger, where RNase HI is from *B. halodurans* and the zinc finger has the ability to bind to specific sequences in the hybrid DNA-RNA. Preferably the ribonuclease, according to the invention, is characterized by being a derivative of RNase HI from *B. halodurans*, the catalytic domain polypeptide, even more preferably comprising a polypeptide encoded by nucleotides 175 to 588 of *rnhA* gene shown in SEQ ID NO: 1. Equally preferably, the ribonuclease according to the invention is characterized by being a derivative of the native RNase HI, which contains a deletion of the DNA-RNA hybrid binding domain. Preferably the ribonuclease, according to the invention, is characterized by being a catalytic domain derivative RNase HI, which contains a substitution of amino acid in the substrate binding region: K81A, K89E and K123A. Most preferably the ribonuclease according to the invention is characterized by being a fusion of the polypeptide encoded by nucleotides 175 to 588 of *rnhA* gene shown in SEQ ID No. 1 and the zinc finger domain being a derivative of zinc finger ZfQQR, preferably a polypeptide encoded by nucleotides from 19 to 303 zinc finger ZfQQR shown in SEQ ID No. 2.

Sequence-specific ribonuclease H can be used in a specific and localized fragmentation of nucleic acids for RNA mass spectrometry, including studies on modification of RNA. The invention can be use to generate fragments of RNA for third generation sequencing. Sequence-specific engineered RNase H can be used to detect or map viral RNA with specific sequences, where the single-stranded RNA is annealed with DNA, and the resulting hybrid is cleaved. The invention can be used for the engineering of proteins by shuffling fragments and ligation of mRNA, where fragments are obtained by digestion with specific engineered ribonuclease H. The invention can be used to direct site-specific cleavage of persistent DNA-RNA hybrids in vivo.

Enzymes with new features can be obtained by constructing fusions of several domains with different functionality. Engineering of a ribonuclease that cleaves the RNA strand of DNA-RNA hybrid in a sequence-dependent manner is based on the fusion of two proteins domains: engineered RNase HI and a DNA-RNA hybrid binding zinc finger. RNase HI from *B. halodurans* is an enzyme that hydrolyzes the RNA strand in a DNA-RNA hybrid in a sequence-independent manner. The zinc finger ZfQQR has the ability to bind to a well-defined sequence in the DNA-RNA hybrid. In one of the embodiments of the fusion enzyme, the domain which exhibits a ribonuclease activity against the RNA strand in a DNA-RNA hybrid is a fragment of a gene from *B. halodurans rnhA* encoding the catalytic domain (termed **cat**). It is a fragment from 175 to 588 nucleotide *rnhA* gene, which corresponds to region from 59 to 196 amino acid residue of the native protein RNase HI from *B. halodurans*. From the native gene a fragment encoding the hybrid binding domain (HBD) was removed, because of its ability to bind DNA-RNA

hybrids independently of the sequence. The fusion of the catalytic domain with ZfQQR was termed **cat-ZfQQR**. The engineered catalytic domain has three amino acid substitutions introduced in the substrate binding region: K81A, K89E and K123A. The substitutions involve the positively charged lysines, which are localized close to the known substrate binding region. Substitutions in the binding site are not intended to inactivate the enzyme, but to cause that the enzyme's substrate binding is dependent upon the presence of the additional DNA-RNA hybrid binding domain. The domain, which confers the sequence specificity of the fusion enzyme, is the zinc finger ZfQQR. In the fusion enzyme, the gene fragment encoding a zinc finger ZfQQR from 19 to 303 nucleotide was used, which corresponds to a region from 7 to 101 amino acid residue of the protein. Additionally, the interdomain linker region of the fusion enzyme was modified to produce two variants, termed **GQ** and **GGKKQ**. GQ is the fusion of the catalytic domain with substitutions in K81A, K89E and K123A with ZfQQR that lacks a fragment encoding amino acids in positions 138-148 of the fusion enzyme. GGKKQ is the fusion of the catalytic domain with substitutions in K81A, K89E and K123A with ZfQQR that lacks a fragment encoding amino acids in positions 138-139 and 141-146 of the fusion enzyme. The descriptions of generated constructs are summarized in Table 1.

Table 1. Description of constructs, their abbreviation used further and in the examples and their references to SEQ ID NOs.

Description	Abbreviation	References to SEQ ID NOs
catalytic domain of RNase HI from *B. halodurans*	**cat**	fragment coded by nucleotides 175 to 588 of SEQ ID No:1
the fusion of catalytic domain with ZfQQR	**cat-ZfQQR**	fragment coded by nucleotides 175 to 588 of SEQ ID No:1 and nucleotide 19 to 303 of SEQ ID No:2
the fusion of the catalytic domain with substitutions in K81A, K89E and K123A with ZfQQR	**catAEA-ZfQQR**	construct coded by SEQ ID No: 3 (nucleotide sequence) and shown SEQ ID No: 4 (amino acid sequence)
the fusion of catalytic domain with substitutions introducing changes in the amino acid sequence of protein in positions K81A, K89E and K123A with zinc finger ZfQQR and the interdomain linker shortened by a fragment encoding amino acids in positions 138-148	**GQ**	construct coded by SEQ ID No: 5 (nucleotide sequence and shown SEQ ID No: 6 (amino acid sequence)
the fusion of catalytic domain with substitutions introducing changes in the amino acid sequence of protein in positions K81A, K89E and K123A with	**GGKKQ**	construct coded by SEQ ID No: 7 (nucleotide sequence) and shown SEQ ID No: 8 (amino acid sequence)

zinc finger ZfQQR and the inter domain linker shortened by a fragment encoding amino acids in positions 138-139 and 141-146		

In the next aspect the invention also provides the method of determining the substrate preference of DNA-RNA hybrid binding protein(s) or its domain(s). Such a method allows to determine the preferred sequence recognized by a protein(s) or its domain(s) and/or bound by a protein domain in the DNA-RNA hybrid.

The invention provides method of determining the sequence preference of DNA-RNA hybrid binding protein(s) or its domain(s), in which the method comprises the following steps:
a) contacting of the purified protein or its domain, with a mixture of a library of DNA-RNA hybrids substrates, wherein DNA-RNA hybrids substrates comprises randomized sequences in the central part, preferably randomized 9 or 10 nucleotide positions, with flanking sequences fixed and allowing a tested protein or its domain to bind with the sequence to which it has an affinity;
b) separating unbound DNA-RNA hybrids by immobilization of protein or its domain, with bound DNA-RNA hybrid, to the resin, preferably glutathione agarose;
c) removing the unbound hybrids;
d) isolating the recombinant protein, together with the associated DNA-RNA hybrids, preferably by adding a buffer containing glutathione;
e) amplification of isolated hybrid using PCR, preferably RT-PCR, wherein in the amplification reaction the primers complementary to known sequences on the flanking sequences of the randomized region are used, and wherein during the PCR reaction on one of the primer the sequence of RNA polymerase promoter is added in order to obtain double-stranded DNA for in vitro transcription with RNA polymerase;
f) reverse transcription is performed using reverse transcriptase that does not possess the RNase H activity and a DNA primer complimentary to 3′ end of the RNA template in order to obtain a DNA - RNA hybrid, and steps a) to f) are preferably repeated.
In preferred embodiment of the method promoter sequence comprises the promoter sequence for T7 RNA polymerase and RNA polymerase is T7 RNA polymerase. Preferably, the protein or its domain is a recombinant protein or recombinant domain. Protein or its domain preferably comprises zinc finger(s). In preferred embodiment the protein or its domain is the fusion of the zinc finger domain and GST, preferably the fusion of the zinc finger domain and GST encoded by SEQ ID No:37. The preferred protein or its domain is the fusion of RNase HI or a derivative thereof, preferably RNase HI is from *B. halodurans*, preferably comprising polypeptide encoded by nucleotides 175 to 588 of *rnhA* gene shown in SEQ ID No:1, and a zinc finger. The zinc

finger has preferably the ability to bind to specific sequence in the DNA-RNA hybrid, the prefer zinc finger is ZfQQR, preferably of sequence encoded by nucleotides from 19 to 303 of the sequence ZfQQR shown in SEQ ID No.2.

As further aspect the invention provides method of obtaining library of DNA-RNA hybrids which comprises the flowing steps:

a) PCR amplification of the library of DNA oligonucleotides containing a degenerate sequence in the central position, flanked by invariant sequences, which include a promoter sequence for RNA polymerase,

b) synthesis of RNA strand using RNA polymerase on the obtained in a) double stranded DNA as a template,

c) reverse transcription of the primer complementary to the invariant sequence present at the 3' end of RNA obtained in b) with a reverse transcriptase that does not possess a RNase H activity, wherein during reverse transcription the RNA strand is not degraded, and the hybrid consisting of a fully complementary RNA and DNA strands are obtained. Preferably, in such method the oligonucleotides contain a degenerate sequence in the central position comprising randomized 9 or 10 nucleotide positions. Even more preferably the promoter sequence comprises the promoter sequence for T7 RNA polymerase and RNA polymerase is T7 RNA polymerase.

To determine the full range of sequences recognized by a sequence-specific DNA-RNA hybrid binding protein and the substrate preference of DNA-RNA hybrid binding protein(s) or its domain(s), in the DNA-RNA hybrids, as described in the invention, a modification of the SELEX method was developed, which enables iterative cycles of selection of a DNA-RNA hybrid sequence preferentially bound by a protein (Figure 10). A new element in this process is the way of amplification and recreation of a library of DNA-RNA hybrids, in order to be used in the next cycle. It involves using PCR amplification of a library of DNA oligonucleotides containing a degenerate sequence in the central position, flanked by invariant sequences, which include a promoter sequence for RNA polymerase, preferably T7 RNA polymerase. The resulting double stranded DNA is used as a template for the synthesis of RNA strand using RNA polymerase, preferably T7 RNA polymerase. A pool of single-stranded RNA is obtained and serves as a template for reverse transcription in order to obtain a library of DNA-RNA hybrids. Reverse transcriptase extends the primer complementary to the invariant sequence present at the 3' end of RNA. During reverse transcription by an enzyme with removed ribonuclease H activity (the reverse transcriptase that does not possess ribonuclease H activity), the RNA strand is not degraded, allowing for the creation of a hybrid consisting of a fully complementary RNA and DNA strands. A library of DNA-RNA hybrids is obtained in this way, because the template

RNA was heterogeneous in the central part of the sequence. The effectiveness of the developed modification of SELEX method was confirmed using zinc finger ZfQQR as ligand, which binds DNA-RNA hybrid. ZFQQR was obtained as a result of engineering and binds the 5 'GGGGAAGAA-3' sequence in the DNA strand of DNA-RNA hybrids (Shi and Berg, Specific DNA-RNA hybrid binding by zinc finger proteins. 1995. Science, vol. 268) For this example, the ZFQQR fusion with Glutathione S-transferase domain (called GST) was used.

All publications and references cited in the description and their references are entirely incorporated herein by reference.

BRIEF DESCRIPTION OF DRAWINGS

To understand the invention better, several the example of embodiments of the invention are shown in figures, in which

Figure 1 shows the sequence of the primers of SEQ ID No:11-25 used in the preparation of the final DNA construct of a gene fusion *rnhA* gene with zinc finger ZfQQR and the preparation of substitutions in the RNase HI from *B. halodurans* gene sequence.

Figure 2 is a sequence of DNA-RNA hybrid shown on SEQ ID No:9 (Fig. 2 (A) - RNA strand) and SEQ ID No:10 (Fig. 2 (B) -DNA strand) containing the binding site for zinc finger ZfQQR, which was used in digestion assays as specific substrate.

Figure 3 shows the digestion products resulting from the cutting of the substrate DNA-RNA hybrid that contains the binding site for the zinc finger, and the dependence of the cleavage on the presence of magnesium and zinc ions. Digestion reaction shown in Lanes 1-14 were performed with 0.05 μM of the radioactively labeled substrate, 0.5 μM of non-labeled substrate, 25 mM Tris (pH 8.0), 100 mM KCl, 2 mM DTT, 30 min at 37 °C with presence or not of different enzyme and presence of 5 mM $MgCl_2$ and/or 20 μM $ZnSO_4$ as indicated below. Lane 1: uncleaved substrate, Lane 2: 12.5 nM of RNase HI from *B. halodurans*, 5 mM $MgCl_2$, 20 μM $ZnSO_4$, Lane 3: 625 nM of **cat**, 5 mM $MgCl_2$, 20 μM $ZnSO_4$, Lane 4: 5 nM **cat-ZfQQR**, 5 mM $MgCl_2$, 20 μM $ZnSO_4$, Lane 5: 25 nM **catAEA-ZfQQR**, 5 mM $MgCl_2$, 20 μM $ZnSO_4$, Lane 6: 12.5 nM of RNase HI from *B. halodurans*, 5 mM $MgCl_2$, Lane 7: 625 nM of **cat,** 5 mM $MgCl_2$, Lane 8: 5 nM **cat-ZfQQR**, 5 mM $MgCl_2$, Lane 9: 25 nM of **catAEA-ZfQQR**, 5 mM $MgCl_2$, Lane 10: 12.5 nM of RNase HI from *B. halodurans*, 20 μM $ZnSO_4$, Lane 11: 625 nM of **cat**, 20 μM $ZnSO_4$, Lane 12: 5 nM of **cat-ZfQQR**, 20 μM $ZnSO_4$, Lane 13: 25 nM of **catAEA-ZfQQR**, 20 μM $ZnSO_4$, Lane 14: Marker size 10-100 nucleotides single-stranded RNA

radioactively labeled isotope phosphorus 33 (USB) where an asterisk (*) marked a unique cleavage site.

Figure 4 shows the digestion products resulting from the cutting of the substrate DNA-RNA hybrid, which contains the binding site for the zinc finger, and the dependence of the cleavage by the variant GQ of the catAEA- ZfQQR on the presence of magnesium and zinc ions. Digestion reaction shown in Lanes 1-14 were performed with 50 nM of **GQ** variant, 0.05 μM of the radioactively labeled substrate, 0.5 μM of non-labeled substrate, 25 mM Tris (pH 8.0), 20 μM $ZnSO_4$, 100 mM KCl, 2 mM DTT, 30 min at 37 °C with various concentrations of $MgCl_2$ as indicated below. Lane 1: uncleaved substrate, Lane 2: 0.05 mM $MgCl_2$, Lane 3: 0.1 mM $MgCl_2$, Lane 4: 0.2 mM $MgCl_2$, Lane 5: 0.5 mM $MgCl_2$, Lane 6: 1 mM $MgCl_2$, Lane 7: 2 mM $MgCl_2$, Lane 8: 5 mM $MgCl_2$, Lane 9: 10 mM $MgCl_2$, Lane 10: Marker size 10-100 nucleotides single-stranded RNA radioactively labeled isotope phosphorus 33 (USB).

Figure 5 shows the digestion products resulting from the cutting of the substrate DNA-RNA hybrid, which contains the binding site for the zinc finger, and the dependence of the cleavage by the variant GGKKQ of the catAEA-ZfQQR on the presence of magnesium and zinc ions. Digestion reaction shown in Lanes 1-14 were performed with 50 nM GGKKQ variant, 0.05 μM of the radioactively labeled substrate, 0.5 μM of non-labeled substrate, 25 mM Tris (pH 8.0), 20 μM $ZnSO_4$, 100 mM KCl, 2 mM DTT, 30 min at 37 °C with various concentrations of $MgCl_2$ as indicated below: Lane 1: uncleaved substrate, Lane 2: 0.05 mM $MgCl_2$, Lane 3: 0.1 mM $MgCl_2$, Lane 4: 0.2 mM $MgCl_2$, Lane 5: 0.5 mM $MgCl_2$, Lane 6: 1 mM $MgCl_2$, Lane 7: 2 mM $MgCl_2$, Lane 8: 5 mM $MgCl_2$, Lane 9: 10 mM $MgCl_2$, Lane 10: Marker size 10-100 nucleotides single-stranded RNA radioactively labeled isotope phosphorus 33 (USB).

Figure 6 shows the mapping of cleavage site generated by **catAEA-ZfQQR**, the **GQ** variant and the **GGKKQ** variant on the substrate strand RNA of the DNA-RNA hybrid containing a binding site for zinc finger ZfQQR. Digestion reaction shown in Lanes 1-14 were performed with 0.05 μM of the radioactively labeled substrate, 0.5 μM of non-labeled substrate, 25 mM Tris (pH 8.0), 100 mM KCl, 2 mM DTT, 30 min at 37 °C with presence or not of different enzyme and presence of appropriate concentration of $MgCl_2$ and/or 20 μM $ZnSO_4$ as indicated below. Lane 1: uncleaved substrate, Lane 2: 35 nM of **catAEA- ZFQQR**, 20 μM $ZnSO_4$, Lane 3: 50 nM of **GQ** variant, 1mM $MgCl_2$, 20 μM $ZnSO_4$ Lane 4: 50 nM of **GGKKQ** variant, 2 mM $MgCl_2$, 20 μM $ZnSO_4$, Lane 5: ribonuclease T1 cleavage of ssRNA, Lane 6: The alkaline hydrolysis of ssRNA substrate.

Figure 7 illustrates cleavage positions of the substrate DNA-RNA hybrids containing the binding site for zinc finger ZfQQR by the **catAEA-ZfQQR**, indicated by arrows above the sequence, the larger black arrow indicates the major cleavage site, the smaller arrows additional sites (top strand RNA, the bottom strand DNA, the binding site for ZfQQR marked with box).

Figure 8 illustrates cleavage positions of the substrate DNA-RNA hybrids containing the binding site for zinc finger ZfQQR by the **GQ** variant of the catAEA-ZfQQR, indicated by arrows above the sequence, the larger black arrow indicates the major cleavage site, the smaller arrows additional sites (top strand RNA, the bottom strand DNA, the binding site for ZfQQR marked with box).

Figure 9 illustrates cleavage positions of the substrate DNA-RNA hybrids containing the binding site for zinc finger ZfQQR by the **GGKKQ** variant of the catAEA-ZfQQR, indicated by arrows above the sequence, the larger black arrow indicates the major cleavage site, the smaller arrows additional sites (top strand RNA, the bottom strand DNA, the binding site for ZfQQR marked with box).

Figure 10 shows a diagram representing the different stages of one round of SELEX procedure modified according to the invention.

Figure 11 shows the sequences of DNA oligonucleotide templates for obtaining single-stranded RNA in the process of in vitro transcription using bacteriophage T7 RNA polymerase. The template A (SEQ ID No:26) contains a binding site for zinc finger ZfQQR. The template B (SEQ ID No:27), does not contain binding site for zinc finger ZfQQR and has a unique cleavage site for restriction enzyme XhoI. The template 9N (SEQ ID No:28), contains nine nucleotide degenerate region (single degenerate nucleotide marked "N").

Figure 12 illustrates the process of obtaining a DNA-RNA hybrid. DNA fragments were resolved in 15% denaturing polyacrylamide gel containing 6 M urea. The 5' ends of 55 nucleotides long DNA oligonucleotide and primer 2 were labeled with radioactive isotope phosphorus 33. Line 1: 0.5 pmol of labeled primer 2 Line 2: 0.25 pmol of reverse transcription product, Line 3: 0.25 pmol of 55 nt labeled DNA oligonucleotide.

Figure 13 illustrates the process of obtaining a DNA-RNA hybrid. DNA fragments were resolved in 15% native polyacrylamide gel. The 5' ends of 55 nucleotides long DNA oligonucleotide and primer 2 were labeled with radioactive isotope phosphorus 33. Line 1: 0.5 pmol of labeled primer 2, Line 2: 0.25 pmol of reverse transcription product, Line 3: 0.25 pmol

of reverse transcription products digested 5 units of ribonuclease H, Line 4: 0.25 pmol of reverse transcription products digested a unit of ribonuclease H, Line 5: 0.25 pmol of 55 nt labeled DNA oligonucleotide.

Figure 14 shows a bar graph illustrating the change in the ratio between double-stranded DNA containing a binding site for zinc finger ZfQQR (A) and double-stranded DNA which doesn't contain a binding site for ZfQQR (B) in successive rounds of the SELEX procedure ("O" means the input, starting mixture of hybrid A with B in the ratio 1:10000, I-V are numbers of successive rounds).

Figure 15 illustrates a sequence logo derived from 40 sequences using WebLogo.

Figure 16 shows the sequences of the DNA-RNA hybrids used to determine the K_D constant for the zinc finger ZfQQR, where Figure 16A is a DNA-RNA hybrid which contains the binding site described in the literature and Figure 16B is a DNA-RNA hybrid with the sequence consensus after five rounds of SELEX.

Figure 17 shows a graph that represents the change of amount of radioactively labeled substrate retained on a nitrocellulose filter, depending on the concentration of zinc finger ZfQQR in the reaction mixture (expressed as percent bound).

DESCRIPTION OF EMBODIMENTS

The examples below are presented only to illustrate the invention and to explain certain aspect of it and not to limit the invention, therefore they should not be identified with its entire scope, which is defined in the enclosed claims.

In the following examples, unless otherwise indicated standard materials and methods are used as described in Sambrook J. et al., "Molecular Cloning: A Laboratory Manual, 2nd edition. 1989. Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory Press, or proceeding in accordance with manufacturers' recommendations for specific materials and methods. As used herein, unless otherwise indicated by standard abbreviations for amino acids and nucleotides or ribonucleotides.

Example 1 Cloning of the *rnhA* gene fragment from *Bacillus halodurans* into the expression vector pET 30b

The vector pET15a carrying the *rnhA* gene (SEQ ID NO:1) (SEQ ID NO:1 is DNA sequence of *rnhA* RNase HI (Gene ID 893801, BH0863) from *B. halodurans* and was obtained from private sources. A fragment of the gene encoding **cat** (catalytic domain) was amplified by using PCR technique in standard conditions performed on the pET15a carrying the *rnhA* gene as the template and 1 U of Phusion polymerase (New England Biolabs) with 50 pmol of each primer Bhcatr (SEQ ID No:12) and Bhcatf (SEQ ID No:11) (see Figure 1) DNA encoding the *rnhA* gene fragment corresponding to 175 – 588 nucleotide and vector DNA pET 30b (Novagen company) was digested with NdeI and KpnI (Fermentas). The reaction mixture containing the DNA was separated on 0.7% agarose gel in TAE buffer and the fragments corresponding to the expected sizes 430 bp and 5313 bp, respectively, were reisolated from the gel using a kit for reisolation (Gel out, A & A Biotechnology). Then, 50 ng reisolated *rnhA* gene fragment and 25 ng of cut vector pET 30b was treated with bacteriophage T4 DNA ligase (Fermentas) and ligase was heat inactivated. Then the ligation mixture was used for bacterial cell transformation. E. coli competent bacteria (Top10 (Invitrogen)), have been transformed with ligation mixture (20 - 200 ng per 50 µl of bacterial cells). The transformants were selected on on LB-agar plates supplemented with 30 µg/ml kanamycin. The selection of transformants containing the desired recombinants was based on analysis of restriction maps, and then the samples were sequenced to confirm the sequence of constructs.

Example 2 Cloning of the gene encoding a zinc finger ZfQQR to the expression vector pET28b

Synthesis of the gene encoding the zinc finger ZfQQR was ordered from Epoch's Life Sciences (see SEQ ID No:2 which is the sequence of the synthesized DNA of a zinc finger ZFQQR gene) on the basis of the amino acid sequence from the article "Shi Y, Berg JM. Specific DNA-RNA hybrid binding proteins. Science. 1995. vol. 268, 282-284). DNA encoding ZfQQR and DNA vector pET28b (Novagen company) was digested with restriction enzymes NcoI and XhoI (enzymes were from Fermentas Company, reactions were carried out in a buffer 2X Yellow according to the manufacturer's instructions, 1 unit of enzyme per 1 mg DNA for 1 h in 37 ° C). The reaction mixture was separated on 0.7% agarose gel in TAE buffer and treated as in Example 1. Then, 50 ng of the reisolated ZfQQR gene and 25 ng of the digested vector pET28b were ligated by T4 DNA ligase (Fermentas, the reaction carried out in buffer supplied by the manufacturer) at room temperature for 1 hour. Ligase was heat inactivated by incubation at 75 ° C for 10 min. Then the ligation mixture was used to transform bacterial cells as in Example 1.

Example 3 Cloning of the gene encoding the zinc finger ZfQQR to the expression vector pET 30b containing a fragment of a gene from *Bacillus halodurans rnhA*

A fragment of the gene encoding ZfQQR from the vector pET28b was amplified by the PCR technique. The reaction was carried out in standard conditions performed on the pET28b carrying the ZfQQR gene as the template and 1 U of Phusion polymerase (New England Biolabs) with 50 pmol of each primer BhZf (SEQ ID No:13) and Kmr (SEQ ID No:15) (see Figure 1). A fragment of the gene encoding the catalytic domain of RNase HI in vector pET 30b was amplified by the PCR technique. The reaction was carried out in standard conditions performed on the pET 30b carrying the cat gene as the template and 1 U of Phusion polymerase (New England Biolabs) with 50 pmol of each primer Kmf (SEQ ID No:14) and Bhcatr (SEQ ID No:12) (see Figure 1). The purified PCR products were phosphorylated with bacteriophage T4 polynucleotide kinase (Fermentas). DNA from both reactions (20 ng) were combined, ligated and ligation mixtures were transformed into bacterial cells as in Example 1.

Example 4 Mutagenesis of the gene encoding the fusion enzyme that introduces amino acid substitutions at positions K81A, K89E and K123A

The ligation of the fragments generated in Example 3 created a construct where the open reading frame contained in the beginning a part of the gene encoding cat and the gene fragment encoding ZfQQR at the end. The obtained DNA construct served as a template to introduce substitutions using PCR technique in the nucleotides encoding amino acid residues at positions 81, 89 and 123 of the protein. Substitutions were introduced in stages, first were substituted nucleotides encoding residues 81 (lysine to alanine substitution) and 89 (substitution of lysine for glutamic acid), only after obtaining such a construct, PCR was carried out to convert the nucleotides encoding the residue in position 123 (substitution of lysine to alanine). The PCR reaction was carried out in standard conditions performed on the pET30b carrying the **cat-ZfQQR** gene as the template and 1 U of Phusion polymerase (New England Biolabs) with 50 pmol of each primer K81Af (SEQ ID No:17), K81Ar (SEQ ID No:16), K89Ef (SEQ ID No:19), K89Er (SEQ ID No:18), K123Af (SEQ ID No:21) and K123Ar (SEQ ID No:20) (see Figure1):

The purified PCR products were treated as in Example 1 and ligation mixtures were transformed into bacterial cells. The final sequence of the gene encoding the **catAEA-ZfQQR** is shown in SEQ ID No:3 and the resulting amino acid sequence of the protein sequence is shown in SEQ ID No:4.

Example 5 Mutagenesis of the gene encoding the fusion enzyme with amino acid substitutions at positions K81A, K89E and K123A that shortens the length of the interdomain linker region

The construct generated in Example 4 carries the open reading frame encoding the **catAEA-ZfQQR** at the end and served as a template to shorten the interdomain linker using PCR technique in the region encoding the interdomain linker 409-449 in the gene on SEQ ID No:3. The PCR reaction was carried out in standard conditions performed on the pET30b carrying the **catAEA-ZfQQR** gene as the template and 1 U of Phusion polymerase (New England Biolabs) with 50 pmol of each primer del11f (SEQ ID No:22) and del11r (SEQ ID No:23) (which were used to generate variant named as GQ), or primers del5f (SEQ ID No:24) and del5r (SEQ ID No:25) (which were used to generate variant named as GGKKQ) respectively (see Figure 1 for primers seqences).

The purified PCR products were treated as in Example 1 and ligation mixtures were transformed into bacterial cells. The final sequence of the gene encoding the variant GQ that is the catAEA-ZfQQR with inter domain linker shortened by a fragment encoding amino acids in positions 138-148 shown in SEQ ID No:5 and the resulting amino acid sequence of the protein sequence shown in SEQ ID No:6 . The final sequence of the gene encoding the variant GGKKQ that is the catAEA- ZfQQR with the inter domain linker shortened by a fragment encoding amino acids in positions 138-139 and 141-146 shown in SEQ ID No:7 and the resulting amino acid sequence of the protein sequence shown in SEQ ID No:8.

Example 6 Expression and purification of proteins by ion exchange chromatography

Plasmid pET15a with the gene encoding the full length RNase HI from *B. halodurans*, pET 30b with the gene encoding **cat**, pET 30b with the gene encoding the **catAEA-ZfQQR** and pET 30b with the gene encoding variant **GQ** and pET30 with the gene encoding variant **GGKKQ** were each transformed into E. coli strain ER2566 (New England Biolabs). The protein expression was induced with IPTG and proteins were purified accoring to standard protocol on Ni-NTA resin (Sigma Aldrich).

Example 7 Nuclease activity assay of the fusion enzyme

The effect of the presence of magnesium and zinc ions in reaction on the enzymatic activity and specificity of the full-length RNase HI from *B. halodurans*, **cat**, **cat-ZfQQR** and **catAEA-ZfQQR** was tested. The activity assay included the presence of 5 mM $MgCl_2$ and/or 20 μM $ZnSO_4$. The 5 'end of the RNA strand in the substrate DNA-RNA hybrid was labeled with

radioactive isotope phosphorus 33. Digestion reaction contained 0.05 μM radiolabeled substrate and 0.5 μM unlabeled substrate:

RNA:

AGAACUAGUGGAUCAACCGGGCUGCAGGAAUUCGAUAUCAAGCUUAUCGAUACC GUGGCGGUUCUUCCCCAAGCC (**SEQ ID No:9**)

DNA:

GCTTGGGGAAGAACCGCCACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCC GGTTGATCCACTAGTTCT (**SEQ ID No:9**)

and 25 mM Tris (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$ and/or 20 μM $ZnSO_4$, 2 mM DTT. Reactions contained: 12,5 nM of the full-length RNase HI from *B. halodurans*, 625 nM of cat, 5 nM of the **cat-ZfQQR** or 25 nM of **catAEA-ZfQQR**. Digestion reactions were carried out in 37 °C and stopped after 30 min by adding formamide to a final concentration of 50%. The digestion products were resolved in 12% denaturing polyacrylamide gel containing 6 M urea (see Figure 3). The gel was dried on a vacuum drier and exposed to Storage Phosphor Screens (GE Healthcare) overnight. The audioradiogram was scanned on a Typhoon Trio scanner (GE Healthcare).

In the presence of 20 μM $ZnSO_4$, and in the absence of $MgCl_2$ the substrate digestion by the **catAEA-ZfQQR** generates products of unique size. Cleavage in this place does not occur when such a substrate is digested by the full-length RNase HI from *B. halodurans*, **cat** and **cat-ZfQQR**.

The effect of linker length reduction on the specificity of the substrate cleavage by variants **GQ** and **GGKKQ** was determined. The conditions assayed included a range of magnesium ion concentration in the presence of zinc in the reaction. The activity assay included the presence of a range of 0.05 mM to 10 mM $MgCl_2$ and 20 μM $ZnSO_4$ and buffer composition as above. The 5 'end of the RNA strand in the substrate DNA-RNA hybrid was labeled with radioactive isotope phosphorus 33. Digestion reaction contained 0.05 μM radiolabeled substrate and 0.5 μM unlabeled substrate (Figure 2), 25 mM Tris (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$ and 20 μM $ZnSO_4$, 2 mM DTT. In the reaction, 50 nM of variant GQ and 50 nM of variant GGKKQ was used. Digestion reactions were carried out as above and result are shown on Figure 4 for GQ and Figure 5 for GGKKQ.For the **GQ** variant of the linker, the optimal concentration of Mg^{2+} in the reaction was 1 mM and for the **GGKKQ** variant it was 2 mM.

In order to accurately determine the cleavage site in the DNA-RNA hybrid substrate that contains a binding site for zinc finger ZfQQR generated by the **catAEA-ZfQQR** and its **GQ** and **GGKKQ** variants, the cleavage products were separated in a polyacrylamide gel with a

resolution allowing the separation of fragments differing by one nucleotide. The 5 'end of the RNA strand in the substrate DNA-RNA hybrid was labeled with radioactive isotope phosphorus 33. The digestion reaction contained 0.05 μM radiolabeled substrate and 0.5 μM unlabeled substrate (see Figure 2) , 25 mM Tris (pH 8.0), 100 mM KCl, 20 μM $ZnSO_4$, 1 mM $MgCl_2$ (in case of variant GQ) and 2 mM $MgCl_2$ (in case of variant GGKKQ) and 2 mM DTT. The reaction contained 0.1 μM catAEA-ZfQQR, 50 nM of variant GQ and 50 nM of variant GGKKQ. Digestion reactions were carried out at 37 ° C and stopped after 1 h by adding formamide to a final concentration of 50%. Size marker with fragments differing by one nucleotide was obtained by alkaline hydrolysis according to Ambion's recommendations (www.ambion.com).The digestion products were resolved in 12% denaturing polyacrylamide gel containing 6 M urea (see Figure 6). The resulting fragments of the identified sequence length was mapped on the sequence of the substrate and determined that the main product of cleavage for the **catAEA-ZfQQR** has the length of 54 nucleotides (see Figure 7). It is located on the opposite strand 7 nucleotides away from the binding site for zinc finger ZfQQR. In case of the **GQ** and **GGKKQ**, the main product of cleavage has the length of 56 nucleotides (see Figure 8 for GQ and Figure 9 for GGKKQ) and it is located on the opposite strand 5 nucleotides away from the binding site for zinc finger ZfQQR.

The new ribonuclease of invention cleaves the RNA strand in DNA-RNA hybrids, and is fusion protein which can comprise the catalytic domain of ribonuclease HI (RNase HI) with a zinc finger DNA- RNA hybrid binding domain, wherein the zinc finger binding domain has the ability to bind to specific sequences in the DNA-RNA hybrid. The preferred obtained fusion proteins are **catAEA-ZfQQR, GQ, GGKKQ.**

It was not obvious that a sequence-specific engineered RNase H could be obtained by fusing a RNase HI domain and a zinc finger because the nuclease domain is a processive enzyme that upon binding to the substrate cleaves mulitple times in several places of the substrate. Moreover, as it was described earlier, the fusion of an unchanged RNase H to a zinc finger does not allow to obtain a sequence-specific enzymeas well as the fusion of the **cat** fragment of RNase HI does not allow to obtain a sequence-specific enzyme. Not to be bound by any theory there are few possible reasons for this - the RNase HI is a processive enzyme that will always cleave in multiple times, the zinc finger in fusion with other domains does not bind its binding sequence in DNA-RNA hybrids, the cleavage conditions were not optimal, the cat domain binds the substrate regardless of the presence of the zinc finger or all of them were important.

Moreover, it was not obvious that the substitutions in the substrate binding region: K81A, K89E and K123A of cat would lead to a sequence-specific enzyme. There are several hypothesis

that may provide an explanation for not obtaining a specific: the **cat** domain can bind the substrate regardless of the presence of the zinc domain, or substitutions in the binding region could have affected the catalysis of the enzyme and produced a variant that is inactive, or substitutions of amino acids in proteins can lead to variants that cannot fold properly and thus that are not soluble. There was no data or suggestions available in prior art that would allow to be certain that the destabilization of binding by the cat domain would enable to obtain an enzyme that cleaves in a defined distance from the binding site.

Example 8 Preparation of substrate DNA-RNA hybrids

Three 78 nucleotide long single-stranded DNA were synthesized named: template A (SEQ ID No:26), template B (SEQ ID No:27)and template 9N (SEQ ID No:28), respectively.

These oligonucleotides were used as a template to create double-stranded DNA using the PCR technique. The reaction was carried out in standard contitions, approximately 0.1 pmol template and 1 unit of Phusion polymerase (New England Biolabs) with 10 pmol of each primer 1 and 2:

primer 1: AATTTAATACGACTCACTATAGGGCTCTAGATCTCACTAAGCATAG (SEQ ID No:33)

primer 2: GAGATCTAGACGGAACATG (SEQ ID No:34)

Primers 1 and 2 were used for amplification of double-stranded DNA in the PCR reaction and primer 2 was also used in reverse transcription reaction.

Reaction conditions: initial denaturation of 98°C 1 min, followed by 15 cycles of 15s denaturation at 98°C, 15s, renaturation at 72 °C (a reduction of the temperature of renaturation with each cycle by 1 °C to final temperature of 58°C) and 2 s extension at 72°C, followed by 10 cycles of 15 s denaturation at 98°C , 15s renaturation at 58°C and 2s extension at 72°C . The reaction mixture was extracted with phenol and the collected aqueous phase was precipitated with 0.5 M sodium acetate pH 4.5 and ethanol. The precipitate was air dried and resuspended in water. Single-stranded RNA transcripts were obtained on a double-stranded DNA template with the promoter sequence for bacteriophage T7 RNA polymerase by using a kit for transcription MEGAshortscript ™ T7 (Ambion). The reaction was carried out in standard conditions with 200 -500 ng of DNA. Transcription reaction was resolved in 15% denaturing polyacrylamide gel containing 6 M urea and TBE buffer and visualized using ethidium bromide under UV light. A band corresponding to the length of 55 nucleotides was excised and elution of RNA from the gel was performed according to standard procedures using Costar Spin X columns (Corning Life Sciences).

DNA-RNA hybrid was obtained on a template of single-stranded RNA using reverse transcriptase with removed RNase H activity. 200 pmol of primer 2 ((SEQ ID No:34) and 200 pmol of single-stranded RNA was incubated 2 min at 70 °C, followed by 2 min on ice. The prepared template was subjected to reverse transcription. The reaction was carried out in 40 µl in a buffer supplied by the manufacturer, with a final concentration of 1 mM dNTP mix, 200 pmol of primer annealed template, 40 units RiboLock (Fermentas) and 400 units RevertAid H minus reverse transcriptase (Fermentas) for 2 h at 42 ° C. The DNA-RNA hybrids from the reaction mixture were separated from the buffer and dNTPs by using the G-25 rasin (Sigma Aldrich) in a standard procedure.

In order to confirm that the developed protocol leads to the formation of complimentary DNA-RNA hybrids, reverse transcription was performed (description as above) using a radioactively labeled with isotope phosphorus 33 primer 2 (SEQ ID No:34). The synthesis of the appropriate size of DNA (the expected length is 55 nucleotides) was confirmed by comparing the size of the resulting DNA and a control 55 nucleotides long DNA oligonucleotide. Samples were resolved in 15% denaturing polyacrylamide gel containing 6 M urea in TBE buffer (see Figure 12). The gel was dried on a vacuum drier and exposed to Storage Phosphor Screens (GE Healthcare) overnight. The audioradiogram was scanned on a Typhoon Trio scanner (GE Healthcare). The creation of DNA-RNA hybrids was confirmed by digesting the product of reverse transcription by ribonuclease H and comparing the migration of the product of the reaction with control 55 nucleotides long DNA oligonucleotide in 15% native polyacrylamide gel in TBE buffer (see Figure 13). Digestion of RNA strands of the DNA-RNA hybrids was carried out in 10 µl in the buffer supplied by the manufacturer with1 and 5 units of RNase H (Fermentas) for 30 min at 37 ° C.

<u>Example 9</u> Cloning, expression and purification of GST fusion protein with the zinc finger ZfQQR

In order to obtain the fusion of the zinc finger domain with GST, a gene encoding ZFQQR was cloned into the expression vector pGEX-4T-1 (Amersham). The synthesis of the gene encoding the zinc finger ZfQQR was ordered from Epoch's Life Sciences on the basis of the amino acid sequence from the article "Shi Y, Berg JM. Specific DNA-RNA hybrid binding proteins. Science. 1995. vol. 268, 282-284). DNA encoding ZfQQR was amplified using PCR technique. The primer ZFf and ZFr were synthesized and used for preparation of DNA construct of the fusion of genes encoding the GST domain and zinc finger ZfQQR, The reaction was carried out in standard conditions and 1 unit of Phusion polymerase (New England Biolabs) with 50 pmol of each primer ZFf and ZFr:

ZFf: GGTTCTGGTGACCCGGG (SEQ ID No:35)

ZFr: CGGGAAAACAGCATTCCAGGTATTAG (SEQ ID No:36)

DNA encoding the zinc finger ZfQQR and the vector pGEX-4T-1 DNA was digested with restriction enzymes SmaI and XhoI (Fermentas). SmaI digestion was performed in a buffer 1X Yellow according to the manufacturer recommendations. After this time the buffer was added to a final concentration 2X Yellow in the reaction, 1 unit of enzyme XhoI for 1 mg DNA for 1 h at 37 ° C. The reaction was separated on 0.7% agarose gel in TAE buffer and the fragments corresponding to the expected sizes were reisolated from the gel using a kit for the reisolation from the agarose gels (Gel Out, A & A Biotechnology). Then, 50 ng of the reisolated DNA encoding zinc finger ZfQQR and 25 ng of the cut vector pGEX-4T-1 was ligated with bacteriophage T4 DNA ligase (Fermentas, the reaction carried out in manufacturer supplied buffer) at room temperature for 1 hour. Ligase was heat inactivated by incubating at 75 ° C for 10 min. Then the ligation mixture was used for transformation into a bacterial strain E. coli Top10 F (Invitrogen). The transformants were selected on100 µg/ml ampicillin. The selection of suitable transformants containing the desired recombinants was based on analysis of restriction maps, and then the clones were sequenced to confirm the sequence of constructs.

The plasmid pGEX-4T-1 gene encoding the zinc finger ZfQQR transformed into E. coli strain BL21 (DE3): (Promega). Transformants from overnight culture were inoculated into liquid LB with 100 mg/ml ampcillin, and incubated at 37 °C for 2 h. After this time, IPTG was added (final concentration 1 mM) and the culture was incubated with shaking at 25 °C for additional 5 hours. After induction the cultures were centrifuged at 4000 rpm at 4 °C for 10 min washed with 2 ml of STE, centrifuged 10,000 g 10 min. The resulting pellet from culture was resuspended in 35 ml PBS and then bacterial cells were disintegrated by single passage through the French press (Constant Systems LTD) at 1360 atmospheres. Lysates were clarified by centrifugation in the ultracentrifuge at 20 000 g at 4 °C for 20 min. The protein was purified on Gluthathione Sepharose rasin (GE Healthcare) according to standard procedures.

<u>Example 10</u> Binding of protein to DNA-RNA hybrid, separation of unbound hybrids and elution of the protein-DNA-RNA hybrid complex from the resin

The binding reaction of DNA-RNA hybrid to zinc finger ZfQQR was performed in 40 µl and contained: 200 pmol of DNA-RNA hybrids, 1 pmol of GST fusion with the zinc finger ZfQQR, 25 mM Tris pH 8.0, 100 mM KCl, 20 µM $ZnSO_4$, 2 mM DTT. The reaction was carried out in room temperature for 30 min. After this time the reaction was added to 7.5 µl of Gluthathione

Sepharose resin and incubated for 30 min at Thermomixer compact (Eppendorf) at 22 °C with shaking (1,400 rpm). In the next stage, the resin was washed three times with 100 µl buffer P (25 mM Tris pH 8.0, 100 mM KCl, 20 µM $ZnSO_4$, 2 mM DTT), each time the sample was centrifugation at 1000 g for 2 min at room temperature and the supernatant removed. The next step was elution, which was repeated twice with 30 µl buffer E and 10 min incubation at room temperature. After each incubation, the sample was centrifuged at 1000 g for 2 min at room temperature and the supernatant was transferred to a tube. 5 µl of the eluate containing the GST fusion with the zinc finger associated with the DNA-RNA hybrid was subjected to amplification using PCR in order to obtain double-stranded DNA, then RNA was transcribed and DNA-RNA hybrids were synthesized by reverse transcription as in Example 8. Obtained DNA-RNA hybrid was the starting material for the next round of SELEX.

Example 11 Control SELEX

A control of the SELEX method was performed on a mixture of DNA-RNA hybrids containing the binding site for zinc finger ZfQQR (hybrid A, is formed on a template A SEQ ID No:26) and a hybrid, which does not have such a binding site, instead a XhoI restriction site (B hybrid, is created the template B SEQ ID No:27). Five rounds of SELEX was conducted on a control pool consisting of a mixture of hybrids A and B in the ratio 1:10000. In order to distinguish the two sequences, 100 ng of DNA resulting from PCR amplification of the eluate after each round was digested with 2 units of enzyme XhoI 16 h at 37 °C. Digestion products were separated on 15% native polyacrylamide gel in TBE buffer. The gel was incubated 2 min in a solution of ethidium bromide at a final concentration of 0.5 µg/ml. The bands were visualized under UV light using a CCD camera for digital archiving of images MultiImager Fluoro-S (BioRad). The intensity of bands corresponding to the size of uncleaved DNA (78 bp) and the digestion products (55 and 23 base pairs) was measured using Quantity One (BioRad) and the relative proportions of the DNA obtained on a template of DNA-RNA hybrids after each round was calculated (see Figure 14). After five rounds of selection with the fusion of GST and the zinc finger ZfQQR, the proportions of DNA carrying the binding site for ZfQQR and without such a sequence have changed to 1:1,7. This means that by performing SELEX procedure, the pool of control sequences was enriched 8500 times by a sequence specifically bound by ZfQQR.

Example 12 in the SELEX library RNA-DNA hybrids

In the next stage, the library of DNA-RNA hybrids containing a central random sequence (generated on the template 9N - SEQ ID No:28) was used to conduct the SELEX procedure and

determine the sequence preference of the zinc finger ZfQQR as in Example 11. In order to determine the sequence of selected hybrids 500 ng of DNA PCR product obtained on the template of the eluate after the five rounds of SELEX was digested with 5 units of XbaI enzyme (Fermentas) in buffer supplied by the manufacturer. Digestion products were separated on 15% native polyacrylamide gel in TBE buffer. The band corresponding to the fragment length of 43 base pairs was excised and isolated from the gel according to standard procedures. In order to obtain concatamers, 100 ng of digested DNA fragments were ligated with bacteriophage T4 DNA ligase (Fermentas) in buffer supplied by the manufacturer at room temperature for 3 hours. Ligase was heat inactivated by incubating at 75 ° C for 10 min. 200 ng of DNA plasmid vector pUC18 (Fermentas) was digested with XbaI enzyme in the buffer supplied by the manufacturer for 3 h at 37 °C . The reaction mixture was separated on 0.7% agarose gel in TAE and the fragments corresponding to the sizes expected reisolated from the gel using a kit for reisolation from agarose gels (Gel out, A & A Biotechnology). Such prepared vector and concatamers were ligated and then transformed into a bacterial strain. The selection of suitable transformants containing the desired recombinants was based on an analysis of the colony color and 12 clones were sequenced and found to contain a total of 42 fragments obtained by the SELEX procedure as indicated in the Table 2.

Table 2. The sequences obtained in the SELEX procedure acquired after sequencing the DNA obtained on a template of DNA-RNA hybrid at the end of five rounds of the modified SELEX procedure performed on the zinc finger ZfQQR and a library of sequences containing 9 nucleotide degenerate region.

Number	Sequence of library	Number	Sequence of library
1	TGGGGACGC	22	GTCAGATGT
2	AGTGCTCGA	23	GAGATCAGT
3	GACGCATGG	24	GCCGAGCGG
4	GGTCTGGAG	25	GCTCGGTGA
5	GAGCGGGAA	26	CGTAGGGAA
6	TCGTTGCAT	27	GTCGGATGG
7	CTAGCGGGT	28	CAGCGGGGT
8	GCCAGCGAG	29	GGGGGGAGG
9	AGAGGGGCA	30	TCGGCCAAG
10	TGGCAGGTT	31	CATGTGTTG
11	GGGTGGGAG	32	GACTGTAGA
12	GGTCCGGGC	33	CTGCCTGAA
13	CTCCGATCA	34	CGGCGGAGA
14	GCAAATGAA	35	CGTACAAGG
15	GGGCGGCGT	36	GGTGGTTGA
16	ACTCTGAGA	37	ATCAGGGGG
17	TGGGAATGG	38	TTGGCGGGG

18	AGCGGAGGG	39	GGGGTCCGA
19	GGATGGAAA	40	GAGAGAGCG
20	GGGCTGTCA	41	TGGCAGCTT
21	ACTCCTGAG	42	CGAGATGGA

On this basis, the ZFQQR binding consensus sequence 5'-GGNCGGNGGG-3' (Figure 15) was obtained using WebLogo (Crooks GE, Hon G, Chandonia JM, Brenner SE WebLogo: A sequence logo generator, Genome Research, 14:1188-1190, (2004), Schneider TD, Stephens RM. 1990. Sequence Logos: A New Way to Display Consensus Sequences. Nucleic Acids Res. 18:6097-6100).

Example 13 Binding of zinc finger ZfQQR to the SELEX consensus sequence

To confirm that the consensus sequence obtained after five rounds of SELEX is bound by the zinc finger, a constant K_D was measured using the nitrocellulose filter binding method. The assay was performed with radioactively labeled substrates described in the literature (LDNA, LRNA) and a consensus sequence (CDNA, CRNA) see Figure 16:

LDNA: TCACTGGGGAAGAAGAATCCTC (SEQ ID No:29)

LRNA: GAGGAUUCUUCUUCCCCAGUGA (SEQ ID No:30)

CDNA: TCACTGGTCGGTGGGAATCCTC (SEQ ID No:31)

CRNA: GAGGAUUCCCACCGACCAGUGA (SEQ ID No:32)

Binding reaction contained 2 nM of labeled substrate, 2μg poly dI-dC as a nonspecific competitor, 25 mM Tris pH 8.0, 100 mM KCl, 20 μM $ZnSO_4$, 2 mM DTT. The binding reaction was incubated for 30 min at room temperature and then filtered immediately through a nitrocellulose filter and washed with 8 volumes of reaction buffer of 25 mM Tris pH 8.0, 100 mM KCl, 20 μM $ZnSO_4$, 2 mM DTT. Measurement of the intensity of the signal coming from the retention of radioactively labeled substrate was made on an audioradiogram of the nitrocellulose filter using a Typhoon Trio scanner and ImageQuant TL software. K_D binding constants were measured and for both substrates were similar, 188 ± 38 nM for the binding of substrate described in the literature and 155 ± 32 nM for the SELEX consensus sequence (Figure 17).

The list of sequences identified in the description:

SEQ ID No:1 - DNA sequence of the gene *rnhA* ribonuclease HI (Gene ID 893801, BH0863) from *B. halodurans* (full length);

SEQ ID No:2 - nucleotide sequence of zinc finger ZfQQR;

SEQ ID No:3 - nucleotide sequence of the gene encoding the enzyme fusion containing a fragment of ribonuclease HI from *B. halodurans* with substitutions K81A, K89E and K123A with zinc finger ZfQQR – named **catAEA-ZfQQR;**

SEQ ID No:4 – amino acid sequence of fusion containing a fragment of ribonuclease HI from *B. halodurans* with substitutions K81A, K89E, K123A and with zinc finger ZfQQR- named **catAEA-ZfQQR;**

SEQ ID No:5- nucleotide sequence of the gene encoding **GQ** variant of catAEA-ZfQQR (the enzyme fusion containing a fragment of ribonuclease HI from *B. halodurans* with substitutions introducing changes in the amino acid sequence of protein in positions K81A, K89E and K123A with zinc finger ZfQQR interdomain linker shortened by a fragment encoding amino acids in positions 138-148) - named GQ;

SEQ ID No:6 - amino acid sequence of the GQ variant of catAEA-ZfQQR the enzyme fusion - named GQ;

SEQ ID No:7 - nucleotide sequence of the **GGKKQ** variant of catAEA-ZfQQR (enzyme fusion containing a fragment of ribonuclease HI from *B. halodurans* with substitutions introducing changes in the amino acid sequence of protein in positions K81A, K89E and K123A with zinc finger ZfQQR with the interdomain linker by a fragment encoding amino acids in positions 138-139 and 141-146)- named GGKKQ;

SEQ ID No:8 - amino acid sequence of GGKKQ variant of the enzyme fusion- named GGKKQ;

SEQ ID No:9 - RNA strand of the substrate that contains the binding sequence for ZfQQR;

SEQ ID No:10 - DNA strand of the substrate that contains the binding sequence for ZfQQR;

SEQ ID No:11 – nucleotide sequence of Bhcatf primer;

SEQ ID No:12 – nucleotide sequence of Bhcatr primer;

SEQ ID No:13 – nucleotide sequence of BhZf primer;

SEQ ID No:14 - nucleotide sequence of Kmf primer;

SEQ ID No:15 - nucleotide sequence of Kmr primer;

SEQ ID No:16 - nucleotide sequence of K81Ar primer;

SEQ ID No:17 - nucleotide sequence of K81Af primer;

SEQ ID No:18 - nucleotide sequence of K89Er primer;

SEQ ID No:19 - nucleotide sequence of K89Ef primer;

SEQ ID No:20 - nucleotide sequence of K123Ar primer;

SEQ ID No:21 - nucleotide sequence of K123Af primer;

SEQ ID No:22 - nucleotide sequence of del11f primer;

SEQ ID No:23 - nucleotide sequence of del11r primer;

SEQ ID No:24 - nucleotide sequence of del5f primer;

SEQ ID No:25 - nucleotide sequence of del5r primer;

SEQ ID No:26 -sequence of the DNA strand of the substrate used for control of the SELEX method that contains the binding site for ZfQQR (Template A);

SEQ ID No:27 -sequence of the DNA strand of the substrate used for control of the SELEX method that does not have a binding site for ZfQQR, instead a XhoI restriction site (Template B);

SEQ ID No:28 -sequence of the DNA strand of the substrate used for construction of the library of DNA-RNA hybrids containing a central nonanucleotide random sequence (Template 9N);

SEQ ID No:29 -nucleotide sequence of the DNA strand of the DNA-RNA hybrid that contains the ZfQQR binding sequence used for determining K_D;

SEQ ID No:30 -nucleotide sequence of the RNA strand of the DNA-RNA hybrid that contains the ZfQQR binding sequence used for determining K_D;

SEQ ID No:31 -nucleotide sequence of the DNA strand of the DNA-RNA hybrid that contains the consensus sequence inferred from SELEX used for determining K_D;

SEQ ID No:32 -nucleotide sequence of the RNA strand of the DNA-RNA hybrid that contains the consensus sequence inferred from SELEX used for determining K_D;

SEQ ID No:33 – primer 1 –primer used for amplification of DNA of template A, B and 9N in the PCR reaction;

SEQ ID No:34 –primer 2 - primer used for amplification of DNA of template A, B and 9N in the PCR reaction;

SEQ ID No:35 – primer ZFf - primer used in PCR for preparation of DNA construct of the fusion gene encoding the GST domain and the gene encoding the zinc finger Zf-QQ;

SEQ ID No:36 - primer ZFr - primer used for preparation of DNA construct of the fusion gene encoding the GST domain and the gene encoding the zinc finger Zf-Q;

SEQ ID No:37- is nucleotide sequence of the gene encoding the fusion of the GST domain and the gene encoding the zinc finger ZfQQR (fusion GST-ZfQQR).

SEQUENCE LISTING

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            20                  25                  30

Thr Gly Glu Val Leu Phe Glu Arg Glu Pro Ile Pro Ile Gly Thr Asn
        35                  40                  45

Asn Met Gly Glu Phe Leu Ala Ile Val His Gly Leu Arg Tyr Leu Lys
    50                  55                  60

Glu Arg Asn Ser Arg Lys Pro Ile Tyr Ser Asp Ser Gln Thr Ala Ile
65                  70                  75                  80

Ala Trp Val Lys Asp Lys Lys Ala Glu Ser Thr Leu Val Arg Asn Glu
                85                  90                  95

Glu Thr Ala Leu Ile Trp Lys Leu Val Asp Glu Ala Glu Glu Trp Leu
            100                 105                 110

Asn Thr His Thr Tyr Glu Thr Pro Ile Leu Ala Trp Gln Thr Asp Lys
        115                 120                 125

Trp Gly Glu Ile Lys Ala Asp Tyr Gly Arg Lys Gly Ser Gly Asp Pro
    130                 135                 140

Gly Lys Lys Lys Gln His Ala Cys Pro Glu Cys Gly Lys Ser Phe Ser
145                 150                 155                 160

Gln Ser Ser Asn Leu Gln Lys His Gln Arg Thr His Thr Gly Glu Lys
                165                 170                 175

Pro Tyr Lys Cys Pro Glu Cys Gly Lys Ser Phe Ser Gln Ser Ser Asn
            180                 185                 190

Leu Gln Lys His Gln Arg Thr His Thr Gly Glu Lys Pro Tyr Lys Cys
        195                 200                 205

Pro Glu Cys Gly Lys Ser Phe Ser Arg Ser Asp His Leu Gln Arg His
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        35                  40                  45

Asn Met Gly Glu Phe Leu Ala Ile Val His Gly Leu Arg Tyr Leu Lys
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Glu Arg Asn Ser Arg Lys Pro Ile Tyr Ser Asp Ser Gln Thr Ala Ile
65                  70                  75                  80

Ala Trp Val Lys Asp Lys Lys Ala Ala Ser Thr Leu Val Arg Asn Glu
                85                  90                  95

Glu Thr Ala Leu Ile Trp Lys Leu Val Asp Glu Ala Glu Glu Trp Leu
            100                 105                 110

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Trp Gly Glu Ile Lys Ala Asp Tyr Gly Gln His Ala Cys Pro Glu Cys
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Ser Gln Ser Ser Asn Leu Gln Lys His Gln Arg Thr His Thr Gly Glu
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Lys Pro Tyr Lys Cys Pro Glu Cys Gly Lys Ser Phe Ser Arg Ser Asp
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        35                  40                  45

Asn Met Gly Glu Phe Leu Ala Ile Val His Gly Leu Arg Tyr Leu Lys
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Glu Arg Asn Ser Arg Lys Pro Ile Tyr Ser Asp Ser Gln Thr Ala Ile
65                  70                  75                  80

Ala Trp Val Lys Asp Lys Lys Ala Ala Ser Thr Leu Val Arg Asn Glu
                85                  90                  95

Glu Thr Ala Leu Ile Trp Lys Leu Val Asp Glu Ala Glu Glu Trp Leu
            100                 105                 110

Asn Thr His Thr Tyr Glu Thr Pro Ile Leu Ala Trp Gln Thr Asp Lys
        115                 120                 125

Trp Gly Glu Ile Lys Ala Asp Tyr Gly Gly Lys Lys Gln His Ala Cys
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Pro Glu Cys Gly Lys Ser Phe Ser Gln Ser Ser Asn Leu Gln Lys His
145                 150                 155                 160

Gln Arg Thr His Thr Gly Glu Lys Pro Tyr Lys Cys Pro Glu Cys Gly
                165                 170                 175

Lys Ser Phe Ser Gln Ser Ser Asn Leu Gln Lys His Gln Arg Thr His
            180                 185                 190

Thr Gly Glu Lys Pro Tyr Lys Cys Pro Glu Cys Gly Lys Ser Phe Ser
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<400> 16
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<400> 21
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agtgagtcgt attaaatt 78

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agtgagtcgt attaaatt 78

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<400> 31
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<213> artificial

<220>
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<220>
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<220>
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<212> DNA
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<220>
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<211> 26
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<220>
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atgttgggtg gttgtccaaa agagcgtgca gagatttcaa tgcttgaagg agcggttttg 300
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acatatttaa atggtgatca tgtaacccat cctgacttca tgttgtatga cgctcttgat 480
gttgttttat acatggaccc aatgtgcctg gatgcgttcc caaaattagt ttgttttaaa 540
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cggccgcatc gtgac 975

Claims

1. A ribonuclease, which cleaves RNA strand in DNA-RNA hybrids, wherein ribonuclease is a fusion protein comprising the catalytic domain of ribonuclease HI (RNase HI) or a derivative thereof with a zinc finger DNA-RNA hybrid binding domain, and wherein the zinc finger binding domain has the ability to bind to specific sequence in the DNA-RNA hybrid.

2. The ribonuclease according to claim 1, wherein the derivative of the catalytic domain of RNase HI comprises a deletion of the RNase HI hybrid binding domain.

3. The ribonuclease according to claim 1 or 2, wherein the catalytic domain of RNase HI is preferably from *Bacillus halodurans*, preferably comprising polypeptide encoded by nucleotides 175 to 588 of the *rnhA* gene shown in SEQ ID No:1.

4. The ribonuclease according to claim 3, wherein the catalytic domain of RNase HI comprises at least one substitution of one amino acid residue in the substrate binding region selected from: K81A, K89E and K123A, and preferably contains all substitutions K81A, K89E and K123A.

5. The ribonuclease according to claims 1-4, wherein the zinc finger domain is a derivative of the zinc finger ZfQQR, preferably a polypeptide encoded by nucleotides from 19 to 303 of the sequence ZfQQR shown in SEQ ID No.2.

6. The ribonuclease according to claims 1-5, wherein it comprises a fusion protein catAEA-ZfQQR as shown in SEQ ID No.4.

7. The ribonuclease according to claims 1-5, wherein it comprises a fusion protein GQ as shown in SEQ ID No.6.

8. The ribonuclease according to claims 1-5, wherein it comprises a fusion protein GGKKQ as shown in SEQ ID No.8.

9. A composition comprising the ribonuclease according to claims 1-8.

10. Use of ribonuclease according to claims 1-8 or composition according to claim 9 for the cleavage of the RNA strand in DNA-RNA hybrids.

11. The use according to claims 10, wherein the cleavage of the RNA strand in DNA-RNA hybrids is located 2-16 nucleotides, preferably 5-7 nucleotides, away from the binding site for the zinc finger.

12. A method of obtaining an engineered variant of RNase HI that cleaves RNA strand in DNA-RNA hybrids comprising the following steps:

a) obtaining the RNase HI catalytic domain that does not bind the substarate but retains the catalytic activity, preferably by the removal of binding domains and/or substitution of amino acids involved in substrate binding;

b) obtaining an engineered RNase HI by making a fusion protein comprising the RNase HI catalytic domain obtained in step a) with a binding domain that has the ability to bind to specific sequences in the DNA-RNA hybrid, preferebly with a zinc finger DNA- RNA hybrid binding domain.

13. The metod of claim 12, wherein the zinc finger domain is a derivative of the zinc finger ZfQQR, preferably a polypeptide encoded by nucleotides from 19 to 303 of the sequence ZfQQR shown in SEQ ID No.2.

14. The method of claim 12-13, wherein the catalytic domain of RNase HI is preferably from *B. halodurans*, preferably comprising a polypeptide encoded by nucleotides 175 to 588 of the *rnhA* gene shown in SEQ ID No:1.

15. The method of claim 12-14, wherein the catalytic domain of RNase HI comprises changes in the substrate binding region, preferably selected from substitutions, deletions or insertions of at least one amino acid residue.

16. A method of determining the sequence preference of DNA-RNA hybrid binding protein(s) or its domain(s), characterized in that the method comprises the following steps:
a) contacting of the purified protein or its domain, with a mixture of a library of DNA-RNA hybrids substrates, wherein DNA-RNA hybrid substrates comprise randomized sequences in the central part, preferably randomized in 9 or 10 nucleotide positions, with flanking sequences fixed and allowing a tested protein or its domain to bind the sequence to which it has an affinity;
b) separating unbound DNA-RNA hybrids by immobilization of protein or its domain, with bound DNA-RNA hybrid, to the resin, preferably glutathione agarose;
c) removing the unbound hybrids;
d) isolating the recombinant protein, together with the associated DNA-RNA hybrids, preferably

by adding a buffer containing glutathione;

e) amplification of the isolated hybrid using PCR, preferably RT-PCR, wherein in the amplification reaction the primers complementary to invariant sequences flanking the randomized region are used, and wherein during the PCR reaction on one of the primer the sequence of RNA polymerase promoter is added in order to obtain double-stranded DNA for *in vitro* transcription with RNA polymerase;

f) reverse transcription is performed using reverse transcriptase that does not possess the RNase H activity and a DNA primer complementary to the 3′ end of the RNA template in order to obtain a DNA-RNA hybrid, and steps a) to f) are preferably repeated.

17. The method of claim 16, wherein the promoter sequence comprises the promoter sequence for T7 RNA polymerase and RNA polymerase is T7 RNA polymerase.

18. The method according to claim 16 or 17, wherein the protein or its domain is a recombinant protein or a recombinant domain.

19. The method according to claims 16-18, wherein the protein or its domain comprises zinc finger(s).

20. The method according to claims 16-19, wherein the protein or its domain is the fusion of the zinc finger domain and the glutathione S-transferase domain (GST), preferably the fusion of the zinc finger domain and GST encoded by SEQ ID No:37.

21. The method according to claims 16-20, wherein protein or its domain is the fusion of RNase HI or a derivative, preferably RNase HI is from *B. halodurans*, preferably comprising polypeptide encoded by nucleotides 175 to 588 of *rnhA* gene shown in SEQ ID No:1 and a zinc finger.

22. The method according to claims 16-19 and 21, wherein the zinc finger has the ability to bind to a specific sequence in the DNA-RNA hybrid.

23. The method according to claims 16-22 wherein the zinc finger is ZfQQR, preferably of sequence encoded by nucleotides from 19 to 303 of the sequence ZfQQR shown in SEQ ID No.2.

24. The method of obtaining a library of DNA-RNA hybrids comprising the flowing steps:

a) PCR amplification of the library of DNA oligonucleotides containing a degenerate sequence in the central position, flanked by invariant sequences, which include a promoter sequence for RNA polymerase,

b) synthesis of RNA strand using an RNA polymerase on the double stranded DNA obtained in a) used as a template,

c) reverse transcription of the primer complementary to the invariant sequence present at the 3' end of RNA obtained in b) with a reverse transcriptase that does not possess the ribonuclease H activity, wherein during the reverse transcription the RNA strand is not degraded, and the hybrid nucleic acid molecule consisting of complementary RNA and DNA strands is obtained.

25. The method of claim 24, wherein oligonucleotides contain a degenerate sequence in the central position comprising randomized 9 or 10 nucleotide positions.

26. The method of claim 24 or 25, wherein the promoter sequence comprises the promoter sequence for T7 RNA polymerase and RNA polymerase is the T7 RNA polymerase.

Fig. 1

Primer name	Sequence	Number of SEQ ID No
Bhcatf	GACGCATATGGCAAAAGAGGAGATTATTTGGG	SEQ ID No:11
Bhcatr	GTGGTACCTTTTCTCCCGTAATCGGC	SEQ ID No:12
BhZf	GGTTCTGGTGACCCGGG	SEQ ID No:13
Kmf	GGGATCGCAGTGGTGAGTAAC	SEQ ID No:14
Kmr	CGGGAAAACAGCATTCCAGGTATTAG	SEQ ID No:15
K81Ar	CCGTCTGGGAATCAGAATAGATC	SEQ ID No:16
K81Af	CAATCGCATGGGTGAAGGATAAAAAAG	SEQ ID No:17
K89Er	CTGCTTTTTTATCCTTCACCC	SEQ ID No:18
K89Ef	CAATCGCATGGGTGAAGGATAAAAAAG	SEQ ID No:19
K123Ar	TAAGATGGGCGTTTCATAGGTATG	SEQ ID No:20
K123Af	GCATGGCAGACCGATAAGTG	SEQ ID No:21
del11f	GGCTCCGGCCAGCACGCGTGCCCGG	SEQ ID No:22
del11r	AGAACCGCTCCCGTAATCGGCCTTAATTTCC	SEQ ID No:23
del5f	CAAAAAACAGCACGCGTGCCC	SEQ ID No:24
del5r	CCCCCGTAATCGGCCTTAATTTCCC	SEQ ID No:25

Fig. 2

(A) RNA:

AGAACUAGUGGAUCAACCGGGCUGCAGGAAUUCGAUAUCAAGCUUAUCGAUA CCGUGGCGGUUCUUCCCCAAGCC (SEQ ID No:9)

(B) DNA:

GCTTGGGGAAGAACCGCCACGGTATCGATAAGCTTGATATCGAATTCCTGCAG CCCGGTTGATCCACTAGTTCT (SEQ ID No:10)

Fig. 3

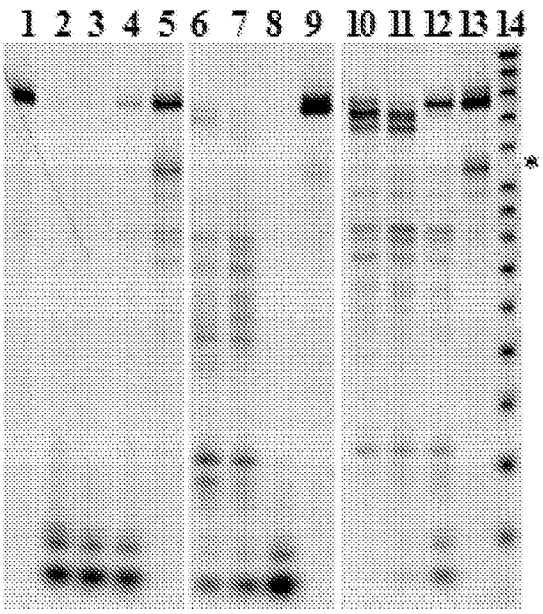

Fig. 4

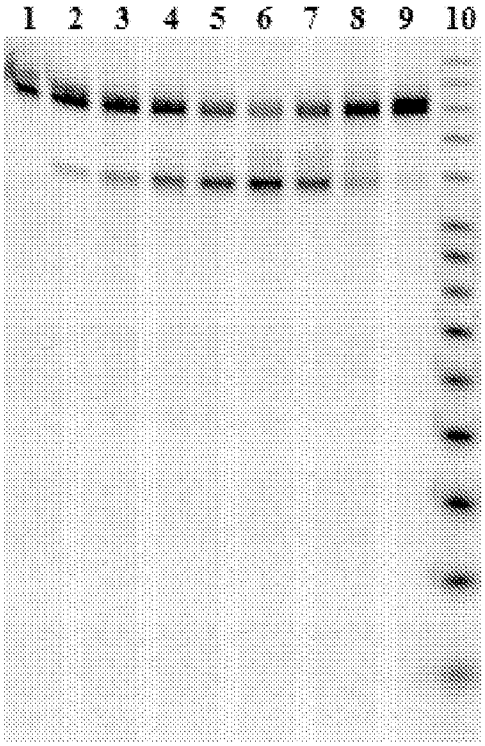

Fig. 5

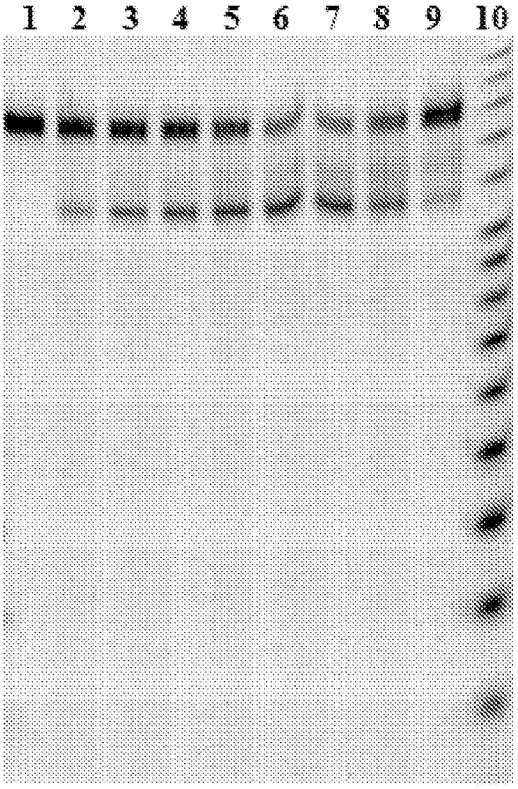

Fig. 6

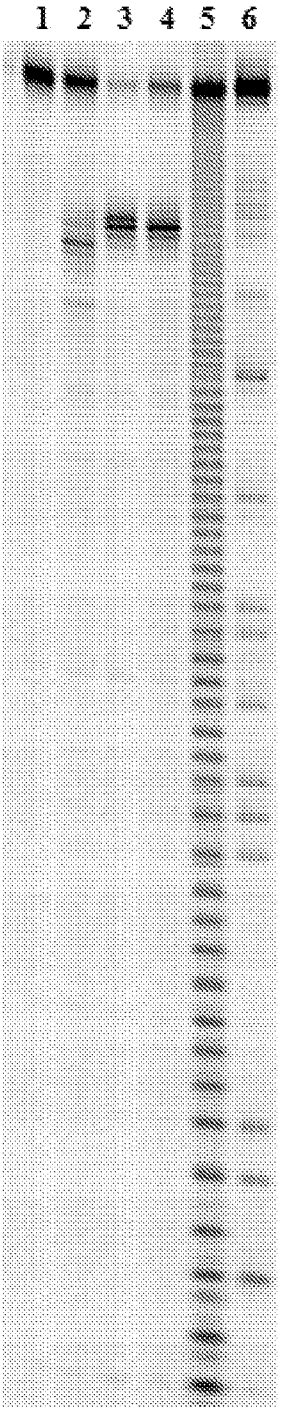

Fig.7

Fig. 8

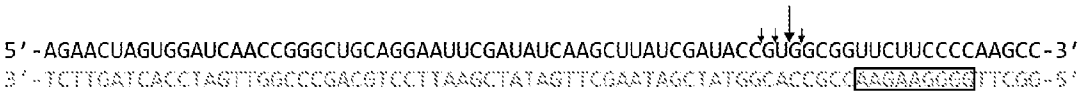

Fig. 9

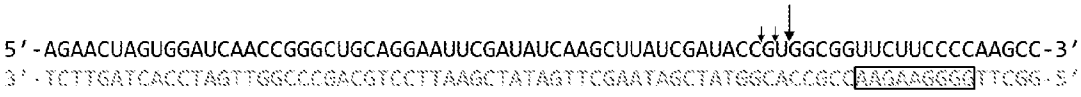

Fig. 10

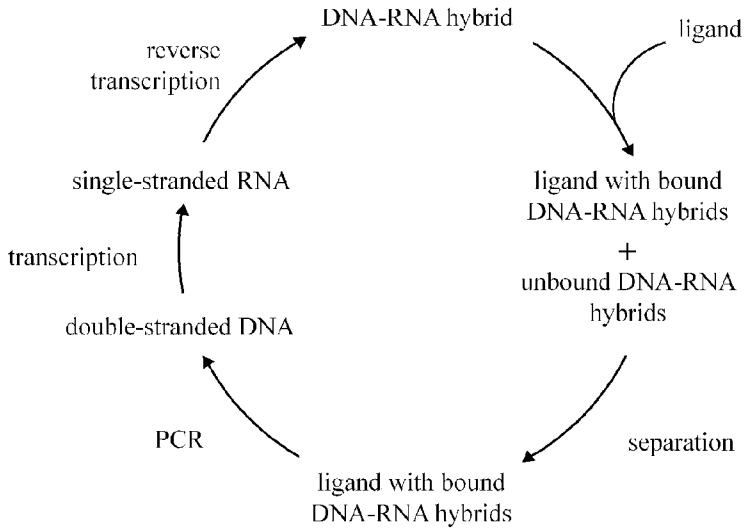

Fig. 11

Template A:

GAGATCTAGACGGAACATGAAGGGGAAGAATTCTATGCTTAGTGAGATCTAGAGCC CTATAGTGAGTCGTATTAAATT (SEQ ID No:26)

Template B:

GAGATCTAGACGGAACATGAAGCTCGAGCCTTCTATGCTTAGTGAGATCTAGAGCC CTATAGTGAGTCGTATTAAATT (SEQ ID No:27)

Template 9N:

GAGATCTAGACGGAACATGTA**NNNNNNNNN**TACTATGCTTAGTGAGATCTAGAGCC CTATAGTGAGTCGTATTAAATT (SEQ ID No:28)

Fig. 12

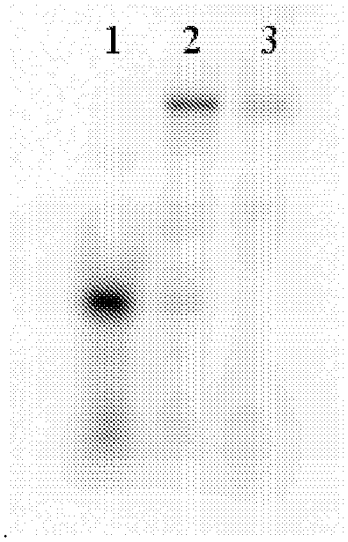

Fig. 13

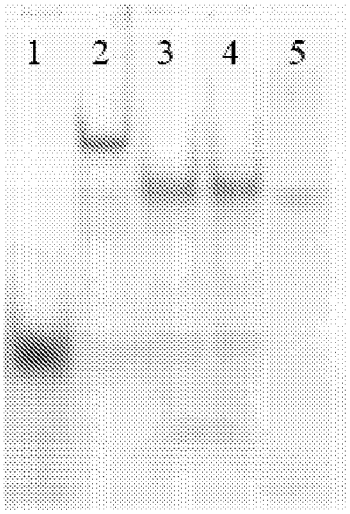

Fig. 14

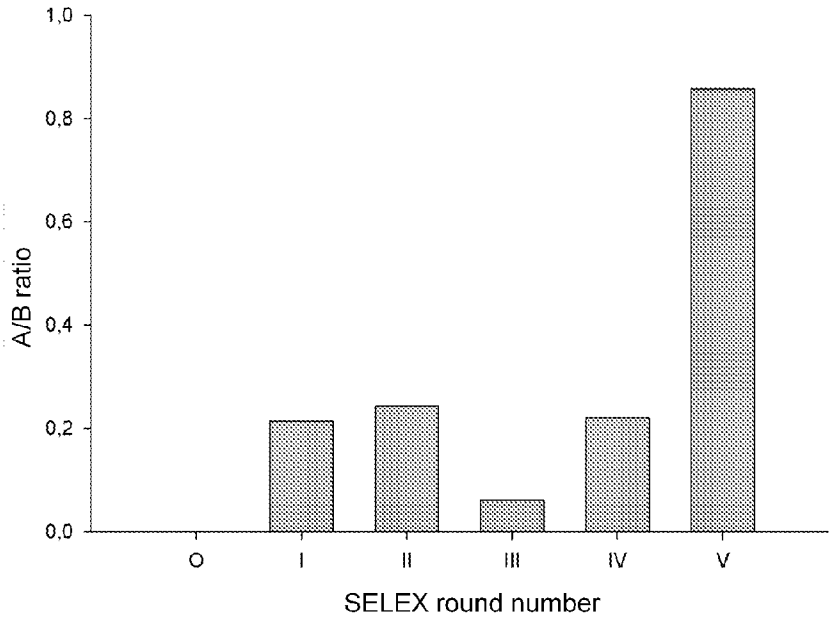

Fig. 15

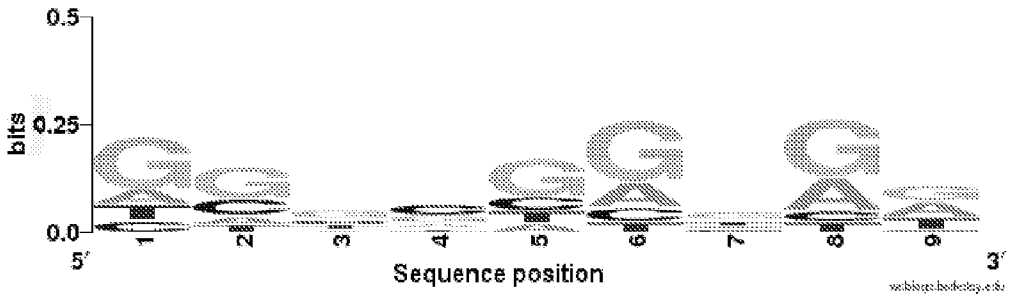

Fig. 16

A)

LDNA: TCACTGGGGAAGAAGAATCCTC (SEQ ID No:29)

LRNA: GAGGAUUCUUCUUCCCCAGUGA (SEQ ID No:30)

B)

CDNA: TCACTGGTCGGTGGGAATCCTC (SEQ ID No:31)

CRNA: GAGGAUUCCCACCGACCAGUGA (SEQ ID No:32)