

(19) **DANMARK**

(10) **DK/EP 2718430 T3**



Patent- og
Varemærkestyrelsen

(12) **Oversættelse af
europæisk patentskrift**

-
- (51) Int.Cl.: **C 12 N 9/22 (2006.01)**
- (45) Oversættelsen bekendtgjort den: **2016-02-08**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2015-12-09**
- (86) Europæisk ansøgning nr.: **12735348.0**
- (86) Europæisk indleveringsdag: **2012-06-07**
- (87) Den europæiske ansøgnings publiceringsdag: **2014-04-16**
- (86) International ansøgning nr.: **PL2012050019**
- (87) Internationalt publikationsnr.: **WO2012169916**
- (30) Prioritet: **2011-06-08 US 201161494494 P** **2011-06-08 PL 39518011**
2011-06-08 PL 39517911 **2011-06-08 US 201161494481 P**
- (84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**
- (73) Patenthaver: **Miedzynarodowy Instytut Biologii Molekularnej I Komorkowej, Ul. Ks. Trojdena 4, 02-109 Warszawa, Polen**
- (72) Opfinder: **BUJNICKI, Janusz Marek, ul. Stanislaw Zaryna 7/100, 02-593 Warszawa, Polen**
SULEJ, Agata Agnieszka, ul. Przy Agorze 17/85, 01-960 Warszawa, Polen
NOWOTNY, Marcin, ul. Inflancka 15/208, 00-189 Warszawa, Polen
SKOWRONEK, Krzysztof Jerzy, ul. Pietkiewicza 39, 05-080 Hornówek, Polen
- (74) Fuldmægtig i Danmark: **Awapatent A/S, Rigensgade 11, 1316 København K, Danmark**
- (54) Benævnelse: **SEKVENSSPECIFIK TEKNISK KONSTRUERET RIBONUCLEASE-H OG FREMGANGSMÅDE TIL BESTEMMELSE AF SEKVENSPREFERENCEN AF DNA-RNA-HYBRIDBINDEDE PROTEINER**
- (56) Fremdragne publikationer:
WO-A1-2009/126632
WO-A1-2010/076939
WO-A1-2011/022427
WO-A2-2007/014181
US-A- 5 270 163
DURAI S ET AL: "Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells", NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 33, no. 18, 26 October 2005 (2005-10-26), pages 5978-5990, XP002403200, ISSN: 0305-1048
Y SHI ET AL: "Specific DNA-RNA hybrid binding by zinc finger proteins", SCIENCE, vol. 268, no. 5208, 14 April 1995 (1995-04-14), pages 282-284, XP55039658, ISSN: 0036-8075, DOI: 10.1126/science.7536342 cited in the application
MARCIN NOWOTNY ET AL: "Crystal Structures of RNase H Bound to an RNA/DNA Hybrid: Substrate Specificity and Metal-Dependent Catalysis", CELL, vol. 121, no. 7, 1 July 2005 (2005-07-01), pages 1005-1016, XP55039777,

Fortsættes ...

ISSN: 0092-8674, DOI: 10.1016/j.cell.2005.04.024

MATRAI JANKA ET AL: "Recent Advances in Lentiviral Vector Development and Applications", MOLECULAR THERAPY, NATURE PUBLISHING GROUP, GB, vol. 18, no. 3, 1 March 2010 (2010-03-01), pages 477-490, XP009143103, ISSN: 1525-0016, DOI: 10.1038/MT.2009.319 [retrieved on 2010-01-19]

DESCRIPTION

TECHNICAL FIELD

[0001] The invention concerns a sequence-specific ribonuclease H, which comprises a fusion of an engineered ribonuclease H (RNase H) 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*.

[0002] Also disclosed is 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 above method 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

[0003] 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.

[0004] 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 H 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 H and the zinc finger, does not lead to a sequence-specific enzyme.

[0005] 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.

[0006] 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.

[0007] 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 Shahbadian 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 Shahbadian 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.

[0008] 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.

[0009] 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

[0010] 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.

[0011] Also disclosed is 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. Additionally, a new and improved method of obtaining a library of DNA-RNA hybrids is disclosed.

[0012] 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.

[0013] In the first aspect, the invention provides a ribonuclease which cleaves the RNA strand in DNA-RNA hybrids, wherein said ribonuclease is a fusion protein comprising a derivative of the catalytic domain of ribonuclease HI (RNase HI) wherein the derivative of the catalytic domain of RNase HI is from *Bacillus halodurans*, comprising polypeptide encoded by nucleotides 175 to 588 of the *mhaA* gene shown in SEQ ID No:1, and wherein the derivative of the catalytic domain of RNase HI comprises at least one substitution of one amino acid residue in the substrate binding domain selected from: K81A, K89E and K123A referring to the

sequence of the polypeptide coded by SEQ ID NO: 1, preferably contains all substitutions K81A, K89E and K123A referring to the sequence of the polypeptide coded by SEQ ID NO: 1, and wherein the derivative of the catalytic domain of RNase HI does not comprise RNase HI hybrid binding domain, with a zinc finger DNA-RNA hybrid binding domain, wherein the zinc finger binding domain has the ability to bind to specific sequence in the DNA-RNA hybrid.

[0014] 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 *in vitro* 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.

[0015] The invention also provides the method of obtaining engineered variant of RNase HI, which cleaves RNA strand in DNA-RNA hybrids comprising the following steps:

1. a) obtaining a derivative of RNase HI catalytic domain, wherein the derivative of the catalytic domain of RNase HI is from *B. halodurans*, comprising polypeptide encoded by nucleotides 175 to 588 of *mhA* gene shown in SEQ ID No:1, and wherein the derivative of the catalytic domain of RNase HI comprises at least one substitution of one amino acid residue in the substrate binding domain selected from: K81A, K89E and K123A referring to the sequence of the polypeptide coded by SEQ ID NO: 1, preferably contains all substitutions K81A, K89E and K123A referring to the sequence of the polypeptide coded by SEQ ID NO: 1, and wherein the derivative of the catalytic domain of RNase HI does not comprise RNase HI hybrid binding domain,
2. b) obtaining an engineered RNase HI by making fusion protein comprising the derivative of the RNase HI catalytic domain obtained in step a) with a zinc finger binding domain that has the ability to bind to specific sequences in the DNA-RNA hybrid. In preferred method of obtaining an engineered variant of 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. The catalytic domain of RNase HI comprises changes in the substrate binding region.

[0016] The ribonuclease that cleaves the RNA strand of the DNA-RNA hybrids, 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. The described ribonuclease is characterized by being a derivative of RNase HI from *B. halodurans*, the catalytic domain polypeptide, comprising a polypeptide encoded by nucleotides 175 to 588 of *mhA* gene shown in SEQ ID NO: 1. The described ribonuclease is characterized by being a derivative of the native RNase HI, which contains a deletion of the DNA-RNA hybrid binding domain. The described ribonuclease 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. The described ribonuclease is characterized by being a fusion of the polypeptide encoded by nucleotides 175 to 588 of *mhA* 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.

[0017] 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 used 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*.

[0018] 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* *mhA* encoding the catalytic domain (termed cat). It is a fragment from 175 to 588 nucleotide *mhA* 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 <i>B. halodurans</i>	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 zinc finger ZfQQR and the inter domain linker shortened by a fragment encoding amino acids in positions 138-139 and 141-146	GGKKQ	construct coded by SEQ ID No: 7 (nucleotide sequence) and shown SEQ ID No: 8 (amino acid sequence)

[0019] Also disclosed is 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.

[0020] In the disclosed method of determining the sequence preference of DNA-RNA hybrid binding protein(s) or its domain(s), the method comprises the following steps:

1. 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;
2. b) separating unbound DNA-RNA hybrids by immobilization of protein or its domain, with bound DNA-RNA hybrid, to the resin, preferably glutathione agarose;
3. c) removing the unbound hybrids;
4. d) isolating the recombinant protein, together with the associated DNA-RNA hybrids, preferably by adding a buffer containing glutathione;
5. 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;
6. f) reverse transcription is performed using reverse transcriptase that does not possess the RNase H activity and a DNA

primer complementary to 3' end of the RNA template in order to obtain a DNA - RNA hybrid, and steps a) to f) are preferably repeated.

For example, in the method promoter sequence comprises the promoter sequence for T7 RNA polymerase and RNA polymerase is T7 RNA polymerase. For example, the protein or its domain is a recombinant protein or recombinant domain. Protein or its domain preferably comprises zinc finger(s). For example, 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 exemplary protein or its domain is the fusion of RNase HI or a derivative thereof, e.g. RNase HI is from *B. halodurans*, e.g. 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 for example the ability to bind to specific sequence in the DNA-RNA hybrid, the exemplary zinc finger is ZfQQR, e.g. of sequence encoded by nucleotides from 19 to 303 of the sequence ZfQQR shown in SEQ ID No.2.

[0021] The description also discloses a method of obtaining library of DNA-RNA hybrids which comprises the following steps:

1. 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,
2. b) synthesis of RNA strand using RNA polymerase on the obtained in a) double stranded DNA as a template,
3. 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. For example, in such method the oligonucleotides contain a degenerate sequence in the central position comprising randomized 9 or 10 nucleotide positions. E.g. the promoter sequence comprises the promoter sequence for T7 RNA polymerase and RNA polymerase is T7 RNA polymerase.

[0022] 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, 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.

BRIEF DESCRIPTION OF DRAWINGS

[0023] To understand the invention better, several examples 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 T 1 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.

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

[0024] 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.

[0025] 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

[0026] 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 (Top 10 (Invitrogen)), have been transformed with ligation mixture (20 - 200 ng per 50 μ l of bacterial cells). The transformants were selected on on LB-agar plates supplemented with 30 μ g/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

[0027] 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*

[0028] 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

[0029] 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):

[0030] 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

[0031] 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 dell 1f (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 sequences).

[0032] 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

[0033] 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 according to standard protocol on Ni-NTA resin (Sigma Aldrich).

Example 7 Nuclease activity assay of the fusion enzyme

[0034] 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 MgCl₂ and/or 20 μM ZnSO₄. 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
GUGGCGGUUCUCCCAAGCC (SEQ ID No:9)

DNA:

GCTTGGGAAGAACCGCCACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCC
GGTTGATCCACTAGTTCT (SEQ ID No:9)

and 25 mM Tris (pH 8.0), 100 mM KCl, 5 mM MgCl₂ and/or 20 μM ZnSO₄, 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 autoradiogram was scanned on a Typhoon Trio scanner (GE Healthcare).

In the presence of 20 μM ZnSO₄, and in the absence of MgCl₂ 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**.

[0035] 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 MgCl₂ and 20 μM ZnSO₄ 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 MgCl₂ and 20 μM ZnSO₄, 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 Mg²⁺ in the reaction was 1 mM and for the **GGKKQ** variant it was 2 mM.

[0036] 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 ZnSO₄, 1 mM MgCl₂ (in case of variant **GQ**) and 2 mM MgCl₂ (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.

[0037] The new ribonuclease cleaves the RNA strand in DNA-RNA hybrids, and is fusion protein which comprises 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**.

[0038] 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 multiple 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 enzyme as 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.

[0039] 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

[0040] 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.

[0041] These oligonucleotides were used as a template to create double-stranded DNA using the PCR technique. The reaction was carried out in standard conditions, 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)

[0042] 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.

[0043] 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).

[0044] 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 resin (Sigma Aldrich) in a

standard procedure.

[0045] 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 autoradiogram 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 with 1 and 5 units of RNase H (Fermentas) for 30 min at 37 ° C.

Example 9 Cloning, expression and purification of GST fusion protein with the zinc finger ZFQQR

[0046] 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)

[0047] 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 on 100 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.

[0048] 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 ampicillin, 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 resin (GE Healthcare) according to standard procedures.

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

[0049] 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 ZnSO₄, 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 ZnSO₄, 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

[0050] 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 Multilimager 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

[0051] 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 reisololation 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

Number	Sequence of library	Number	Sequence of library
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

[0052] On this basis, the ZFQR binding consensus sequence 5'-GGNCGNGGG-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 ZFQR to the SELEX consensus sequence

[0053] 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: GAGGAUUCUUCUCCCCAGUGA (SEQ ID No:30)

CDNA: TCACTGGTGGGTGGGAATCCTC (SEQ ID No:31)

CRNA: GAGGAUCCCACCGACCAGUGA (SEQ ID No:32)

[0054] Binding reaction contained 2 nM of labeled substrate, 2 μ g poly dI-dC as a nonspecific competitor, 25 mM Tris pH 8.0, 100 mM KCl, 20 μ M ZnSO₄, 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 μ M ZnSO₄, 2 mM DTT. Measurement of the intensity of the signal coming from the retention of radioactively labeled substrate was made on an autoradiogram 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 \pm 38 nM for the binding of substrate described in the literature and 155 \pm 32 nM for the SELEX consensus sequence (Figure 17).

The list of sequences identified in the description:

[0055]

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 K81 Ar primer;

SEQ ID No:17 - nucleotide sequence of K81 Af 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

[0056]

<110> IBMIK

<120> Sequence-specific engineered ribonuclease H and the method for determining the sequence preference of DNA-RNA hybrid binding proteins

<130> PZ/1574/AGR/PCT

<160> 37

<170> PatentIn version 3.3

<210> 1

<211> 688

<212> DNA

<213> Bacillus halodurans

<400> 1

```

atggcaagt caaaatacta tgcgtatgg aatggacgga agcccgcat ttatacgagc    60
tggctctgcat gtgaagctca agtaaaagga tataccggcg ccaaatttaa atcctatcct    120
tccaaggaag aagcagaggc tgcctttaga ggagaagagg caacaccgaa gcttgcaaaa    180
gaggagatta tttgggagag cctgtctgta gatgttgga gccaaaggaa tcccggaatt    240
gtggaatata aagcgttga tacgaaaacg ggagaagtcc ttttgaacg agagccgatt    300
ccgatcggga caaacaatat gggtagtctt ctgcgatcg ttcacgggct tcgttacctt    360
aaggaaacgga acagtcgtaa gccgatctat tctgattccc agacggcaat caaatgggtg    420
aaggataaaa aagcaaaatc gaccctcgtg cgcaatgaag aaacagcgtc tatttggaaag    480
ctttagatg aagcggagga gtggctaaac actcatacct atgaaacgcc catcttaaaa    540
tggcagaccg ataagtgagg ggaataaag gccgattacg ggagaaaagg taccgacgac    600
gacgacaagg ccatggogat atcggatccg aattcgagct ccgtcgacaa gcttgcggcc    660
gcactcgagc accaccacca ccaccact                                     688

```

<210> 2

<211> 305

<212> DNA

<213> artificial

<220>

<223> ZfQQR nucleotide sequence

<400> 2
 ccattgaaaa actgcgtaac gttctgggtg acccgggtaa aaaaaaacag cacgcgtgcc 60
 cggaaatgcyg taaatctttc tctcagctct ctaacctgca gaaacaccag cgtaccaca 120
 ccgggtgaaa accgtacaaa tgtccagaat gtggcaaaaag ctttagtcaa agttctaatac 180
 ttcaaaaaa tcaacgcacg cataccggcg agaagccata taagtgtccg gactgcccga 240
 aaagctctc ccgctctgat cacctccagc gtcacacgcy cactcatcag aacaaaaaac 300
 tcgag 305

<210> 3
 <211> 720
 <212> DNA
 <213> artificial

<220>
 <223> fusion catAEA-ZfQQR - nucleotide sequence

<400> 3
 atggcaaaag aggagattat ttgggagagc ctgtctgtag atgttggcag ccaagggaaat 60
 cccggaattg tggaatataa agcgtttgat acgaaaacgg gagaagtcc ttttgaacga 120
 gagccgattc cgatcgggac aaacaatatg ggtgagtttc tcgcatcgt tcacgggctt 180

 cgttacctta aggaacggaa cagtcgtaag ccgatctatt ctgattccca gacggcaatc 240
 gcatgggtga aggataaaaa agcagaatcg accctcgtgc gcaatgaaga aacagcgtt 300
 atttggaagc ttgtagatga agcggaggag tggctaaaca ctcataccta tgaacgccc 360
 atcttagcat ggcagaccga taagtggggg gaaattaagg ccgattacgg gagaaaagg 420
 tctggtgacc cgggtaaaaa aaaacagcac gcgtgcccgg aatgcggtaa atctttctc 480
 cagctctcta acctgcaaaa acaccagcgt acccaacacc gtgaaaaacc gtacaaatgt 540
 ccagaatgtg gcaaaagctt tagtcaaatg tctaattctc aaaaacatca acgcacgcat 600
 accggcgaga agccatataa gtgtccggag tgcggcaaaa gcttctccc ctctgatcac 660
 ctcacgctc atcacgccc acatcagaac aaaaaactcg agcaccacca ccaccaccac 720

<210> 4
 <211> 240
 <212> PRT
 <213> artificial

<220>
 <223> amino acid sequence of fusion catAEA-ZfQQR

<400> 4
 Met Ala Lys Glu Glu Ile Ile Trp Glu Ser Leu Ser Val Asp Val Gly
 1 5 10 15
 Ser Gln Gly Asn Pro Gly Ile Val Glu Tyr Lys Gly Val Asp Thr Lys
 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
 210 215 220

Gln Arg Thr His Gln Asn Lys Lys Leu Glu His His His His His His
 225 230 235 240

<210> 5

<211> 687

<212> DNA

<213> artificial

<220>

<223> fusion of variant GQ of catAEA-ZfQQR - nucleotide sequence

<400> 5

```

atggcaaaag aggagattat ttgggagagc ctgtctgtag atgttggcag ccaagggaaat    60
cccggaattg tggaatataa aggcgttgat acgaaaacgg gagaagtcct ttttgaacga    120
gagccgattc cgatcgggac aaacaatatg ggtgagtctc tcgcgatcgt tcacgggctt    180
cgttacctta aggaacggaa cagtcgtaag cccgatctatt ctgattccca gacggcaatc    240
gcatgggtga aggataaaaa agcagcatcg accctcgtgc gcaatgaaga aacagcgcctt    300
atttggaagc ttgtagatga agcggaggag tggctaaaca ctcatacctg tgaaacgccc    360
atcttagcat ggcagaccga taagtggggg gaaattaagg ccgattacgg gcagcacgcg    420
tgcccggaat gcggtaaatc tttctctcag tcttctaacc tgcagaaaaca ccagcgtacc    480
cacaccggtg aaaaaccgta caaatgtcca gaatgtggca aaagcttag tcaaagtctc    540
aatcttcaaa aacatcaacg cacgcatacc ggcgagaagc catataagtg tccggagtgc    600
ggcaaaagct tctcccgcctc tgatcacctc cagcgtcctc agcgcactca tcagaacaaa    660
aaactcgagc accaccacca ccaccac
    
```

<210> 6

<211> 229

<212> PRT

<213> artificial

<220>

<223> fusion of variant GQ of catAEA-ZfQQR - amino acid sequence

<400> 6

```

Met Ala Lys Glu Glu Ile Ile Trp Glu Ser Leu Ser Val Asp Val Gly
 1          5          10          15
Ser Gln Gly Asn Pro Gly Ile Val Glu Tyr Lys Gly Val Asp Thr Lys
 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 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 Gln His Ala Cys Pro Glu Cys
130          135          140
Gly Lys Ser Phe Ser Gln Ser Ser Asn Leu Gln Lys His Gln Arg Thr
145          150          155          160
His Thr Gly Glu Lys Pro Tyr Lys Cys Pro Glu Cys Gly Lys Ser Phe
165          170          175
Ser Gln Ser Ser Asn Leu Gln Lys His Gln Arg Thr His Thr Gly Glu
180          185          190
Lys Pro Tyr Lys Cys Pro Glu Cys Gly Lys Ser Phe Ser Arg Ser Asp
195          200          205
His Leu Gln Arg His Gln Arg Thr His Gln Asn Lys Lys Leu Glu His
210          215          220
His His His His His
225
    
```

<210> 7

<211> 696

<212> DNA

<213> artificial

<220>

<223> fusion of variant GGKKQ of catAEA-ZfQQR - nucleotide sequence

<400> 7

```

atggcaaaag aggagattat ttgggagagc ctgtctgtag atgttggcag ccaagggaaat    60
cccggaattg tggaaataaa aggcgttgat acgaaaacgg gagaagtctt ttttgaacga    120
gagccgattc cgatcgggac aaacaatatg ggtgagtttc tcgcatcgtt tcacgggctt    180
cgttacctta aggaacggaa cagtcgtaag ccgatctatt ctgattccca gacggcaatc    240
gcatgggtga aggataaaaa agcagcatcg accctcgtgc gcaatgaaga aacagcgctt    300
atttgaagc ttgtagatga agcggaggag tggctaaaca ctcataccta tgaaacgccc    360
atcttagcat ggcagaccga taagtggggg gaaattaagg ccgattacgg gggcaaaaaa    420
cagcacgcgt gcccggaatg cggtaaatct ttctctcagt cttctaacct gcagaaacac    480
cagcgtacct acaccgggtga aaaaccgtac aaatgtccag aatgtggcaa aagctttagt    540
caaagtctta atcttcaaaa acatcaacgc acgcataccg gcgagaagcc atataagtgt    600
ccggagtgcg gcaaaagctt ctcccgtct gatcacctcc agcgtcatca gcgcactcat    660
cagaacaaaa aactcgagca ccaccaccac caccac
    
```

<210> 8

<211> 232

<212> PRT

<213> Artificial

<220>

<223> fusion of variant GGKKQ of catAEA-ZfQQR - amino acid sequence

<400> 8

```

Met Ala Lys Glu Glu Ile Ile Trp Glu Ser Leu Ser Val Asp Val Gly
 1          5          10          15
Ser Gln Gly Asn Pro Gly Ile Val Glu Tyr Lys Gly Val Asp Thr Lys
 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 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
130          135          140
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
195          200          205
Arg Ser Asp His Leu Gln Arg His Gln Arg Thr His Gln Asn Lys Lys
210          215          220
Leu Glu His His His His His His
225          230
    
```

<210> 9

<211> 75

<212> RNA

<213> Artificial

<220>

<223> RNA strand that contains the binding sequence for ZfQQR

<400> 9
 agaacuagug gaucaaccgg gcugcaggaa uucgauauca agcuuauca uaccguggcg 60
 guucuucucc aagcc 75

<210> 10
 <211> 74
 <212> DNA
 <213> artificial

<220>
 <223> DNA strand of the substrate that contains the binding sequence for ZFQR

<400> 10
 gcttggggaa gaaccgccac ggtatcgata agcttgatat cgaattcctg cagcccggtt 60
 gatccactag ttct 74

<210> 11
 <211> 32
 <212> DNA
 <213> artificial

<220>
 <223> primer

<400> 11
 gacgcatatg gcaaaagagg agattattg gg 32

<210> 12
 <211> 26
 <212> DNA
 <213> artificial

<220>
 <223> primer

<400> 12
 gtggtacctt ttctccgta atcggc 26

<210> 13
 <211> 17
 <212> DNA
 <213> artificial

<220>
 <223> primer

<400> 13
 ggtctggtg acccggg 17

<210> 14
 <211> 21
 <212> DNA
 <213> artificial

<220>
 <223> primer

<400> 14
 gggatcgcag tggtagtaa c 21

<210> 15
 <211> 26
 <212> DNA

<213> artificial

<220>

<223> primer

<400> 15

cgggaaaaca gcattccagg tattag 26

<210> 16

<211> 23

<212> DNA

<213> artificial

<220>

<223> primer

<400> 16

ccgtctggga atcagaatag atc 23

<210> 17

<211> 27

<212> DNA

<213> artificial

<220>

<223> primer

<400> 17

caatcgcacg ggtgaaggat aaaaaag 27

<210> 18

<211> 21

<212> DNA

<213> artificial

<220>

<223> primer

<400> 18

ctgcttttt atccttcacc c 21

<210> 19

<211> 27

<212> DNA

<213> artificial

<220>

<223> primer

<400> 19

caatcgcacg ggtgaaggat aaaaaag 27

<210> 20

<211> 24

<212> DNA

<213> artificial

<220>

<223> primer

<400> 20

taagatgggc gttcatagg tatg 24

<210> 21

<211> 20

<212> DNA

<213> artificial

<220>

<223> primer

<400> 21

gcatggcaga ccgataagtg 20

<210> 22

<211> 25

<212> DNA

<213> artificial

<220>

<223> primer

<400> 22

ggctccggcc agcacgctg cccgg 25

<210> 23

<211> 31

<212> DNA

<213> artificial

<220>

<223> primer

<400> 23

agaaccgctc ccgtaatcg ccttaattc c 31

<210> 24

<211> 21

<212> DNA

<213> artificial

<220>

<223> primer

<400> 24

caaaaaacag cacgcgtgcc c 21

<210> 25

<211> 25

<212> DNA

<213> artificial

<220>

<223> primer

<400> 25

ccccgtaat cggccttaat ttccc 25

<210> 26

<211> 78

<212> DNA

<213> artificial

<220>

<223> contains the binding site for ZfQQR

<400> 26

gagatctaga cggaacatga aggggaagaa ttctatgctt agtgagatct agagccctat 60
 agtgagtcgt attaaatt 78
 <210> 27
 <211> 78
 <212> DNA
 <213> artificial
 <220>
 <223> do not contain a binding site for ZfQQR
 <400> 27
 gagatctaga cggaacatga agctcgagcc ttctatgctt agtgagatct agagccctat 60
 agtgagtcgt attaaatt 78
 <210> 28
 <211> 78
 <212> DNA
 <213> artificial
 <220>
 <223> template with nonanucleotide random sequence
 <220>
 <221> misc_feature
 <222> (22)..(30)
 <223> n is a, c, g, or t
 <400> 28
 gagatctaga cggaacatgt anhhhhnnnn tactatgctt agtgagatct agagccctat 60
 agtgagtcgt attaaatt 78
 <210> 29
 <211> 22
 <212> DNA
 <213> artificial
 <220>
 <223> DNA strand of the DNA-RNA hybrid
 <400> 29
 tcactgggga agaagaatcc tc 22
 <210> 30
 <211> 22
 <212> RNA
 <213> artificial
 <220>
 <223> RNA strand of the DNA-RNA hybrid
 <400> 30
 gaggauucuu cuuucccagu ga 22
 <210> 31
 <211> 22
 <212> DNA
 <213> artificial
 <220>
 <223> DNA strand of the DNA-RNA hybrid
 <400> 31

tcactggtcg gtgggaatcc tc 22

<210> 32

<211> 22

<212> RNA

<213> artificial

<220>

<223> RNA strand of the DNA-RNA hybrid

<400> 32

gaggauuccc accgaccagu ga 22

<210> 33

<211> 46

<212> DNA

<213> artificial

<220>

<223> starter

<400> 33

aatttaatac gactcactat agggctctag atctcactaa gcatag 46

<210> 34

<211> 19

<212> DNA

<213> artificial

<220>

<223> starter

<400> 34

gagatctaga cggaacatg 19

<210> 35

<211> 17

<212> DNA

<213> artificial

<220>

<223> starter

<400> 35

ggttctggtg acccggg 17

<210> 36

<211> 26

<212> DNA

<213> artificial

<220>

<223> starter

<400> 36

cgggaaaaca gcattccagg tattag 26

<210> 37

<211> 975

<212> DNA

<213> artificial

<220>

<223> fusion of the GST domain and zinc finger ZfQQR

<400> 37

```

atgtccccta tactaggtta ttggaaaatt aagggccttg tgcaaccac tcgacttett    60
ttggaatatac ttgaagaaaa atatgaagag catttgatg agcgcgatga aggtgataaa    120
tggcgaaaca aaaagtttga attgggtttg gagtttcca atcttcctta ttatattgat    180
ggtgatgta aattaacaca gtctatggcc atcatacgtt atatactga caagcacacac    240
atggtgggtg gttgtccaaa agagcgtgca gagattcaa tgcttgaagg agcggttttg    300
gatattagat acgggtgttc gagaattgca tatagtaaag accttgaaac tctcaaaagt    360
gattttctta gcaagctacc tgaatgctg aaaaatgttc aagatcgttt atgtcataaa    420
acatatattaa atggtgatca tgtaaccat cctgacttca tgttgatga cgctcttgat    480
gttgtttat acatggacc aatgtgcctg gatgcgttcc caaaattagt ttgttttaa    540
aaacgtattg aagctatccc acaaatgat aagtacttga aatccagcaa gtatatagca    600
tggcctttgc agggctggca agccacgttt ggtggtggcg accatcctcc aaaatcggat    660
ctggttccgc gtggatccc ggaattccg ggtaaaaaaa aacagcacgc gtgccggaa    720
tgcgtaaat ctttctctca gtcttctaac ctgcagaaac accagcgtac ccacaccggt    780
gaaaaaccgt acaaatgtcc agaatgtggc aaaagcttta gtcaaaagttc taatcttcaa    840
aaacatcaac gcacgcatac cggcgagaag ccatataagt gtccggagtg cggcaaaagc    900
ttctccgct ctgacacact ccagcgtcat cagcgcactc atcagaacaa aaaactcgag    960
cggccgcatc gtgac                                     975

```

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- [WO2010076939A1 \[0004\] \[0004\] \[0006\]](#)
- [WO03087341A2 \[0004\] \[0004\] \[0006\]](#)
- [WO2009146179A1 \[0004\] \[0004\] \[0006\]](#)
- [WO2007014181A2 \[0004\]](#)
- [WO2007014182A2 \[0004\]](#)
- [US2006057590A \[0008\] \[0008\]](#)
- [JP54059392B \[0008\] \[0008\]](#)

Non-patent literature cited in the description

- **ELLINGTON, A.D.SZOSTAK, J.W.**Nature, 1990, vol. 346, 818-822 [0005]
- **HUIZENGA DESZOSTAK JW.**Biochemistry, 1995, vol. 34, 2656-65 [0005]
- **BLACKWELL TKWEINTRAUB H.**Science, 1990, vol. 250, 1104-1110 [0005]
- **HERSKOVITZ M.A. et al.**Endoribonuclease RNase III is essential in Bacillus subtilisMol Microbiol., 2000, vol. 38, 51027-33 [0007]
- **DASGUPTA S. et al.**Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the Escherichia coli endoribonuclease RNase III—the effect of dsRNA binding on gene expressionMol Microbiol., 1998, vol. 28, 3629-40 [0007]
- **SHAHBABIAN et al.**RNase Y, a novel endoribonuclease, initiates riboswitch turnover in Bacillus subtilisThe EMBO Journal, 2009, vol. 28, 3523-3533 [0007]
- **SHIBERG**Specific DNA-RNA hybrid binding by zinc finger proteinsScience, 1995, vol. 268, [0022]
- **SHI YBERG JM**Specific DNA-RNA hybrid binding proteinsScience, 1995, vol. 268, 282-284 [0027] [0046]
- **CROOKS GEHON GCHANDONIA JMBRENNER SE**WebLogo: A sequence logo generatorGenome Research, 2004, vol. 14, 1188-1190 [0052]
- **SCHNEIDER TDSTEPHENS RM**Sequence Logos: A New Way to Display Consensus SequencesNucleic Acids Res., 1990, vol. 18, 6097-6100 [0052]

P A T E N T K R A V

1. Ribonuclease, som kløver RNA-strengen i DNA-RNA-hybrider, hvor ribonucleasen er et fusionsprotein omfattende
et derivat af det katalytiske domæne af ribonuclease-HI (RNase-HI),
5 hvor derivatet af det katalytiske domæne af RNase-HI er fra *Bacillus halodurans*, omfattende polypeptid kodet for af nukleotiderne 175 til 588 af *rnhA*-genet vist i SEQ ID No:1, og
hvor derivatet af det katalytiske domæne af RNase-HI omfatter mindst én substitution af én aminosyrerest i det substratbindende domæne valgt blandt: K81A, K89E og
10 K123A refererende til sekvensen af polypeptidet kodet af SEQ ID NO: 1, fortrinsvist indeholdende alle substitutionerne K81A, K89E og K123A refererende til sekvensen af polypeptidet kodet af SEQ ID NO: 1, og
hvor derivatet af det katalytiske domæne af RNase-HI, ikke omfatter et RNase-HI-hybridbindende domæne,
15 med et zinkfinger-DNA-RNA-hybridbindende domæne, hvor det zinkfingerbindende domæne har evnen til at binde til en specifik sekvens i DNA-RNA-hybriden.
2. Ribonuclease ifølge krav 1, hvor zinkfingerdomænet er et derivat af zinkfingereren ZfQQR, fortrinsvis et polypeptid kodet for af nukleotiderne fra 19 til 303 af sekvensen ZfQQR vist i SEQ ID No.2.
20 3. Ribonuclease ifølge krav 1-2, omfattende et fusionsprotein catAEA-ZfQQR som vist i SEQ ID No.4, eller et fusionsprotein GQ som vist i SEQ ID No.6, eller omfattende et fusionsprotein GGKKQ som vist i SEQ ID No.8.
4. Sammensætning omfattende en ribonuclease ifølge krav 1-3.
5. Anvendelse af en ribonuclease ifølge krav 1-3 eller en sammensætning ifølge krav
25 4, til *in vitro* kløvning af RNA-strengen i DNA-RNA-hybrider, fortrinsvist, hvor kløvningen af RNA-strengen i DNA-RNA-hybriderne er placeret 2-16 nukleotider, fortrinsvist 5-7 nukleotider, væk fra det bindende sted for zinkfingeren.
6. Fremgangsmåde til tilvejebringelse af en teknisk konstrueret variant af RNase-HI, som kløver RNA-strengen i DNA-RNA-hybrider, omfattende de følgende trin at:
30 a) tilvejebringe et derivat af det RNase-HI katalytiske domæne, hvor derivatet af det katalytiske domæne af RNase-HI er fra *Bacillus halodurans*, omfattende et polypeptid kodet for af nukleotiderne 175 til 588 af *rnhA*-genet vist i SEQ ID No:1, og
hvor derivatet af det katalytiske domæne af RNase-HI omfatter mindst én substitution af én aminosyrerest i det substratbindende domæne valgt blandt: K81A, K89E og
35 K123A refererende til sekvensen af polypeptidet kodet af SEQ ID NO: 1, fortrinsvist indeholdende alle substitutionerne K81A, K89E og K123A refererende til sekvensen af polypeptidet kodet af SEQ ID NO: 1, og
hvor derivatet af det katalytiske domæne af RNase-HI ikke omfatter et RNase-HI-hybridbindende domæne,

b) tilvejebringe en teknisk konstrueret RNase-HI, ved at fremstille et fusionsprotein, som omfatter derivatet af det RNase-HI katalytiske domæne tilvejebragt i trin a) med et zinkfingerbindende domæne, som har evnen til at binde til specifikke sekvenser i DNA-RNA-hybriden,

- 5 fortrinsvist, hvor zinkfingerdomænet er et derivat af zinkfingeren ZfQQR, fortrinsvist et polypeptid kodet for af nukleotiderne fra 19 til 303 af sekvensen ZfQQR vist i SEQ ID No.2.

DRAWINGS

Fig. 1

Primer name	Sequence	Number of SEQ ID No
Bhcatf	GACGCATATGGCAAAAAGAGGAGATTATTTGGG	SEQ ID No:11
Bheatr	GTGGTACCTTTTCTCCCGTAATCGGC	SEQ ID No:12
BhZf	GGTCTGGTGACCCGGG	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
del1f	GGCTCCGGCCAGCACGCGTGCCCGG	SEQ ID No:22
del1r	AGAACCGCTCCCGTAATCGGCCTTAATTCC	SEQ ID No:23
del5f	CAAAAAACAGCACGCGTGCCC	SEQ ID No:24
del5r	CCCCCGTAATCGGCCTTAATTCCC	SEQ ID No:25

Fig. 2

(A) RNA:

AGAACUAGUGGAUCAACCGGGCUGCAGGAAUUCGAUAUCAAGCUUAUCGAUA
CCGUGGCGGUUCUUCCECAAGCC (SEQ ID No:9)

(B) DNA:

GCTTGGGGAAGAACCGCCACGGTATCGATAAGCTTGATATCGAATTCCTGCAG
CCCGGTTGATCCACTAGTTCT (SEQ ID No:10)

Fig. 3

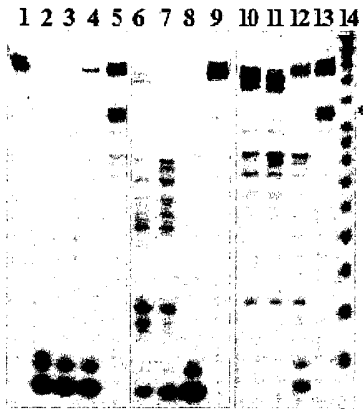


Fig. 4

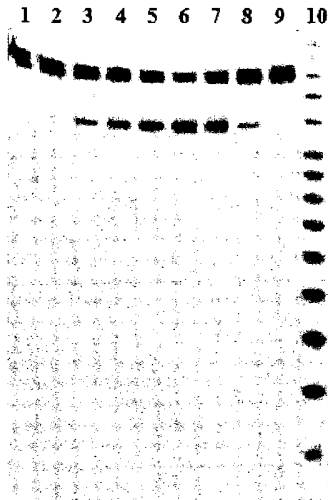


Fig. 5

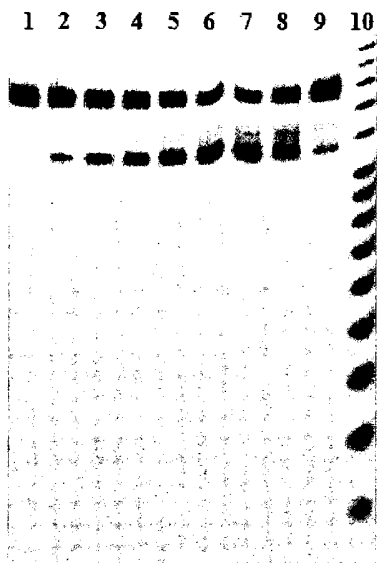


Fig. 6

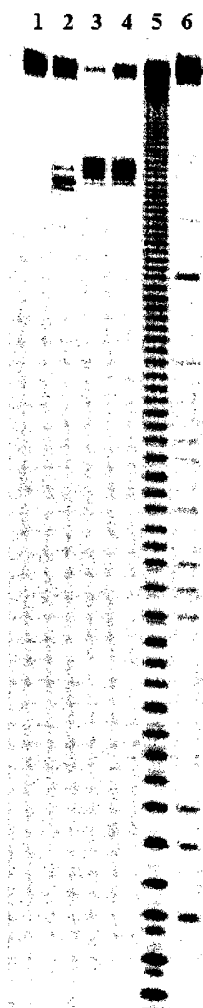


Fig. 7

5' - AGAACUAGUGGAUCAACCGGGCUGCAGGAAUUCGAUUAUCAAGCUUAUCGAUACCGUGGCCGGUUCUCCCAAGCC - 3'
 3' - TCTTGATCACCTAGTTGGCCGACGTCCTTAAGCTATAGTTCGAATAGCTATGGCACCGCCUAGAAGGGGTTCGG - 5'

Fig. 8

5' - AGAACUAGUGGAUCAACCGGGCUGCAGGAAUUCGAUUAUCAAGCUUAUCGAUACCGUGGCCGGUUCUCCCAAGCC - 3'
 3' - TCTTGATCACCTAGTTGGCCGACGTCCTTAAGCTATAGTTCGAATAGCTATGGCACCGCCUAGAAGGGGTTCGG - 5'

Fig. 9

5' - AGAACUAGUGGAUCAACCGGGCUGCAGGAAUUCGAUUAUCAAGCUUAUCGAUACCGUGGCCGGUUCUCCCAAGCC - 3'
 3' - TCTTGATCACCTAGTTGGCCGACGTCCTTAAGCTATAGTTCGAATAGCTATGGCACCGCCUAGAAGGGGTTCGG - 5'

Fig. 10

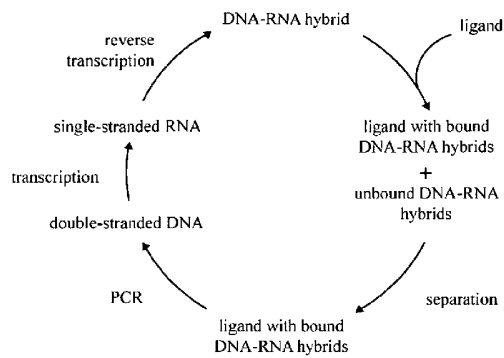


Fig. 11

Template A:

GAGATCTAGACGGAACATGAAGGGGAAGAATTCTATGCTTAGTGAGATCTAGAGCC
 CTATAGTGAGTCGTATTAATTAATT (SEQ ID No:26)

Template B:

GAGATCTAGACGGAACATGAAGCTCGAGCCTTCTATGCTTAGTGAGATCTAGAGCC
 CTATAGTGAGTCGTATTAATTAATT (SEQ ID No:27)

Template 9N:

GAGATCTAGACGGAACATGTANNNNNNNNTACTATGCTTAGTGAGATCTAGAGCC
 CTATAGTGAGTCGTATTAATTAATT (SEQ ID No:28)

Fig. 12

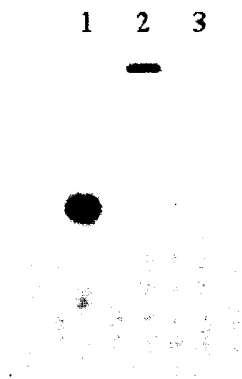


Fig. 13

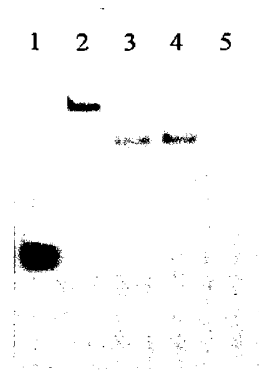


Fig. 14

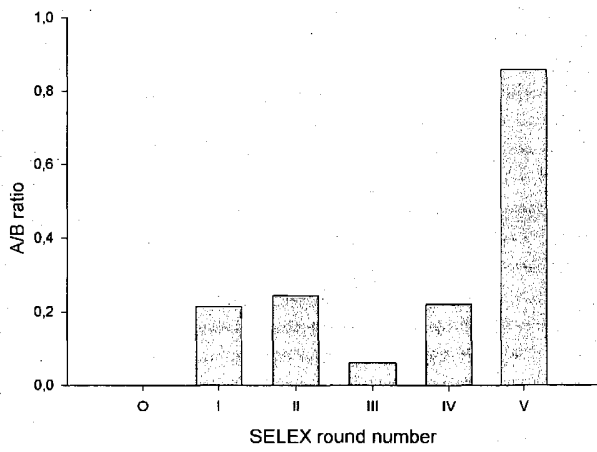


Fig. 15

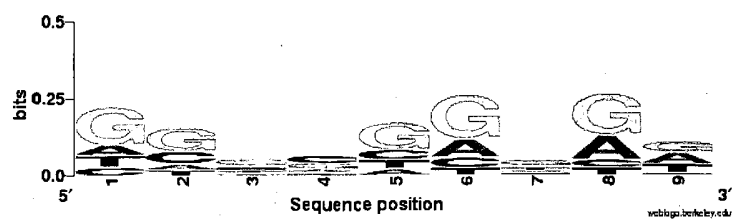


Fig. 16

A)

LDNA: TCACTGGGGAAGAAGAATCCTC (SEQ ID No:29)

LRNA: GAGGAUUCUUCUCCCCAGUGA (SEQ ID No:30)

B)

CDNA: TCACTGGTCGGTGGGAATCCTC (SEQ ID No:31)

CRNA: GAGGAUCCCACCGACCAGUGA (SEQ ID No:32)