



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/12, 5/10, 5/16, C12Q 1/68,</b> <b>C12P 19/34, C07K 14/47</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/06845</b> <b>(43) International Publication Date:</b> 19 February 1998 (19.02.98)
<b>(21) International Application Number:</b> PCT/US97/14254 <b>(22) International Filing Date:</b> 14 August 1997 (14.08.97)  <b>(30) Priority Data:</b> 60/024,057      16 August 1996 (16.08.96)      US 08/794,546      3 February 1997 (03.02.97)      US 08/795,395      4 February 1997 (04.02.97)      US 08/910,731      13 August 1997 (13.08.97)      US  <b>(71) Applicant:</b> LIFE TECHNOLOGIES, INC. [US/US]; 9800 Medical Center Drive, Rockville, MD 20850 (US).  <b>(71)(72) Applicants and Inventors:</b> CHATTERJEE, Deb, K. [US/US]; 6 Forest Ridge Court, Potomac, MD 20878 (US). SHANDILYA, Harini [IN/US]; 6115 Twain Drive, New Market, MD 21774 (US).  <b>(74) Agents:</b> ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MAMMALIAN RIBONUCLEASE INHIBITORS AND USE THEREOF  <b>(57) Abstract</b> <p>The invention relates to the cloning of a gene encoding rat liver RI, and its expression in a cellular host. In addition, the invention relates to the successful cloning of a gene encoding porcine liver RI, and its expression in a cellular host. The invention also relates to the cloning and expression of human liver RI, and the cloning and expression of chimeric mammalian RIs, particularly chimeric porcine/human liver RIs, which may be thermostable. The invention also relates to methods and kits for use in producing nucleic acid molecules and polypeptides using the RIs of the invention, and to nucleic acid molecules and polypeptides produced using these methods and kits.</p>		

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## Mammalian Ribonuclease Inhibitors and Use Thereof

### FIELD OF THE INVENTION

The present invention is in the fields of molecular biology and protein chemistry. The invention relates to the successful cloning of a full length cDNA of porcine liver ribonuclease inhibitor (RI) gene and its expression in a cellular host. In addition, the invention relates to the cloning of full length rat liver RI, and its expression in a cellular host. The invention also relates to the successful cloning of full length human liver RI, and to the cloning of chimeric mammalian RIs, particularly a chimeric porcine/human RI, which demonstrate increased thermostability relative to non-human RIs.

### BACKGROUND OF THE INVENTION

RI is a cytoplasmic protein ubiquitously present in a variety of mammalian tissues. It inhibits a variety of ribonucleases (RNases) by binding tightly to both intracellular and extracellular RNases by forming a 1:1 complex (Roth, J.S., *Methods Cancer Res.* 3:153-243 (1967); Blackburn, P., *et al.*, *J. Biol. Chem.* 252:5904-5910 (1977); Blackburn, P., *J. Biol. Chem.* 254:12484-12487 (1979); Blackburn, P., and Moore, S., *Enzymes*, 3rd Ed. (1982), vol. 15, pp. 317-433; Lee, F.S., *et al.*, *Biochemistry* 27:8545-8553 (1988)). Some evidence indicates that tissues which are highly active in protein synthesis have a high excess of RI over RNase. Conversely, in catabolically active tissues that do not accumulate RNA, the ratio of RI to RNase is low (Kraft, N., and Shortman, K., *Biochim. Biophys. Acta.* 217:164-175 (1970)). The biological function of RI has been implicated to be in (a) regulation of RNA turnover by controlling cytoplasmic RNase activity, (b) safeguarding against non-cytoplasmic RNases that mislocalized to the cytoplasm, and (c) regulation of angiogenin, a protein that induces blood vessel growth and contains RNase activity. *In vitro*, RI is useful in a variety of molecular biology applications where RNase contamination is a potential problem. Examples of these applications include reverse transcription of mRNA, cell-free translation systems, preparation of RNase-free antibodies, and *in vitro* virus replication. Ideally, RI to be used in these kinds of applications will be capable of inhibiting a large number of RNases, such as eukaryotic RNase A, RNase B and RNase C, as well as prokaryotic RNases.

RI has been purified to homogeneity from several mammalian tissues, including placenta (Blackburn, P., *et al.*, *J. Biol. Chem.* 252:5904-5910 (1977); Blackburn, P., *J. Biol. Chem.*

254:12484-12487 (1979)), brain (Burton, L.E., *et al.*, *Int. J. Pept. Protein Res.* 16:359-364 (1980)) and liver (Burton, L.E., and Fucci, N.P., *Int. J. Pept. Protein Res.* 18:372-379 (1982); Hofsteenge, J., *et al.*, *Biochemistry* 27:8537-8544 (1988)). The protein has an apparent molecular mass of approximately 50 kilodaltons (kD). The primary amino acid sequence of various RIs, such as human placental (Lee, F.S., *et al.*, *Biochemistry* 27:8545-8553 (1988)), porcine liver (Hofsteenge, J., *et al.*, *Biochemistry* 27:8537-8544 (1988)) and rat lung (Kawanomoto, M., *et al.*, *Biochim. Biophys. Acta.* 1129:335-338 (1992)) is known. The crystal structure of porcine RI has been published (Kobe, B., and Deisenhofer, J., *Nature* 366:751-756 (1993)). The human placental RI has been successfully cloned and expressed in *E. coli* (Lee, F.S., *et al.*, *Biochemistry* 27:8545-8553 (1988); Promega Catalog 1993/1994). A complete rat lung RI cDNA has been described (Kawanomoto *et al.*, *Biochim. Biophys. Acta.* 1129:335-338 (1992)); this cDNA has been used to study the distribution of the RI mRNA in various tissues.

The complete coding sequence of rat lung RI is known, but recombinant rat lung RI has not been expressed in either *E. coli* or any other known expression host (Kawanomoto, M., *et al.*, *Biochim. Biophys. Acta* 1129:335-338 (1992)). Several attempts to isolate a cDNA clone for rat liver RI have similarly been unsuccessful (*Id.*).

The complete amino acid sequence of porcine RI has been determined by direct sequencing of the purified protein. In addition, a partial cDNA sequence of porcine kidney RI has been described by Vicentini, A.M., *et al.* (*Biochemistry* 29:8827-8834 (1990)). The cDNA lacks 241 nucleotides at the 5'-end of the coding sequence corresponding to the first 81 amino acids of porcine kidney RI. However, a synthetic complete porcine kidney RI coding sequence has been prepared by ligating a synthetic oligonucleotide encoding amino acid residues 1-81 of porcine RI, the sequence of which is based on the amino acid sequence of porcine liver RI, to a restriction fragment of the incomplete cDNA which corresponds to amino acid residues 82-456 of the above-mentioned cDNA. This protein has been expressed in *Saccharomyces cerevisiae*.

From the foregoing, it will be clear that there is a need in the art for recombinantly produced RNase inhibitors that are active against a broad range of RNases from both eukaryotic and prokaryotic sources.

## BRIEF SUMMARY OF THE INVENTION

The invention relates to the successful cloning of a full length cDNA of porcine liver RI gene and its expression in a cellular host.

More particularly, the invention relates to a composition comprising an isolated nucleic acid molecule comprising a nucleotide sequence (SEQ ID NO:1) encoding the complete coding sequence of porcine ribonuclease inhibitor (RI). In another embodiment, this nucleic acid molecule is operably linked to a promoter for expression of porcine RI. In another embodiment, the invention relates to a vector comprising this latter construct, which when introduced into a cellular host leads to expression of porcine RI. In another embodiment, the invention relates to a host cell comprising the above-described vector and to recombinant hosts comprising the porcine RI sequence. The invention also relates to a method of obtaining a porcine RI polypeptide (such as that depicted in SEQ ID NO:2), comprising obtaining the host cell or recombinant host described above and isolating a porcine RI polypeptide from it.

The invention also relates to the cloning of a cDNA encoding rat liver RI, and its expression in a cellular host.

More particularly, the invention relates to a composition comprising an isolated nucleic acid molecule comprising a nucleotide sequence encoding the complete coding sequence of rat liver RI (SEQ ID NO:3). The invention also relates to a molecule wherein the coding sequence is operably linked to a promoter for expression of rat liver RI. In another embodiment, the invention relates to a vector comprising the above described nucleic acid molecule which, when introduced into a cellular host, leads to expression of rat liver RI. In another embodiment, the invention relates to a host cell transformed with the above described vector and to recombinant hosts containing the rat RI sequence. The invention also relates to a method for obtaining a rat liver RI polypeptide (such as that depicted in SEQ ID NO:4), comprising culturing this host cell or recombinant host and isolating a rat liver RI polypeptide from it.

The invention also relates to isolated nucleic acid molecules comprising a polynucleotide encoding a human liver RI. The invention also relates to molecules in which the human liver RI sequence is operably linked to a promoter for expression of human liver RI. Preferred nucleic acid molecules include those wherein the polynucleotide encoding the human liver RI has a nucleotide sequence at least 99% identical to a reference sequence selected from the group consisting of: (a) the nucleotide sequence set forth in SEQ ID NO:5, wherein T can also be U; (b) a nucleotide

sequence encoding the human liver RI polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in NRRL Deposit No. B-21810; and (c) a nucleic acid sequence complementary to (a) or (b). The invention also relates to isolated nucleic acid molecules comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having  
5 a nucleotide sequence identical to the nucleotide sequence of the above-described nucleic acid molecules. The invention also relates to vectors comprising the human liver RI sequence, to host cells comprising such vectors, and to recombinant hosts comprising human liver RI sequences. The invention also relates to methods for producing a human liver RI polypeptide (such as that depicted in SEQ ID NO:6), the methods comprising culturing the host cells or recombinant hosts of the  
10 invention and isolating a human liver RI polypeptide. The invention also relates to a human liver RI polypeptide produced according to these methods.

In another embodiment, the invention relates to isolated nucleic acid molecules comprising a polynucleotide encoding a chimeric mammalian RI, which may be thermostable, wherein the polynucleotide comprises a substantial portion of a nucleotide sequence encoding a RI from a first  
15 mammalian species (preferably a non-human species such as porcine, rat, equine, bovine, ovine, murine, canine, feline, ape, monkey, etc.) and a substantial portion of a nucleotide sequence encoding a RI from a second mammalian species which is different from the first mammalian species (preferably a human, the polynucleotide preferably encoding a human liver RI or a human placental RI). The invention also relates to molecules in which the chimeric RI sequence is operably linked  
20 to a promoter for expression of the chimeric RI. Nucleic acid molecules according to the invention may include those comprising a polynucleotide encoding a chimeric porcine/human RI, such as those wherein the polynucleotide encoding the chimeric mammalian RI has a nucleotide sequence at least 90% or at least 95% identical to a reference sequence selected from the group consisting of: (a) the nucleotide sequence set forth in SEQ ID NO:7, wherein T can also be U; and (b) a nucleic acid  
25 sequence complementary to (a). The invention also relates to isolated nucleic acid molecules comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to the nucleotide sequence of the above-described nucleic acid molecules. The invention also relates to vectors containing chimeric RI sequences, to host cells comprising such vectors, and to recombinant hosts containing the chimeric  
30 RI sequences.

The invention also relates to methods for producing a chimeric mammalian RI polypeptide, which may be thermostable, the methods comprising culturing the host cells of the invention and

isolating a chimeric mammalian RI polypeptide. The invention also relates to a chimeric mammalian RI polypeptide produced according to these methods, which may be a chimeric porcine/human RI polypeptide (such as that depicted in SEQ ID NO:8) and which may be thermostable.

The invention also relates to any of the described nucleic acid molecules which may further  
5 comprise one or more nucleotide sequences encoding one or more binding peptides. Such sequences encoding binding peptides may be located 5' to the translation start site of the polynucleotide encoding the RI or chimeric RI. Binding peptides according to this aspect of the invention may include an OmpA signal sequence, a GST tag, a HIS tag, a thioredoxin tag or a hemagglutinin (HA) tag. Vectors containing these modified molecules, host cells comprising such vectors and  
10 recombinant hosts containing these modified molecules are also contemplated by the invention.

In another aspect, the invention relates to methods for producing a nucleic acid molecule comprising mixing a template nucleic acid molecule, which may be an RNA molecule such as a mRNA or polyA<sup>+</sup> RNA molecule, with one or more of the RI polypeptides of the invention (preferably chimeric RI such as a chimeric porcine/human RI polypeptide) under conditions sufficient  
15 to synthesize a nucleic acid molecule from the template molecule. According to this aspect of the invention, the mixture may further comprise one or more thermostable DNA polymerases such as *Tne*, *Tma*, *Taq*, *Pfu*, *Tth*, *Tfi*, VENT, DEEPVENT, *Pwo* or *Tfl* polymerases, or mutants, variants and derivatives thereof. The mixtures may also comprise one or more polypeptides having reverse transcriptase activity, such as M-MLV reverse transcriptase, RSV reverse transcriptase, AMV  
20 reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase or HIV reverse transcriptase. These polypeptides having reverse transcriptase activity may be substantially reduced in RNase H activity; preferred such polypeptides include M-MLV H<sup>-</sup> reverse transcriptase, RSV H<sup>-</sup> reverse transcriptase, AMV H<sup>-</sup> reverse transcriptase, RAV H<sup>-</sup> reverse transcriptase, MAV H<sup>-</sup> reverse transcriptase and HIV H<sup>-</sup> reverse transcriptase. The mixtures used in these methods may also  
25 optionally comprise one or more polypeptides having RNA polymerase activity. According to the invention, the methods for producing a nucleic acid molecule may be performed at temperatures of from about 45°C to about 70°C, from about 50°C to about 65°C, from about 55°C to about 60°C, from about 56°C to about 60°C, from about 57°C to about 60°C, from about 58°C to about 60°C, from about 59°C to about 60°C, or at a temperature of 50°C or greater, 51°C or greater, 52°C or  
30 greater, 53°C or greater, 54°C or greater, 55°C or greater, 56°C or greater, 57°C or greater, 58°C or greater, 59°C or greater, 60°C or greater, 61°C or greater, 62°C or greater, 63°C or greater, 64°C or greater or 65°C or greater.

The invention also relates to nucleic acid molecules produced according to these methods, which may be DNA molecules (including cDNA molecules), RNA molecules or DNA-RNA hybrid molecules, and which may be single-stranded or double-stranded.

5 In another aspect, the invention relates to methods for *in vitro* production of a polypeptide comprising mixing a mRNA or polyA<sup>+</sup> RNA molecule with one or more of the RIs of the invention (such as a porcine liver, rat liver, human liver or chimeric mammalian (particularly a chimeric porcine/human) RI polypeptide), under conditions sufficient to translate a polypeptide from the mRNA or polyA<sup>+</sup> RNA molecule. The invention also relates to polypeptides produced according to these methods.

10 The invention is also directed to kits comprising one or more containers containing one or more of the above-described RIs of the invention. Kits according to this aspect of the invention may further comprise additional containers containing, for example, one or more of the thermostable DNA polymerases, polypeptides having reverse transcriptase activity or polypeptides having RNA polymerase activity described above. Additional kits of the invention may further comprise one or  
15 more containers containing a cell-free protein translation mixture.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** is a map of plasmid pRatRI.

20 **Figure 2** is a map of plasmid pRERatRI.

**Figure 3** is a map of plasmid pPorcineRI.

**Figure 4** is a map of plasmid pHumanRI.

**Figure 5** is a map of plasmid pPorcineHuRI.



## DETAILED DESCRIPTION OF THE INVENTION

**Definitions**

In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

**Cloning vector.** A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, provide tetracycline resistance or ampicillin resistance.

**Expression vector.** A vector similar to a cloning vector but which is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (*i.e.*, operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either constitutive, repressible or inducible.

**Substantially pure.** As used herein, this term means that the desired purified protein is free from contaminating cellular components which would substantially inhibit the ribonuclease inhibiting activity of the protein. For example, a "substantially purified" or "substantially pure" RI means a preparation of RI polypeptide wherein at least 50%, preferably at least 70%, and more preferably at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of contaminating proteins (*i.e.*, those that are not RI proteins) have been removed from the preparation. "Substantially pure" does not indicate that the protein must be completely free of all contaminants.

**Substantially RNase Free.** The terms "substantially RNase free" and "substantially free of RNase" are defined herein as having less than 10%, preferably about or less than 1% and most preferably about or less than 0.1%, of the RNase activity found when purifying ribonuclease inhibitor with an RNase affinity column. For assays to determine RNase activity and ribonuclease inhibitor activity, see Goldman *et al.*, *Prep. Biochem.* 11:49-67 (1981).

**Recombinant Host.** According to the invention, a recombinant host may be any prokaryotic or eukaryotic microorganism which contains the desired cloned genes on an expression vector or

cloning vector. This term is also meant to include those microorganisms that have been genetically engineered to contain the desired gene(s) in the chromosome or genome of that organism.

**Recombinant vector.** Any cloning vector or expression vector which contains the desired cloned gene(s).

5       **Host.** Any prokaryotic or eukaryotic microorganism that is the recipient of a replicable expression vector or cloning vector. A "host," as the term is used herein, also includes prokaryotic or eukaryotic microorganisms that can be genetically engineered by well known techniques to contain desired gene(s) on its chromosome or genome. For examples of such hosts, see Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

10       **Promoter.** A DNA sequence generally described as the 5' region of a gene, located proximal to the start codon. The transcription of an adjacent gene(s) is initiated at the promoter region. Promoters can be inducible, repressible or constitutive. Genes that are under the control of an inducible or repressible promoter are transcribed at levels that vary in response to the external environment. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. If a promoter is a repressible promoter, then the rate of transcription decreases in response to repressor (or negative regulator). It is possible for a promoter to be both inducible and repressible. Transcription from such a promoter will be inhibited in response to a repressor; this inhibition may be reversed by the action of an inducing agent. An example of such a promoter that is well known by those of ordinary skill in the art is the *lac* promoter. A description of the regulation of the *lac* promoter may be found in Lewin, *Genes V*, Oxford University Press, New York (1994). In contrast, if the promoter is a constitutive promoter, the rate of transcription is not regulated by the external environment.

20       **Repression.** Repression is the inhibition of transcription effected by the binding of repressor protein to a specific site on DNA.

25       **Induction.** Induction is the switching on of transcription as a result of interaction of an inducer with a positive or negative regulator.

30       **Positive Regulation of Transcription.** A mechanism of control of gene expression where a gene is not transcribed unless a positive regulator, or activator, allows initiation of transcription.

**Negative Regulation of Transcription.** A mechanism of control of gene expression where a gene is transcribed unless transcription is prevented by the action of a negative regulator, or repressor.

**Repressor.** A protein which prevents transcription by binding to a specific site on DNA.

**Operator.** The site on DNA at which a repressor protein binds to prevent transcription from initiating at the adjacent promoter.

**Inducer.** A molecule that triggers gene transcription by binding to a regulator protein such  
5 as a repressor.

**Gene.** A DNA sequence that contains information needed for expressing a polypeptide or protein.

**Structural gene.** A DNA sequence that is transcribed into messenger RNA (mRNA) that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

10 **Expression.** Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

**Chimeric.** A chimeric molecule is a molecule that is composed of a portion of a molecule obtained from one species of organism fused or otherwise joined to a portion of a molecule obtained  
15 from another species of organism. For example, a chimeric porcine/human enzyme may be made by linking, usually using genetic engineering techniques, one or more portions of the porcine form of the enzyme to one or more portions of the human form of the enzyme. Analogously, a chimeric porcine/human polynucleotide may be made by linking one or more portions of the porcine form of a polynucleotide (*e.g.*, a polypeptide-encoding gene) to one or more portions of the human form of  
20 the polynucleotide. Chimeric polypeptides may then be obtained by transfecting a host cell with one or more of these chimeric polynucleotides and culturing the host cells under conditions favoring the expression of the chimeric polynucleotides by the host cells.

## **Nucleic Acid Molecules and Polypeptides**

### ***Porcine and Rat Liver RIs***

25 The invention relates to a composition comprising an isolated nucleic acid molecule comprising a nucleotide sequence encoding the complete coding sequence of porcine ribonuclease inhibitor (RI).

The invention also relates to a composition comprising an isolated nucleic acid molecule comprising a nucleotide sequence encoding the complete coding sequence of rat ribonuclease  
30 inhibitor (RI).

In a preferred embodiment, a sequence encoding either rat or porcine RI is operably linked to a promoter for expression of the RI protein. In another preferred embodiment, a sequence encoding either rat liver or porcine liver RI is operably linked to a promoter for expression of the RI protein.

5 In another preferred embodiment, the invention relates to a composition comprising an isolated nucleic acid molecule comprising a nucleotide sequence of porcine liver RI, wherein said nucleotide sequence is selected from the group consisting of:

(a) the sequence set forth in SEQ ID NO:1, wherein T can also be U;

(b) a nucleotide sequence encoding the porcine RI polypeptide having the complete amino  
10 acid sequence encoded by the cDNA clone contained in NRRL Deposit No. B-21612 (deposited with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 USA);

(c) a nucleotide sequence complementary to (a) or (b); and

(d) fragments of (a) or (b) or (c) which fall within a portion of SEQ ID NO:1, wherein  
15 said fragments are at least 18 bases in length, and will hybridize to porcine genomic DNA encoding liver RI under stringent hybridization conditions. Preferably, the fragments fall within the portion of SEQ ID NO:1 that encodes the first 81 amino acids of porcine liver RI.

In another preferred embodiment, the invention relates to a composition comprising an isolated nucleic acid molecule comprising a nucleotide sequence of rat liver RI, wherein said  
20 nucleotide sequence is selected from the group consisting of:

(a) the sequence set forth in SEQ ID NO:3, wherein T can also be U;

(b) a nucleotide sequence encoding the rat RI polypeptide having the complete amino acid  
25 sequence encoded by the cDNA clone contained in NRRL Deposit No. B-21613 (deposited with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 USA);

(c) a nucleotide sequence complementary to (a) or (b); and

(d) fragments of (a), (b) or (c) which fall within a portion of SEQ ID NO:3, wherein said  
fragments are at least 18 bases in length, and will hybridize to rat RI under stringent hybridization conditions.

30 By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium

phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

Such nucleic acid molecules and fragments thereof are useful as DNA probes for detecting expression of RI in porcine or rat tissue by northern blot analysis, or as primers for amplification of  
5 a target sequence by the polymerase chain reaction (PCR).

Since the nucleotide sequences of porcine and rat liver RI are provided, generating such DNA fragments would be routine to the skilled artisan. For example, restriction endonuclease cleavage or shearing by sonication could easily be used to generate fragments of various sizes. Alternatively, the DNA fragments of the present invention could be generated synthetically according to known  
10 techniques.

The present invention also relates to vectors which include the isolated DNA molecules of the present invention, host cells which are genetically engineered with the recombinant vectors, and the production of porcine liver or rat liver RI polypeptides (such as those depicted in SEQ ID NOs: 2 and 4, respectively) by recombinant techniques.

15 Eukaryotic and prokaryotic hosts that may be used for cloning and expressing the enzymes of the invention are well known in the art. Vectors which replicate in such host cells are also well known. The following vectors, which are commercially available, are provided by way of example. Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A,  
20 available from Stratagene; ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia; and pUC18, pUC19 and pPROEX1, available from Life Technologies, Inc. (LTI; Rockville, Maryland). Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia (Piscataway, New Jersey). These vectors are listed solely by way of illustration of the  
25 many commercially available and well known vectors that are available to those of skill in the art for use in accordance with this aspect of the present invention. It will be appreciated that any other plasmid or vector suitable for, for example, introduction, maintenance, propagation or expression of a polynucleotide or polypeptide of the invention in a host may be used in this aspect of the invention.

Preferred prokaryotic hosts include, but are not limited to, bacteria of the genus *Escherichia*,  
30 *Bacillus*, *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, *Xanthomonas*, etc. Two of the most preferred prokaryotic hosts are *E. coli* DH10B and DH5αF'IQ (available from LTI).

Host cells can be genetically engineered to incorporate nucleic acid molecules and express polypeptides of the present invention. For instance, recombinant constructs may be introduced into host cells using well known techniques of infection, transduction, transfection, and transformation. The polynucleotides may be introduced alone or with other polynucleotides. Such other  
5 polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention.

Thus, for instance, polynucleotides of the invention may be transfected into host cells with another, separate, polynucleotide encoding a selectable marker, using standard techniques for co-transfection and selection in, for instance, mammalian cells. In this case the polynucleotides generally  
10 will be stably incorporated into the host cell genome.

Alternatively, the polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. The vector construct may be introduced into host cells by the aforementioned techniques. Generally, a plasmid vector is introduced as DNA in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. Electroporation also may  
15 be used to introduce polynucleotides into a host. If the vector is a virus, it may be packaged *in vitro* or introduced into a packaging cell and the packaged virus may be transduced into cells. A wide variety of techniques suitable for making polynucleotides and for introducing polynucleotides into cells in accordance with this aspect of the invention are well known and routine to those of skill in the art. Such techniques are reviewed at length in Sambrook *et al.* cited above, which is illustrative  
20 of the many laboratory manuals that detail these techniques.

In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, or a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The vectors, in the case of phage and  
25 viral vectors also may be and preferably are introduced into cells as packaged or encapsulated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells.

The present invention also encompasses the expression of rat liver or porcine liver RI in  
30 prokaryotic and eukaryotic cells. Therefore, preferred among vectors, in certain respects, are those for expression of the polynucleotides and polypeptides of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to

the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors provide for specific expression. Such specific expression may be inducible expression or expression only in certain types of cells or  
5 both inducible and cell-specific. Particularly preferred among inducible vectors are vectors that can be induced for expression by environmental factors that are easy to manipulate, such as temperature and nutrient additives. A variety of vectors suitable to this aspect of the invention, including constitutive and inducible expression vectors for use in prokaryotic and eukaryotic hosts, are well known and employed routinely by those of skill in the art.

10 The engineered host cells can be cultured in conventional nutrient media, which may be modified as appropriate for, *inter alia*, activating promoters, selecting transformants or amplifying genes. Culture conditions, such as temperature, pH and the like, previously used with the host cell selected for expression generally will be suitable for expression of polypeptides of the present invention as will be apparent to those of skill in the art.

15 In order to express the porcine or rat RI polypeptides of the invention, the polynucleotides encoding these genes generally will be inserted into the vector using standard techniques so that they are operably linked to the promoter for expression. The polynucleotide will be positioned so that the transcription start site is located appropriately 5' to a ribosome binding site. The ribosome binding site will be 5' to the AUG that initiates translation of the polypeptide to be expressed. Generally,  
20 there will be no other open reading frames that begin with an initiation codon, usually AUG, and lie between the ribosome binding site and the initiating AUG. Also, generally, there will be a translation stop codon at the end of the polypeptide and there will be a polyadenylation signal and a transcription termination signal appropriately disposed at the 3' end of the transcribed region.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the  
25 periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional coding sequence which codes for additional  
30 amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a tag sequence, such as a peptide or leader peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the

invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in the pPROEX1 vector (LTI), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci., USA* 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The polypeptide may also be fused to the (hemagglutinin) HA tag, which corresponds to an epitope derived of influenza hemagglutinin protein, described by Wilson *et al.*, *Cell* 37:767 (1984), for instance. The fusion of the HA tag to the target protein allows easy detection or isolation of the recombinant protein with an antibody that recognizes the HA epitope.

The sequence encoding the target protein may also be fused with sequence encoding the outer membrane protein A (OmpA) signal secretion sequence (21 amino acids) (Humphreys, D. P., *et al.*, *J. Biol. Chem.* 270:28210-28215 (1995). Other tags that may be used for this purpose include the glutathione S-transferase (GST) tag and the thioredoxin tag (Chopra, A. K., *et al.*, *Gene (Amsterdam)* 144: 81-85 (1994). Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, regions may be added to the polypeptide to facilitate enhanced expression as well as purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art.

The DNA molecule inserted in the expression vector is operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. It will be understood that numerous promoters not mentioned are suitable for use in this aspect of the invention are well known and readily may be employed by those of skill in the manner illustrated by the discussion and the examples herein. Polynucleotides of the invention, encoding the polypeptides of the invention, generally will be inserted into the vector using standard techniques so that they are operably linked to the promoter for expression. The polynucleotide will be positioned so that the transcription start site is located appropriately 5' to a ribosome binding site. The ribosome binding site will be 5' to the AUG that initiates translation of the polypeptide to be expressed. Generally, there will be no other open reading frames that begin with an initiation codon, usually AUG, and lie between the ribosome binding site and the initiating AUG. Also, generally, there will be a translation stop codon at the end of the polypeptide and there



will be a polyadenylation signal and a transcription termination signal appropriately disposed at the 3' end of the transcribed region.

In general, expression constructs will contain sites for transcription initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, in accordance with many commonly practiced procedures, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria. Hosts for of a great variety of expression constructs are well known, and those of skill will be enabled by the present disclosure readily to select a host for expressing a polypeptides in accordance with this aspect of the present invention.

Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, where the selected promoter is inducible, expression of the RI of the invention is induced by appropriate means (*e.g.*, temperature shift or exposure to chemical inducer) and cells are cultured for an additional period.

In a preferred embodiment, the plasmid pPROEX1 is used for the expression of either rat or porcine RI. pPROEX1 contains the gene for the *lacI*<sup>q</sup> repressor, which blocks expression of the inserted gene of interest (here, the gene encoding porcine or rat RI) by binding to the *lac* operator unless an inducer such as isopropylthiogalactoside (IPTG) is present.

Of course, it will be understood by those of skill in the art that other inducers besides IPTG can be utilized where the RI gene is under the control of a *lacI*<sup>q</sup>-repressible promoter, including but not limited to other thiogalactosides.

Where porcine RI is to be expressed, it is particularly preferred that the host cell expressing  
5 the porcine RI be a cell that constitutively expresses the repressor which controls porcine RI expression (see Example 2). Therefore, a particularly preferred method of expressing porcine RI comprises transforming a vector, which comprises the gene encoding porcine RI under the control of the lac promoter, into a host cell which constitutively expresses the lac repressor, and then inducing expression of porcine RI after the cells have grown to a sufficient density, such as an A590  
10 of between about 0.6 and 1.0.

It will thus be understood by those of ordinary skill that other expression systems may be utilized, so long as the expression of porcine liver RI is tightly controlled. In such an expression system, the host cell harboring the porcine RI gene can be grown to a desired cell density, at which point expression of porcine RI can be initiated.

15 In order to obtain the RI, cells typically are then harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents; such methods are well known to those skilled in the art.

20 The rat liver RI or porcine liver RI polypeptide can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. Most preferably, affinity chromatography is employed for purification. Well  
25 known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells.  
30 Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the

invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

### ***Human Liver RIs and Chimeric Mammalian RIs***

The invention also relates to nucleic acid molecules, vectors, host cells and recombinant hosts comprising human liver and chimeric RIs. The description above related to vectors, cloning, expression, protein purification, etc., also apply equally to the human and chimeric RIs of the invention. Specifically, using the techniques described in more detail below in Example 7, the invention relates to a full-length cDNA clone of human liver RI. Also provided by the present invention, as described in detail below in Example 8, are chimeric mammalian RIs comprising a substantial portion of a RI polypeptide from a first mammalian species linked to a substantial portion of a RI polypeptide from a second mammalian species.

Thus, the invention relates to isolated nucleic acid molecules comprising a polynucleotide encoding a human liver RI. Preferred nucleic acid molecules include those wherein the polynucleotide encoding the human liver RI has a nucleotide sequence at least 90%, preferably at least 95% and most preferably at least about 99%, identical to a reference sequence selected from the group consisting of: (a) the nucleotide sequence set forth in SEQ ID NO:5, wherein T can also be U; (b) a nucleotide sequence encoding the human liver RI polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in NRRL Deposit No. B-21810 (deposited with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 USA); and (c) a nucleic acid sequence complementary to (a) or (b). Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping by *in situ* hybridization with chromosomes, for detecting expression of the human liver RI gene in tissue samples, and for the production of human liver RI, among other uses detailed below.

In addition, the invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a chimeric mammalian RI, particularly wherein the polynucleotide comprises a substantial portion of a nucleotide sequence encoding a RI from a first mammalian species and a substantial portion of a nucleotide sequence encoding a RI from a second mammalian species which is different from the first mammalian species. As used herein, the term "a substantial portion of a nucleotide sequence" means at least 100 contiguous nucleotides, at least 150 contiguous nucleotides, at least 200 contiguous nucleotides or at least 250-500 contiguous nucleotides, of a nucleotide sequence encoding a given RI. According to the invention, the first mammalian species is preferably

non-human and the second mammalian species is preferably human, although nucleic acid molecules in which both mammalian species are non-human (but which are different from each other) are also intended to be encompassed by the invention. Examples of such nucleic acid molecules include, but are not limited to, those encoding a porcine/human RI, a rat/human RI, a murine/human RI, a  
5 bovine/human RI, an equine/human RI, an ovine/human RI, a canine/human RI, a feline/human RI, an ape/human RI, a monkey/human RI, etc. In a particularly preferred embodiment, the polynucleotide or portion thereof encoding a human RI encodes a human liver RI (SEQ ID NO:5) or a human placental RI (Lee, F.S., *et al.*, *Biochemistry* 27:8545-8553 (1988)).

It has unexpectedly been found in the present invention that chimeric mammalian RIs  
10 comprising a portion (*e.g.*, the carboxy terminal portion) of a human RI, such as a human liver or placental RI, makes the RI thermostable. By a "thermostable RI" is intended a RI polypeptide or fragment thereof that retains at least about 70%, 75%, 80%, 85%, 90%, 95% or 99% of its RI activity (as determined by activity assays described below in the Examples) upon exposure to or incubation at temperatures of at least about 45°C to about 60°C, or at least about 51°C, 52°C,  
15 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, or 59°C, for about 1-5 minutes. Thus, the present invention also relates to a chimeric mammalian RI wherein the chimeric mammalian RI is thermostable. The chimeric RI comprises the portion or fragment of RI involved with thermostability. Such a thermostable portion, according to the invention, can be fused to RIs or to portions of RI which may have other beneficial features (*e.g.*, specificity for a larger group or different types of RNases).

20 More particularly, the invention relates to nucleic acid molecules comprising a polynucleotide encoding a chimeric porcine/human RI. Such nucleic acid molecules may have any nucleotide sequence encoding a chimeric porcine/human RI according to the description above, including, for example, a nucleotide sequence at least about 90%, at least about 95% or at least about 99% identical to a reference sequence selected from the group consisting of: (a) the nucleotide sequence  
25 set forth in SEQ ID NO:7, wherein T can also be U; and (b) a nucleic acid sequence complementary to (a). It will be apparent to the skilled artisan, however, that equivalent polynucleotides encoding a chimeric porcine/human RI, but having nucleotide sequences that may be different from that set forth in SEQ ID NO:7, may be prepared by the present methods and are therefore encompassed by the present invention. Since determined nucleotide sequences encoding a human liver RI polypeptide  
30 and two non-human mammalian RI polypeptides (specifically, porcine and rat liver RIs) are provided in SEQ ID NOs:5, 1 and 3, respectively, generating polynucleotides encoding a chimeric mammalian RI, such as a chimeric porcine/human RI or a chimeric rat/human RI, having nucleotide sequences

different from that set forth in SEQ ID NO:7, could be accomplished using methods that are well-known in the art and that therefore would be routine to the skilled artisan.

Nucleic acid molecules of the present invention which encode a human liver RI polypeptide or a chimeric mammalian RI polypeptide may include, but are not limited to, those encoding the amino acid sequence of the mature polypeptides by themselves; the coding sequence for the mature polypeptides and additional coding sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptides, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example introns and non-coding 5' and 3' sequences, such as the transcribed, untranslated regions (UTRs) or other 5' flanking sequences that may play a role in transcription (*e.g.*, via providing ribosome- or transcription factor-binding sites), mRNA processing (*e.g.*, splicing and polyadenylation signals) and stability of mRNA; the coding sequence for the mature human liver RI polypeptide (*e.g.*, SEQ ID NO:5) or chimeric mammalian RI polypeptide (*e.g.*, SEQ ID NO:7) operably linked to a regulatory DNA sequence, particularly a heterologous regulatory DNA sequence such as a promoter or enhancer; and the coding sequence for the mature human liver RI polypeptide or chimeric mammalian RI polypeptide linked to one or more coding sequences which code for amino acids that provide additional functionalities. Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain embodiments of this aspect of the invention, the marker amino acid sequence may be a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described for instance in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86:821-824 (1989), hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37: 767 (1984). Yet another useful marker peptide for facilitation of purification of human liver RI or a chimeric mammalian RI is glutathione S-transferase (GST) encoded by the pGEX fusion vector (*see, e.g.*, Winnacker, *From Genes to Clones*, New York: VCH Publishers, pp. 451-481 (1987), or HSA (human Serum Albumin) binding domain encoded by pAff2c (Nilsson *et al.*, *BioTechniques* 22:744-751 (1997)). Other such fusion proteins include a human liver RI polypeptide (*e.g.*, SEQ ID NO:5) or chimeric mammalian RI polypeptide (*e.g.*, SEQ ID NO:7) fused to immunoglobulin Fc at the N- or C-terminus.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of a human RI polypeptide or a chimeric mammalian RI polypeptide. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism (*see* Lewin, B., ed., *Genes II*, , John Wiley & Sons, New York (1985)). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of a human liver RI polypeptide or chimeric mammalian RI polypeptide or portions thereof. Also especially preferred in this regard are conservative substitutions.

The present invention is directed to nucleic acid molecules at least about 90% or at least about 95% identical to the nucleic acid sequence set forth in SEQ ID NOs:5 and 7, or to the nucleic acid sequences of the deposited cDNAs, irrespective of whether they encode polypeptides having human liver RI activity or chimeric mammalian RI activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having human liver RI activity or chimeric mammalian RI activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having human liver RI or chimeric mammalian RI activity include, *inter alia*, (1) isolating the human liver RI gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (*e.g.*, "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the human liver RI gene, as described for human gene localization in Verma *et al.*, *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and (3) northern blot analysis for detecting human liver RI mRNA expression in specific tissues or chimeric mammalian RI expression in, for example, the tissues of a transgenic animal.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least about 90% or at least about 95% identical to the nucleic acid sequences of the deposited cDNAs or the nucleic acid sequences set forth in SEQ ID NOs:5 and 7 will encode polypeptides having human

liver RI or chimeric mammalian RI polypeptide structure and/or activity. In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized by one of ordinary skill in the art that, for such nucleic acid molecules that are not degenerate  
5 variants, a reasonable number will also encode polypeptides having human liver RI or chimeric mammalian RI polypeptide structure and/or activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or unlikely to significantly affect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid). For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U.,  
10 *et al.*, *Science* 247:1306-1310 (1990), and the references cited therein.

The invention also relates to isolated nucleic acid molecules comprising polynucleotides which hybridize under stringent hybridization conditions, as defined above, to a portion of a polynucleotide having a nucleotide sequence identical to the nucleotide sequence of the above-described nucleic acid molecules. By a polynucleotide which hybridizes to a "portion" of a  
15 polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides, and more preferably at least about 20 nucleotides, still more preferably at least about 30 nucleotides, and even more preferably about 30-70 nucleotides of the reference polynucleotide. These hybridizing polynucleotides are useful, for example, as probes and primers as discussed above.

Of course, polynucleotides hybridizing to a larger portion of the reference polynucleotide  
20 (e.g., a nucleic acid molecule comprising a human liver RI or a chimeric mammalian RI gene having the nucleotide sequences set forth in SEQ ID NOs:5 and 7, respectively), for instance, a portion about 50-200 nucleotides in length, or even to the entire length of the reference polynucleotide, are also useful as probes according to the present invention, as are polynucleotides corresponding to most, if not all, of the nucleotide sequences set forth in SEQ ID NOs:5 and 7. By a portion of a  
25 polynucleotide of "at least 20 nucleotides in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the nucleotide sequences as set forth in SEQ ID NOs:5 and 7). As indicated, such portions are useful diagnostically either as a probe according to conventional DNA hybridization techniques or as primers for amplification of a target sequence by the polymerase chain reaction (PCR), as described, for instance,  
30 in *Molecular Cloning, A Laboratory Manual*, 2nd edition, Sambrook, J., Fritsch, E. F. and Maniatis, T., eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), the entire disclosure of which is hereby incorporated herein by reference.

Since determined nucleotide sequences encoding a human liver RI polypeptide and a chimeric porcine/human RI polypeptide are provided in SEQ ID NOs:5 and 7, respectively, generating polynucleotides which hybridize to a portion of the human liver RI cDNA molecule or a chimeric porcine/human RI cDNA molecule would be routine to the skilled artisan. For example, restriction  
5 endonuclease cleavage or shearing by sonication of the above-described cDNA clones could easily be used to generate DNA portions of various sizes which are polynucleotides that hybridize to a portion of the human liver RI cDNA molecule or the chimeric porcine/human cDNA molecule. Alternatively, the hybridizing polynucleotides of the present invention could be generated synthetically according to known techniques.

10 The invention also relates to such nucleic acid molecules which may further comprise one or more nucleotide sequences encoding one or more binding peptides, which sequences are preferably located 5' to the translation start site of the polynucleotides encoding the human liver RI or the chimeric mammalian RI. Binding peptides according to this aspect of the invention may include those described in detail above.

15 The invention also relates to these nucleic acid molecules, with or without the sequences encoding one or more binding peptides, wherein the nucleic acid molecules encoding the human liver RI or chimeric mammalian RI are operably linked to a promoter for expression of human liver RI or a chimeric mammalian RI. Examples of such promoters, and methods of operably linking the nucleic acid molecules of the invention to these promoters, are as described above.

20 The invention also relates to vectors comprising the above-described nucleic acid molecules, which may be expression vectors comprising the nucleic acid molecule operably linked to a promoter, which may be an inducible promoter under the control of a repressor such as *lacI*<sup>q</sup>. The invention also relates to host cells comprising these nucleic acid molecules or vectors. Particularly preferred host cells include those described above, particularly *Escherichia coli* cells and most particularly  
25 *Escherichia coli* strains DH10B and DH5 $\alpha$ F'IQ (available from LTI, Rockville, Maryland).

The invention also relates to methods for producing a human liver RI polypeptide, and to methods for producing a chimeric mammalian RI polypeptide (particularly a chimeric porcine/human RI polypeptide) which may be thermostable. Methods according to this aspect of the invention may comprise culturing one or more of the above-described host cells under conditions suitable for the  
30 expression of the nucleic acid molecule encoding the human liver RI, or the chimeric mammalian RI, by the host cells, and isolating a human liver RI polypeptide or a chimeric mammalian RI polypeptide. Techniques for introducing nucleic acids into a host cell, culturing the host cell and isolating a



polypeptide produced by the host cell are as generally described above, and more fully in the Examples below.

The invention also relates to a human liver RI polypeptide produced according to these methods. In particular, the invention provides an isolated human liver RI polypeptide having the amino acid sequence encoded by a polynucleotide having the nucleotide sequence set forth in SEQ ID NO:5 or by the deposited cDNA clone, the complete amino acid sequence set forth in SEQ ID NO:6, or a peptide or polypeptide comprising a portion of the above polypeptides. In addition, the invention relates to a chimeric mammalian RI polypeptide produced according to these methods, particularly a chimeric porcine/human (preferably human liver or placental) RI polypeptide which may be thermostable. In particular, the invention provides an isolated chimeric mammalian RI polypeptide having the amino acid sequence encoded by a polynucleotide having the nucleotide sequence set forth in SEQ ID NO:7, the complete amino acid sequence set forth in SEQ ID NO:8, or a peptide or polypeptide comprising a portion of the above polypeptides.

As used herein, the terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least two amino acids coupled by (a) peptidyl linkage(s). The term "polypeptide" is used herein to denote chains comprising ten or more amino acid residues. As is commonly recognized in the art, all oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus.

It will be recognized by those of ordinary skill in the art that some amino acid sequences of the human liver RI polypeptide or chimeric mammalian polypeptide can be varied without significant effect on the structure or function of the polypeptide. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine structure and activity. In general, it is possible to replace residues which form the tertiary structure, provided that residues performing a similar function are used. In other instances, the type of residue may be completely unimportant if the alteration occurs at a non-critical region of the polypeptide.

Thus, the invention further includes variants or mutants of the human liver RI polypeptide or chimeric mammalian RI polypeptide, including allelic variants, which show substantial structural homology to or activity of a human liver RI or chimeric mammalian RI polypeptide. Such variants or mutants may include deletions, insertions, inversions, repeats, and type substitutions (for example, substituting one hydrophilic residue for another, but not strongly hydrophilic for strongly

hydrophobic as a rule). Small changes or such "neutral" amino acid substitutions will generally have little effect on activity.

Typical conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxylated residues Ser and Thr; exchange  
5 of the acidic residues Asp and Glu; substitution between the amidated residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among the aromatic residues Phe and Tyr.

Thus, the fragment, derivative or analog of the polypeptides having amino acid sequences as set forth in SEQ ID NOs:6 or 8, or those encoded by polynucleotides having nucleic acid sequences  
10 as set forth in SEQ ID NOs:5 or 7 or by the deposited cDNA clone, may be (i) those in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue), and such substituted amino acid residue may be encoded by the genetic code or may be an amino acid (*e.g.*, desmosine, citrulline, ornithine, etc.) that is not encoded by the genetic code; (ii) those in which one or more of the amino acid residues  
15 includes a substituent group (*e.g.*, a phosphate, hydroxyl, sulfate or other group) in addition to the normal "R" group of the amino acid; (iii) those in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) those in which additional amino acids are fused to the mature polypeptide, such as an immunoglobulin Fc region peptide, a leader or secretory sequence, a  
20 sequence which is employed for purification of the mature polypeptide (such as GST) or a proprotein sequence. Such fragments, derivatives and analogs are intended to be encompassed by the present invention, and are within the scope of those skilled in the art from the teachings herein and the state of the art at the time of invention.

The polypeptides of the present invention are preferably provided in an isolated form, and  
25 preferably are substantially purified. A recombinantly produced version of the human liver RI polypeptide or chimeric mammalian RI polypeptide can be substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988).

The polypeptides of the present invention include those which are at least 90% identical, more preferably at least 95% identical, and most preferably at least 96%, 97%, 98% or 99% identical to  
30 the polypeptides encoded by polynucleotides having nucleic acid sequences as set forth in SEQ ID NOs:6 or 8 or by the deposited cDNA, or to the polypeptides having the complete amino acid sequences set forth in SEQ ID NOs:5 or 7. The present polypeptides also include portions of the

above-described polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a RI polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the RI polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino (N-) or carboxy (C-) terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

The invention also relates to a general method of producing ribonuclease inhibitor which is substantially free of RNase activity. Such methods apply to purification of any ribonuclease inhibitor, regardless of the source. Thus, the general method of the invention relates to isolating substantially RNase free ribonuclease inhibitor from a biological sample (*e.g.*, cells, tissue, skin, brain, liver, kidney, *etc.*) obtained from any mammalian source (*e.g.*, human, rat, porcine, canine, ovine, feline, monkey, ape, bovine, equine, chimeric mammalian, *etc.*). In particular, the method of the invention for obtaining substantially RNase free ribonuclease inhibitor comprises (a) obtaining a biological sample from a mammalian source which contains a ribonuclease inhibitor, (b) mixing said sample with a binding partner, with the proviso that said binding partner is not an RNase, and (c) isolating ribonuclease inhibitor bound to said binding partner. In accordance with the invention, the binding partner may be any molecule, other than RNase, which reversibly binds ribonuclease inhibitor. Preferably, such binding partner is an antibody or an antibody fragment (*e.g.*, Fab) having specificity for ribonuclease inhibitor, although other suitable binding partners will be readily apparent to one of skill in the art. In the preferred method of the invention, ribonuclease inhibitor is isolated or purified by affinity chromatography, wherein said binding partner is attached to a solid support (*e.g.*, any column matrix including but not limited to sepharose, agarose, hydroxyapatite, *etc.*).

The invention also provides a general method for obtaining recombinant ribonuclease inhibitor which is substantially free of RNase activity. Such a method is suitable for isolating and

purifying any recombinant ribonuclease inhibitor, regardless of the type of the ribonuclease inhibitor (*e.g.*, human, bovine, porcine, rat, bovine, canine, feline, equine, ovine, monkey, ape, chimeric mammalian, *etc.*) and regardless of the recombinant host used to express the ribonuclease inhibitor. In this method of the invention, substantially RNase free recombinant ribonuclease inhibitor is  
5 obtained by (a) culturing a recombinant host under conditions sufficient to express ribonuclease inhibitor, (b) mixing the ribonuclease inhibitor with a binding partner, with the proviso that the binding partner is not an RNase, and (c) isolating the ribonuclease inhibitor bound to the binding partner. In accordance with this aspect of the invention, the binding partner may be any molecule which binds to the ribonuclease inhibitor (*e.g.*, antibodies and fragments thereof) provided however  
10 that said binding partner is not RNase.

Preferably, the recombinant ribonuclease is expressed in a modified form which allows selective binding to the binding partner. Such modifications which allow the isolation of a desired protein with a binding partner will be apparent to one skilled in the art. Preferably, the RI is modified to comprise a peptide to form a fusion protein which specifically binds to the binding partner  
15 (referred to herein as a "binding peptide"). Such peptide tags are well known in the art. Preferred peptide tags include His tag, thioredoxin tag, hemagglutinin tag, GST tag, HSA binding tag, and OmpA signal sequence tag. As will be understood, the binding partner which recognizes and binds to the binding peptide may be any molecule or compound including metal ions (*e.g.*, metal affinity columns), antibodies or fragments thereof and any protein or peptide which binds the binding peptide.  
20 Thus, the invention relates to a method of obtaining substantially RNase free recombinant ribonuclease inhibitor comprising (a) culturing a recombinant host under conditions sufficient to express a ribonuclease inhibitor which comprises a binding peptide, (b) mixing the ribonuclease inhibitor with a binding partner which is capable of binding to the binding peptide, and (c) isolating the ribonuclease inhibitor bound to said binding partner. Preferably, the binding partner which binds  
25 the binding peptide is bound to a solid support (*e.g.*, affinity chromatography).

The method of the invention may also comprise removing the binding peptide from RI after isolating or purifying the recombinant RI. Such removal may be accomplished by well known chemical and enzymatic techniques.

### Uses

30 The RIs of the invention may be used for a variety of purposes, in industrial, clinical, forensic, research and other settings. The RIs of the present invention can be used as molecular weight

markers on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art. In addition, the RIs of the invention may be used in synthesis of nucleic acid molecules, *in vitro* synthesis of polypeptides, and *in situ* transcription (IST) (Chang, H., *J. Immunol. Methods*. 176:235-243 (1994)).

## 5           **Production of Nucleic Acid Molecules**

The RIs of the present invention may be used in methods for the production of nucleic acid molecules. Methods according to this aspect of the invention may comprise, for example, mixing a template nucleic acid molecule, which may be an RNA molecule such as a mRNA or polyA<sup>+</sup> RNA molecule, with one or more of the RI polypeptides of the invention (such as one or more human liver  
10 RI polypeptides or chimeric mammalian, particularly porcine/human, RI polypeptides) under conditions sufficient to synthesize a nucleic acid molecule from the template nucleic acid molecule. Preferably, thermostable RIs are used in this method. Appropriate conditions for production of nucleic acid molecules are known in the art (*see, e.g.,* Sambrook, J., *et al., Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press  
15 (1987)), and will be familiar to one of ordinary skill.

The mixtures may further comprise one or more polypeptides having reverse transcriptase activity, such as M-MLV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase or HIV reverse transcriptase. These polypeptides having reverse transcriptase activity may be substantially reduced in RNase H  
20 activity; preferred such polypeptides include M-MLV H<sup>-</sup> reverse transcriptase, RSV H<sup>-</sup> reverse transcriptase, AMV H<sup>-</sup> reverse transcriptase, RAV H<sup>-</sup> reverse transcriptase, MAV H<sup>-</sup> reverse transcriptase and HIV H<sup>-</sup> reverse transcriptase. Optimal concentrations, formulations, etc., for use of polypeptides having reverse transcriptase activity, which may be substantially reduced in RNase H activity, in production of nucleic acid molecules according to the methods of the present invention  
25 are disclosed, for example, in co-pending U.S. Application No. 60/044,589, filed April 22, 1997, the disclosure of which is incorporated by reference herein in its entirety.

According to this aspect of the invention, the mixture may further comprise one or more thermostable DNA polymerases such as *Tne*, *Tma*, *Taq*, *Pfu*, *Tth*, *Tfi*, VENT, DEEPVENT, *Pwo* or *Tfl* polymerases, or mutants, variants and derivatives thereof. Optimal concentrations, formulations,  
30 etc., for use of thermostable DNA polymerases in production of nucleic acid molecules according to the methods of the present invention are disclosed, for example, in co-pending U.S. Application

Nos. 08/689,815, filed August 14, 1996, 08/778,082, filed January 2, 1997, and 08/801,720, filed February 14, 1997, the disclosures of which are incorporated by reference herein in their entireties.

The mixtures used in the present methods may also optionally comprise one or more polypeptides having RNA polymerase activity.

5       According to the invention, the methods for producing a nucleic acid molecule may be conducted using automated thermocycling, particularly since the human liver RIs and chimeric mammalian RIs (such as the chimeric porcine/human RI) of the invention are thermostable. Thus, the methods of the invention may be performed at temperatures of from about 45°C to about 70°C, from about 50°C to about 65°C, from about 55°C to about 60°C, from about 56°C to about 60°C,  
10   from about 57°C to about 60°C, from about 58°C to about 60°C, from about 59°C to about 60°C, or at temperatures of about 50°C or greater, about 51°C or greater, about 52°C or greater, about 53°C or greater, about 54°C or greater, about 55°C or greater, about 56°C or greater, about 57°C or greater, about 58°C or greater, about 59°C or greater, about 60°C or greater, about 61°C or greater, about 62°C or greater, about 63°C or greater, about 64°C or greater or about 65°C or  
15   greater.

The invention also relates to nucleic acid molecules produced according to these methods, which may be DNA molecules (including cDNA molecules or libraries), RNA molecules or DNA-RNA hybrid molecules. Nucleic acid molecules produced according to the methods of the invention may be full-length nucleic acid molecules (*e.g.*, full-length cDNAs or RNAs) or fragments thereof,  
20   and may be single-stranded or double-stranded.

In a preferred aspect of the invention, the method of nucleic acid synthesis concerns the use of mRNA as a template to make cDNA or a cDNA library. Such method may comprise (a) contacting an mRNA template or population of mRNA molecules (obtained from a cell or tissue of biological sample) with one or more RI's of the invention and (b) incubating said mixture under  
25   conditions sufficient to make one or more DNA molecules (*e.g.* cDNA) complementary to all or a portion of said template or said population of molecules. The synthesized DNA molecules may then be used as templates for further nucleic acid synthesis to make, for example, double stranded DNA molecules (*e.g.* double stranded cDNA) from the single stranded templates. In a preferred aspect, the synthesis of DNA molecules is accomplished by well known amplification techniques such as  
30   polymerase chain reaction (PCR).

### ***In Vitro Polypeptide Production***

In an alternative use, the RIs of the present invention may be used in methods for the *in vitro* production of polypeptides. Methods according to this aspect of the invention may comprise, for example, mixing a mRNA or polyA<sup>+</sup> RNA molecule with one or more of the RIs of the invention  
5 (such as a porcine liver, rat liver, human liver or chimeric mammalian (particularly a chimeric porcine/human) RI polypeptide), under conditions sufficient to translate a polypeptide from the mRNA or polyA<sup>+</sup> RNA molecule. Such conditions may comprise mixing the RNA molecule and one or more RIs of the invention with one or more cell-free protein translation systems that are known in the art, such as rabbit reticulocyte lysate, wheat germ extract, etc. (*see* Walter, P., and Blobel, G.,  
10 *J. Cell Biol.* 91:557 (1981); Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 18.79-18.80 (1987); Kaufman, P.B., *et al.*, *Handbook of Molecular and Cellular Methods in Biology and Medicine*, Boca Raton, Florida: CRC Press, pp. 318-322 (1995)). Alternatively, the RIs of the invention may be used to produce polypeptides in automated or semi-automated protein synthesis methodologies  
15 that will be familiar to the skilled artisan.

The invention also relates to polypeptides produced according to these methods. Polypeptides produced by these methods may include full-length polypeptides or any fragment thereof.

### ***In Vitro Transcription***

20 In an alternative use, the RIs of the present invention may be used in methods for the *in vitro* production of a nucleic acid molecule from a template nucleic acid molecule template (*i.e.*, *in vitro* transcription). Methods according to this aspect of the invention may comprise, for example, mixing a nucleic acid molecule (which may be single-stranded or double-stranded and which is preferably a DNA molecule, a cDNA or an RNA molecule such as a mRNA molecule) with one or more of the  
25 RIs of the invention (such as a porcine liver, rat liver, human liver or chimeric mammalian (particularly a chimeric porcine/human) RI polypeptide), under conditions sufficient to produce a nucleic acid molecule from the template. Such conditions may comprise mixing the template nucleic acid molecule (preferably a DNA or cDNA molecule) and one or more RIs of the invention with one or more nucleotides (such as those described above) and one or more RNA polymerases that are  
30 known in the art (*see* Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press (1987); Kaufman, P.B., *et al.*,

*Handbook of Molecular and Cellular Methods in Biology and Medicine*, Boca Raton, Florida: CRC Press (1995)), to produce one or more mRNA molecules. Alternatively, these conditions may comprise mixing the template nucleic acid molecule (preferably an mRNA molecule) and one or more RIs of the invention with one or more nucleotides (such as those described above), one or more polypeptides having reverse transcriptase activity and/or one or more polypeptides having DNA polymerase activity, particularly those described above and most particularly those polypeptides having RT and/or DNA polymerase activity which are also thermostable, to produce one or more DNA molecules which may be single-stranded or double-stranded. In addition to isolated nucleic acid molecules, such methods may also be performed using whole cells or tissues or extracts thereof, if the nucleotides included in the reaction mix are also detectably labeled (*e.g.*, with fluorescent or radioactive labels), these methods of the invention may be used to label specific nucleic acid sequences in fixed cells or tissues (*see* Chang, H., *J. Immunol. Meth.* 176(2):235-243 (1994)).

### Kits

The invention is also directed to kits comprising one or more containers containing one or more of the RIs of the invention. Kits according to this aspect of the invention may further comprise additional containers containing, for example, one or more of the thermostable DNA polymerases, polypeptides having reverse transcriptase activity or polypeptides having RNA polymerase activity described above. Additional kits of the invention may further comprise one or more containers containing a cell-free protein translation mixture, such as a rabbit reticulocyte lysate or a wheat germ extract. The kits of the invention may also optionally comprise one or more additional containers containing other reagents necessary for synthesis and analysis of nucleic acids or polypeptides, such as one or more buffers, one or more detergents, one or more deoxyribonucleoside triphosphates, one or more ribonucleoside triphosphates, agents for electrophoresis (*e.g.*, acrylamide or agarose), and the like.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.



## Examples

### *Example 1: Cloning and Expression of Rat Liver RI in E. coli*

To clone the gene encoding rat liver RI, total mRNA was isolated from rat liver, and first strand cDNA synthesis was accomplished using the protocol as previously described (Nathan, M. *et al.*, *Focus* 17:78-80 (1995)). The oligonucleotide used at the 5'-end of the gene was 5' TAA TAG **CAT ATG** AGT CTT GAC ATC CAG TGT GAG 3' (SEQ ID NO:9). The oligonucleotide used at the 3'-end of the gene was 5' TTA TTA **GGA TCC** TTA TCA GGA AAT GAT CCT CAG GGA TGG CC 3' (SEQ ID NO:10). An *Nde*I and a *Bam*HI site were created in the oligonucleotide (bold and underlined) for easy cloning into an expression vector. The oligonucleotides were designed based on the cDNA sequence of rat lung RI cDNA, and were obtained from Life Technologies, Inc. (LTI; Rockville, Maryland).

PCR was done using the PCR Supermix (LTI), which contains *Taq* DNA polymerase, plus about 10-20 ng of cDNA and oligonucleotides at a concentration of 2.5  $\mu$ M. A Perkin-Elmer 9600 PCR machine was used to carry out the following cycling procedure: 1 cycle of 94°C for 5 min.; 30 cycles of 94°C for 20 sec./55°C for 20 sec./72°C for 1 min.; 1 cycle of 72°C for 5 min.

The PCR fragment was digested with *Nde*I and *Bam*HI and cloned into the pPROEX1 expression vector digested with *Nde*I and *Bam*HI, which was obtained from LTI. This plasmid was then transformed into *E. coli* DH10B, a strain which was obtained from LTI. Six clones were picked to test whether they contained the cloned PCR product. Five out of the six clones contained the cloned fragment. One of the clones, pRatRI (Fig. 1), was grown to express the rat liver RI, the amino acid sequence of which is given in SEQ ID NO:4. Proteins isolated from induced cultures (40 ml) grown at 30°C and 37°C were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, U.K., *Nature* 227:680-685 (1970)).

The pPROEX1 vector encodes 23 amino acid residues prior to the *Nde*I site. This region contains six histidine residues, a spacer region and a TEV-protease site. Thus, cloning at the *Nde*I site created a fusion protein containing an additional 23 amino acids at the amino terminus. Both the induced culture grown at 30°C and the induced culture grown at 37°C produced a protein slightly larger than 50 kD (about 52 kD), although RI purified from rat liver has a molecular weight of about 50 kD. The increase in the size of rat RI expressed from *E. coli* was due to the additional 24 amino acids corresponding to vector sequence. Although total RI protein production was higher at 37°C

than at 30°C, more soluble protein was produced at 30°C. Therefore, the cells appeared to produce more inclusion bodies at 37°C than at 30°C.

Since the construction in pPROEX1 produced a fusion protein, an attempt to produce an unfused authentic rat liver RI was made. The *NdeI-BamHI* fragment from pRatRI was subcloned into pRE1 at the *NdeI-BamHI* sites. Plasmid pRE1, obtained from Dr. McKenney (Reddy, P. *et al.*, *Nucleic Acids Res.* 17:10473-10488 (1989)), is an expression vector containing a lambda pL promoter. The ligated material was introduced into CJ374 containing the repressor CI857 in a compatible chloramphenicol resistance plasmid (pCJ136). Six clones were tested for the presence of the cloned fragment, and six out of six clones contained the insert. One of the clones, pRERatRI (Fig. 2), was used to study the level of expression of RI. The recombinant host cell comprising pRERatRI, *E. coli* DH5 $\alpha$ F'IQ(pRERatRI), was deposited on August 9, 1996, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 USA, as Deposit No. NRRL B-21613.

A 50 ml culture was grown in rich medium (20 g NZ amine, 10 g Yeast Extract, 5g NaCl, 2.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.4 g MgSO<sub>4</sub>, 5 ml glycerol, pH 7.2), containing both ampicillin and chloramphenicol at 30°C to an A590 of about 0.8. The culture was induced at 42°C for 30 min and outgrown at 30°C. Samples were withdrawn for analysis by SDS-PAGE at different times. No induced protein around 50 kD was detected at any induction time point up to four hours. Surprisingly, rat RI was only produced as a fusion protein. In order to find out whether fusion is necessary for expression of rat liver RI, the RI coding sequence was fused with an OmpA signal sequence (21 amino acids) and cloned in pRE1 (Fig. 2). The cells were grown as described above, and the samples were analyzed by SDS-PAGE. An expressed protein of about 50 kD was detected in the induced sample but not in the uninduced samples. Since the expected size of fused protein was 52 kD, and the induced soluble protein was about 50 kD, it was apparent that the protein was made as a fused protein and later processed to authentic RI without the signal sequence. This confirms that rat RI protein expresses well when produced as a fusion protein in *E. coli*. However, the fusion partner is dispensable following expression, as was shown with the OmpA-RI fusion.

**Example 2: Cloning and Expression of Porcine Liver RI**

In order to obtain the gene encoding porcine liver RI, total mRNA was isolated from porcine liver, and first strand cDNA synthesis was accomplished using the protocol as previously described (Nathan, M. *et al.*, *Focus* 17:78-80 (1995)). Because the complete sequence of the porcine liver RI gene had not been established (Vincentini *et al.*, *Biochemistry* 29: 8827-8834 (1990), the oligonucleotide used at the 5'-end of the gene corresponded to the amino-terminal amino acid sequence of porcine liver RI protein, while the oligonucleotide used at the 3'-end of the gene corresponded to the carboxy-terminal sequence of the porcine kidney RI gene. The nucleotide sequences of the oligonucleotide used at the 5'-end of the gene, which included an *Nde*I site (bold and underlined) was 5' TAT TAT **CATATG** AAC CTG/C GAC/T ATC/T CAC/T TGC/T GA 3' (SEQ ID NO:11). The oligonucleotide used at the 3'-end of the gene (bold and underlined) was 5' TAT TAT **AAGCTT** GCC CAA AAG GTG TTT TAC TAA GTA G 3' (SEQ ID NO:12).

PCR was carried out as described above in Example 1. The PCR products and the expression vector pPROEX1 were digested with *Nde*I and *Hind*III. The PCR products were then ligated into the vector, and the ligated material was introduced into DH10B, obtained from LTI. Surprisingly, very few clones were found. The PCR was repeated, and the fragment cloned again into pPROEX1. Only two out of 30 clones tested were found to have the insert. However, upon digestion with *Nde*I and *Hind*III, both clones generated a fragment of only about 1250 bp, rather than the 1500 bp of the PCR fragment. To confirm that the result was not a gel artifact, restriction digestion analysis was performed. The results suggest that the insert was indeed smaller than the PCR product. Interestingly, the two clones generated an identical restriction digestion profile.

The cloned fragment was also sequenced from both ends (see Example 3). The sequencing results indicated that both the amino and the carboxy termini of porcine liver RI were intact. These experiments indicate that the region deleted from the PCR fragment was internal to the porcine RI gene. The clone produced a protein of about 48 kD in size, smaller than the expected size of slightly greater than 50 kD. This smaller protein displayed no RI activity. From all of these experiments it was apparent that porcine RI is inhibitory to the growth of *E. coli*, and that the cloning procedure that was successful for obtaining the gene for rat liver RI was unsuitable for obtaining porcine liver RI. This result was surprising; as rat and porcine RI have 75% sequence identity, the clones were expected to have similar properties when expressed in *E. coli*. The reasons that porcine RI was

difficult to clone and express in *E. coli* are unclear, but one theory is that porcine RI is so toxic to *E. coli* that the cell cannot express sufficient repressor in time to completely inhibit RI expression.

The 1500 bp PCR product of the porcine RI gene was then cloned into the vector pUC18 digested by *Nde*I and *Hind*III, which lacks a promoter to drive the gene. These clones were stable.

- 5 The *Nde*I-*Hind*III fragment containing the porcine RI gene was then subcloned into pPROEX1 digested with *Nde*I and *Hind*III, and the resulting plasmid was introduced into DH5 $\alpha$ F'IQ, a strain which constitutively expresses *lacI*<sup>q</sup> so that the repressor is present even before the introduction of the expression plasmid containing an additional copy of *lacI*<sup>q</sup>. Twelve transformants were tested for the insert. 12 out of 12 clones generated a correct sized fragment. One of the clones, pPorcineRI
- 10 (Fig. 3), was tested further for expression of porcine RI, the amino acid sequence of which is given in SEQ ID NO:2. The recombinant host cell comprising pPorcineRI, *E. coli* DH5 $\alpha$ F'IQ(pPorcineRI), was deposited on August 9, 1996, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 USA, as Deposit No. NRRL B-21612.

- E. coli* DH5 $\alpha$ F'IQ containing pPorcineRI was grown to an A590 of between about 0.6-1.0
- 15 in a rich medium (20 g NZ amine, 10 g Yeast Extract, 5 g NaCl, 2.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.4 g MgSO<sub>4</sub>, 5 ml glycerol, pH 7.2) and 100  $\mu$ g/ml ampicillin at 30°C. The culture was induced with IPTG (1.5mM) for 3hrs. The culture was spun down and the pellets were stored at -70°C until used. The clone produced a protein slightly larger than 50 kD as expected. Thus, by the unusual procedure of preprotecting the expression of porcine RI, porcine liver RI was successfully cloned and expressed
- 20 in *E. coli*. Upon induction, however, the growth rate of *E. coli* expressing porcine RI diminished considerably, whereas no inhibition of growth was observed in *E. coli* expressing rat liver RI.

The problems encountered in cloning and expressing porcine RI in *E. coli* allow a prediction that porcine RI will be active against *E. coli* RNases in addition to RNaseA, B and C.

### **Example 3: DNA Sequence Analysis of Porcine Liver RI**

- 25 The nucleotide sequence of the 243 nucleotides (encoding 81 amino acids) at the 5'-end of the RI gene (SEQ ID NO:1, nucleotides 1-243; SEQ ID NO:2, amino acids 1-81) was obtained by methods known in the art. The amino acid sequence deduced from the nucleotide sequence of the cDNA matched perfectly with the amino acid sequence derived from direct sequencing of purified porcine RI.

**Example 4: Purification of RI**

All steps were carried out at 4°C or on ice unless specified. The cells containing the recombinant plasmid were suspended at a 1:3 ratio (grams of cells : ml buffer) in a buffer consisting of 50 mM Tris-HCl pH 8.0, 100 mM KCl, and 10 mM beta-mercaptoethanol ( $\beta$ -ME). The cell  
5 suspension was subjected to sonication using a sonicator (Heat Systems) until 80% of the cells were cracked as measured by A590. The cell debris was clarified by centrifugation. The supernatant was filtered through a 5  $\mu$ m acrodisc filter before loading onto a Ni-NTA-agarose affinity column equilibrated with 20mM Tris-HCl, pH 8.0, 100mM KCl, 10mM  $\beta$ -ME, 10% glycerol. The column was washed with 10 column volumes of equilibration buffer containing 20 mM imidazole. The RI  
10 was eluted using a linear gradient of imidazole ranging from 20 mM to 150 mM in the same buffer. The fractions were analyzed by SDS-PAGE for the presence of a 52 kD protein, the apparent size of the fused RI.

Fractions containing the expected 52 kD protein were pooled and dialyzed in a buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol (DTT), 10% glycerol. The dialyzed  
15 material was loaded onto a MonoQ column (Pharmacia) equilibrated with the same buffer. The column was washed with 10 column volumes of the same buffer. The RI was eluted using a linear gradient of KCl ranging from 0 mM to 500 mM in the same buffer.

The fractions were analyzed by SDS-PAGE, and fractions containing a 52 kD protein were pooled. The pooled material was dialyzed in a storage buffer containing 20 mM Tris-HCl, pH 7.5,  
20 50 mM KCl, 10 mM DTT, and 50% glycerol. The sample was tested for RNase inhibitor activity.

**Example 5: Assay for RNase Inhibitor Activity**

The activity of RI was determined essentially as described (Blackburn, P., *et al.*, *J. Biol. Chem.* 252:5904-5910 (1977)). RNase inhibitor activity was expressed by its ability to inhibit the activity of 5 ng of RNaseA (as measured by hydrolysis of yeast RNA) by 50%.

**Example 6: Stabilization of RNase Inhibitor Activity**

The addition of a chelator (e.g., EDTA) was found to stabilize storage of RNase inhibitor purified over a Ni-NTA agarose affinity column (see Example 4). Samples stored without chelator were found stable at 4°C for less than one month, while RI stored in the presence of a chelator were  
5 stable for greater than 6 months at 4°C. Thus, the invention relates generally to the use of a chelator to stabilize enzymes or proteins purified by a metal (e.g., Ni) column. The chelator effectively binds or removes the metal in the purified protein or enzyme, thus stabilizing the protein or enzyme.

**Example 7: Cloning and Expression of Human Liver RI**

Human liver cDNA was used as the template to amplify the RNase inhibitor gene. The  
10 oligonucleotides used were 5' TAT TAT CAT ATG AGC CTG GAC ATC CAG AGC CTG GA 3' (SEQ ID NO:13) (5' end) and 5' TAT TAG AAT TCA AGC TTA TCA GGA GAT GAC CCT CAG GGA 3' (SEQ ID NO:14) (3' end). An *NdeI* and a *HindIII* were created in the oligonucleotide for easy cloning into an expression vector. PCR was done using LTI's PCR supermix, 25 ng of cDNA, and oligonucleotides at a concentration of 1.5 µM. A Perkin Elmer 9600 thermocycler was used to  
15 perform the PCRs using the following cycling protocol: 1 cycle at 94°C for 5 minutes; 20 cycles of 94°C for 20 seconds/55°C for 20 seconds/72°C for 1 minute; and 1 cycle at 72°C for 5 minutes.

The 1400 bp PCR product was digested with *NdeI* and *HindIII* and was cloned into the vectors pUC18 or pPROEX1. The PCR product was then ligated into the appropriately digested vector. The ligated material was introduced into competent DH5αF'IQ *E. coli*. Four of the eight  
20 clones in pUC18 had the desired insert whereas none of the eight in pPROEX was correct. The human liver RI was then subcloned into the expression vector pPROEX 1 as an *NdeI/HindIII* fragment, to produce pHumanRI (Figure 4). All of the eight clones tested had the correct insert. When the clone was analyzed for protein expression by SDS-PAGE, a protein of about 52 kD was found to be produced. Upon sequencing, this protein was found to have the amino acid sequence  
25 set forth in SEQ ID NO:6.

The recombinant host cell comprising pHumanRI, *E. coli* DH5αF'IQ(pHumanRI), was deposited on August 12, 1997, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 USA, as Deposit No. NRRL B-21810.

**Example 8: Cloning and Expression of Chimeric Porcine/Human RI**

Sequence analysis of both the porcine RI and the human RI showed the presence of a single *StuI* site present ~700 bp away from the start of the RI gene. Oligonucleotides were designed to amplify the amino end and the carboxy end of the human liver RI. A *StuI* site was created in both  
5 the oligos. The oligonucleotides were 5' GTG GCC TCC AAG GCC TCG CTG CGG 3' (SEQ ID NO:15) to amplify the carboxy end and 5' CCG CAG CGA GGC CTT GGA GGC CAC 3' (SEQ ID NO:16) to amplify the amino end. The amino end of the porcine RI was amplified and the ~700 bp fragment was digested with the restriction enzymes *NdeI* and *StuI*. The human liver RI clone in the vector pPROEX I was digested with *StuI* and *HindIII* and the ~700 bp fragment was isolated. The  
10 plasmid pUC18 was digested with *NdeI* and *HindIII* and the three fragments were ligated. The ligated material was introduced into competent DH5 $\alpha$ F'IQ *E. coli*. None of the eight clones tested had the right insert. Since the previous attempt involved ligating three fragments, which can prove problematic, a different strategy was used. The amino end of the porcine RI was amplified and the ~700 bp fragment was isolated after digesting it with *NdeI* and *StuI*. The human liver RI cloned into  
15 pUC18 was also digested with *NdeI* and *StuI* and the larger fragment which has the amino end deleted was isolated. The two fragments were ligated and introduced into competent DH5 $\alpha$ F'IQ *E. coli*. Of the 12 clones tested, only one had the amino end of the porcine RI and the carboxy end of the human RI, as confirmed by restriction digestion analysis. This porcine/human chimeric RI was then subcloned into the expression vector pPROEX I as an *NdeI*/ *HindIII* fragment. However,  
20 SDS-PAGE analysis of the expressed protein indicated that a ~32 kD protein was expressed, rather than the expected ~52 kD protein. The expression of this truncated protein may have been due to the *StuI* enzyme not cutting the PCR product; the ligation of the blunt fragment would then cause a frame shift resulting in a premature stop and thus expression of a truncated protein. Since this clone still had the *StuI* site, it was digested with *NdeI* and *StuI* and the ~700 bp fragment was ligated  
25 to the human liver RI cloned into pUC18 which was digested with *NdeI* and *StuI* to delete the amino end. Five of the nine clones tested were correct and the gene was then subcloned into the expression vector pPROEX I as an *NdeI*/*HindIII* fragment, to produce plasmid pPorcineHuRI (Figure 5). SDS-PAGE analysis confirmed that a protein of ~52 kD was expressed by this construct. The clone was sequenced to confirm that the construct had the amino end of porcine RI and the carboxy end of  
30 human RI; the amino acid sequence of this chimeric polypeptide is set forth in SEQ ID NO:8.

**Example 9: Purification and Characterization of Chimeric Porcine/Human RI**

All steps were carried out at 4°C or on ice unless stated. Host cells comprising plasmid pPorcineHuRI (see Example 8 and Figure 5) were suspended in a buffer consisting of 50 mM Tris (pH 8.0), 100 mM KCl, and 10 mM  $\beta$ -mercaptoethanol ( $\beta$ -ME). The cell suspension was  
5 homogenized under pressure in a gauhin homogenizer until 85% of the cells were cracked as measured by A590. The cell debris was removed by centrifugation and the supernatant was loaded onto a chelating column (AF Chelate 650 M from TosoHaas) that was charged with nickel and equilibrated with 20 mM Tris pH 8.0, 100 mM KCl, 10 mM  $\beta$ -ME and 10% glycerol. The column was washed with 10 column volumes of equilibration buffer containing 20 mM imidazole. The RI  
10 was eluted with a linear gradient of imidazole from 20 mM to 150 mM in the same buffer. The fractions were analyzed by SDS-PAGE for the presence of the ~52 kD RI protein.

The fractions were pooled and dialyzed in a buffer containing 20 mM Tris (pH 8.0), 0.5 mM EDTA, 8 mM dithiothreitol (DTT) and 10% glycerol. The dialyzed material was loaded onto an anion exchange column (Source 30Q; Pharmacia) equilibrated in the same buffer. The column was  
15 washed with 10 column volumes of the same buffer and was eluted using a linear gradient of KCl from 0 mM to 500 mM in the same buffer.

The fractions were analyzed by SDS-PAGE and those containing the ~52 kD protein were pooled. This was dialyzed into a storage buffer containing 20 mM Tris (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 8 mM DTT and 50% glycerol. Samples were tested for RNase inhibitor activity  
20 essentially by their ability to inhibit the hydrolysis of cyclic 2',3'-CMP by RNaseA (Blackburn, P., *J. Biol. Chem.* 254:12484-12487 (1979)). One unit of inhibitor was that amount required to inhibit by 50% the activity of 5 ng of RNaseA.

**Example 10: Thermostability of RIs**

To determine the utility of the cloned RIs in high-temperature (*i.e.*, above about 45°C)  
25 reactions, 800 units of either cloned porcine RI or chimeric porcine/human RI were incubated on ice, or at 50°C, 55°C or 60°C for 5 minutes in a buffer containing 50 mM Bicine (pH 8.2), 115 mM potassium acetate, 8% glycerol and 2.5 mM manganese diacetate. The residual activity was determined by adding 2  $\mu$ g RNase A and measuring the inhibition of hydrolysis of 0.2 mM cCMP by RNaseA.



The cloned porcine RI was found to be inactivated by incubation for five minutes at 55°C, while the chimeric porcine/human RI retained nearly 100% of its activity under these conditions. However, the chimeric porcine/human RI was inactivated by incubation for five minutes at 60°C. Similar thermostability assays indicated that rRNasin (recombinant human placental RI; Promega) retained its activity also retained its activity after incubation for five minutes at 55°C, while it was inactivated at 60°C. These results indicate that the cloned porcine/human chimeric RI of the invention is more thermostable than is full-length porcine RI. Moreover, the thermostability of the porcine/human chimeric RI of the invention appears to be similar to that of a cloned human placental RI. These findings suggest that chimeric RIs comprising at least a portion of the human RI sequence may be useful in higher-temperature reactions, such as PCRs or RT-PCRs using automated thermocycling, which would be precluded by the use of thermolabile mammalian enzymes such as porcine RI.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- 5 (A) NAME: Life Technologies, Inc.  
(B) STREET: 9800 Medical Center Drive  
(C) CITY: Rockville  
(D) STATE: Maryland  
(E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 20850

## (i) APPLICANT:

- 10 (A) NAME: Chatterjee, Deb K.  
(B) STREET: 6 Forest Ridge Court  
(C) CITY: North Potomac  
(D) STATE: Maryland  
15 (E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 20878

## (i) APPLICANT:

- 20 (A) NAME: Shandilya, Harini  
(B) STREET: 6115 Twain Drive  
(C) CITY: New Market  
(D) STATE: Maryland  
(E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 21774

- 25 (ii) TITLE OF INVENTION: Mammalian Ribonuclease Inhibitors and Use  
Thereof

- (iii) NUMBER OF SEQUENCES: 16

## (iv) COMPUTER READABLE FORM:

- 30 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (vi) PRIOR APPLICATION DATA:

- 35 (A) APPLICATION NUMBER: US (To be assigned)  
(B) FILING DATE: 13-AUG-1997

## (vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/795,395  
(B) FILING DATE: 04-FEB-1997

## (vi) PRIOR APPLICATION DATA:

- 40 (A) APPLICATION NUMBER: US 08/794,546  
(B) FILING DATE: 03-FEB-1997

## (vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 60/024,057  
(B) FILING DATE: 16-AUG-1996

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1371 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 1..1368

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	ATG AAC CTC GAT ATT CAT TGC GAG CAG CTG AGC GAC GCC CGG TGG ACA	48
	Met Asn Leu Asp Ile His Cys Glu Gln Leu Ser Asp Ala Arg Trp Thr	
	1 5 10 15	
15	GAG CTC CTG CCG CTG CTC CAG CAG TAT GAG GTG GTC AGG CTC GAC GAC	96
	Glu Leu Leu Pro Leu Leu Gln Gln Tyr Glu Val Val Arg Leu Asp Asp	
	20 25 30	
	TGC GGC CTC ACG GAG GAG CAC TGC AAG GAC ATC GGT TCT GCC CTC CGG	144
	Cys Gly Leu Thr Glu Glu His Cys Lys Asp Ile Gly Ser Ala Leu Arg	
20	35 40 45	
	GCC AAC CCC TCC CTG ACC GAG CTC TGC CTC CGC ACC AAC GAG CTG GGC	192
	Ala Asn Pro Ser Leu Thr Glu Leu Cys Leu Arg Thr Asn Glu Leu Gly	
	50 55 60	
25	GAT GCC GGC GTG CAC CTG GTG CTG CAG GGC CTG CAG AGC CCC ACC TGC	240
	Asp Ala Gly Val His Leu Val Leu Gln Gly Leu Gln Ser Pro Thr Cys	
	65 70 75 80	
	AAG ATC CAG AAG CTC AGC CTG CAG AAC TGC TCC CTG ACC GAG GCG GGC	288
	Lys Ile Gln Lys Leu Ser Leu Gln Asn Cys Ser Leu Thr Glu Ala Gly	
	85 90 95	
30	TGC GGG GTC CTG CCC AGC ACG CTG CGC TCC CTG CCC ACG CTG CGG GAG	336
	Cys Gly Val Leu Pro Ser Thr Leu Arg Ser Leu Pro Thr Leu Arg Glu	
	100 105 110	
	CTG CAT CTC AGC GAC AAC CCA CTG GGG GAC GCC GGC CTG CGG CTG CTC	384
	Leu His Leu Ser Asp Asn Pro Leu Gly Asp Ala Gly Leu Arg Leu Leu	
35	115 120 125	
	TGT GAG GGG CTC CTG GAC CCC CAG TGC CAC CTG GAG AAG CTG CAG TTG	432
	Cys Glu Gly Leu Leu Asp Pro Gln Cys His Leu Glu Lys Leu Gln Leu	
	130 135 140	
40	GAG TAC TGC CGC CTG ACG GCC GCC AGC TGC GAG CCC CTG GCC TCG GTG	480
	Glu Tyr Cys Arg Leu Thr Ala Ala Ser Cys Glu Pro Leu Ala Ser Val	
	145 150 155 160	

	CTC AGG GCC ACG CGG GCC TTG AAG GAG CTC ACG GTG AGC AAC AAC GAC	528
	Leu Arg Ala Thr Arg Ala Leu Lys Glu Leu Thr Val Ser Asn Asn Asp	
	165 170 175	
5	ATC GGC GAG GCC GGC GCC CGG GTG CTG GGC CAG GGT CTG GCA GAC TCT	576
	Ile Gly Glu Ala Gly Ala Arg Val Leu Gly Gln Gly Leu Ala Asp Ser	
	180 185 190	
	GCC TGC CAG CTG GAG ACG CTC AGG CTG GAG AAC TGC GGT CTC ACG CCA	624
	Ala Cys Gln Leu Glu Thr Leu Arg Leu Glu Asn Cys Gly Leu Thr Pro	
	195 200 205	
10	GCC AAC TGC AAA GAC CTG TGC GGA ATT GTG GCC TCC CAG GCC TCG CTG	672
	Ala Asn Cys Lys Asp Leu Cys Gly Ile Val Ala Ser Gln Ala Ser Leu	
	210 215 220	
15	AGG GAG CTT GAC CTG GGC AGC AAC GGG CTG GGC GAC GCG GGC ATA GCC	720
	Arg Glu Leu Asp Leu Gly Ser Asn Gly Leu Gly Asp Ala Gly Ile Ala	
	225 230 235 240	
	GAG CTG TGC CCC GGG CTC TTG AGC CCC GCC TCC CGC CTC AAG ACC CTG	768
	Glu Leu Cys Pro Gly Leu Leu Ser Pro Ala Ser Arg Leu Lys Thr Leu	
	245 250 255	
20	TGG CTC TGG GAG TGT GAC ATC ACC GCC AGT GGC TGC AGA GAC CTC TGC	816
	Trp Leu Trp Glu Cys Asp Ile Thr Ala Ser Gly Cys Arg Asp Leu Cys	
	260 265 270	
	CGT GTC CTC CAG GCC AAG GAG ACC CTG AAG GAG CTC AGT CTG GCG GGC	864
	Arg Val Leu Gln Ala Lys Glu Thr Leu Lys Glu Leu Ser Leu Ala Gly	
	275 280 285	
25	AAC AAG CTG GGC GAC GAG GGC GCC CGG CTG CTG TGC GAG AGC CTG CTG	912
	Asn Lys Leu Gly Asp Glu Gly Ala Arg Leu Leu Cys Glu Ser Leu Leu	
	290 295 300	
30	CAG CCC GGC TGC CAG CTG GAG TCC CTG TGG GTG AAG TCC TGC AGC CTC	960
	Gln Pro Gly Cys Gln Leu Glu Ser Leu Trp Val Lys Ser Cys Ser Leu	
	305 310 315 320	
	ACG GCG GCC TGC TGC CAG CAC GTC AGC TTG ATG CTG ACC CAG AAC AAG	1008
	Thr Ala Ala Cys Cys Gln His Val Ser Leu Met Leu Thr Gln Asn Lys	
	325 330 335	
35	CAT CTC CTG GAA CTT CAG TTG AGC AGC AAC AAG CTG GGT GAC TCT GGC	1056
	His Leu Leu Glu Leu Gln Leu Ser Ser Asn Lys Leu Gly Asp Ser Gly	
	340 345 350	
	ATC CAG GAG CTG TGC CAG GCC CTG AGC CAG CCG GGC ACC ACA CTG CGG	1104
	Ile Gln Glu Leu Cys Gln Ala Leu Ser Gln Pro Gly Thr Thr Leu Arg	
	355 360 365	
40	GTG CTC TGT CTT GGG GAC TGT GAG GTG ACC AAC AGC GGC TGC AGC AGC	1152
	Val Leu Cys Leu Gly Asp Cys Glu Val Thr Asn Ser Gly Cys Ser Ser	
	370 375 380	

CTC GCC TCG CTC CTG CTG GCC AAC CGC AGC CTG CGA GAG CTG GAC CTG 1200  
 Leu Ala Ser Leu Leu Leu Ala Asn Arg Ser Leu Arg Glu Leu Asp Leu  
 385 390 395 400  
 5 AGC AAC AAC TGT GTG GGC GAC CCG GGC GTC CTG CAG CTG CTG GGG AGC 1248  
 Ser Asn Asn Cys Val Gly Asp Pro Gly Val Leu Gln Leu Leu Gly Ser  
 405 410 415  
 CTG GAG CAG CCG GGC TGC GCC CTG GAG CAG CTG GTC CTG TAC GAC ACC 1296  
 Leu Glu Gln Pro Gly Cys Ala Leu Glu Gln Leu Val Leu Tyr Asp Thr  
 420 425 430  
 10 TAC TGG ACG GAG GAG GTG GAG GAC CGC CTG CAG GCC CTG GAG GGG AGC 1344  
 Tyr Trp Thr Glu Glu Val Glu Asp Arg Leu Gln Ala Leu Glu Gly Ser  
 435 440 445  
 AAG CCC GGC CTG AGG GTC ATC TCC TGA 1371  
 Lys Pro Gly Leu Arg Val Ile Ser  
 15 450 455

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 456 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asn Leu Asp Ile His Cys Glu Gln Leu Ser Asp Ala Arg Trp Thr  
 1 5 10 15  
 25 Glu Leu Leu Pro Leu Leu Gln Gln Tyr Glu Val Val Arg Leu Asp Asp  
 20 25 30  
 Cys Gly Leu Thr Glu Glu His Cys Lys Asp Ile Gly Ser Ala Leu Arg  
 35 40 45  
 30 Ala Asn Pro Ser Leu Thr Glu Leu Cys Leu Arg Thr Asn Glu Leu Gly  
 50 55 60  
 Asp Ala Gly Val His Leu Val Leu Gln Gly Leu Gln Ser Pro Thr Cys  
 65 70 75 80  
 Lys Ile Gln Lys Leu Ser Leu Gln Asn Cys Ser Leu Thr Glu Ala Gly  
 85 90 95  
 35 Cys Gly Val Leu Pro Ser Thr Leu Arg Ser Leu Pro Thr Leu Arg Glu  
 100 105 110  
 Leu His Leu Ser Asp Asn Pro Leu Gly Asp Ala Gly Leu Arg Leu Leu  
 115 120 125  
 Cys Glu Gly Leu Leu Asp Pro Gln Cys His Leu Glu Lys Leu Gln Leu

	130	135	140
	Glu Tyr Cys Arg Leu Thr Ala Ala Ser Cys Glu Pro Leu Ala Ser Val		
	145	150	155 160
5	Leu Arg Ala Thr Arg Ala Leu Lys Glu Leu Thr Val Ser Asn Asn Asp		
		165	170 175
	Ile Gly Glu Ala Gly Ala Arg Val Leu Gly Gln Gly Leu Ala Asp Ser		
		180	185 190
	Ala Cys Gln Leu Glu Thr Leu Arg Leu Glu Asn Cys Gly Leu Thr Pro		
		195	200 205
10	Ala Asn Cys Lys Asp Leu Cys Gly Ile Val Ala Ser Gln Ala Ser Leu		
		210	215 220
	Arg Glu Leu Asp Leu Gly Ser Asn Gly Leu Gly Asp Ala Gly Ile Ala		
		225	230 235 240
15	Glu Leu Cys Pro Gly Leu Leu Ser Pro Ala Ser Arg Leu Lys Thr Leu		
		245	250 255
	Trp Leu Trp Glu Cys Asp Ile Thr Ala Ser Gly Cys Arg Asp Leu Cys		
		260	265 270
	Arg Val Leu Gln Ala Lys Glu Thr Leu Lys Glu Leu Ser Leu Ala Gly		
		275	280 285
20	Asn Lys Leu Gly Asp Glu Gly Ala Arg Leu Leu Cys Glu Ser Leu Leu		
		290	295 300
	Gln Pro Gly Cys Gln Leu Glu Ser Leu Trp Val Lys Ser Cys Ser Leu		
		305	310 315 320
25	Thr Ala Ala Cys Cys Gln His Val Ser Leu Met Leu Thr Gln Asn Lys		
		325	330 335
	His Leu Leu Glu Leu Gln Leu Ser Ser Asn Lys Leu Gly Asp Ser Gly		
		340	345 350
	Ile Gln Glu Leu Cys Gln Ala Leu Ser Gln Pro Gly Thr Thr Leu Arg		
		355	360 365
30	Val Leu Cys Leu Gly Asp Cys Glu Val Thr Asn Ser Gly Cys Ser Ser		
		370	375 380
	Leu Ala Ser Leu Leu Leu Ala Asn Arg Ser Leu Arg Glu Leu Asp Leu		
		385	390 395 400
35	Ser Asn Asn Cys Val Gly Asp Pro Gly Val Leu Gln Leu Leu Gly Ser		
		405	410 415
	Leu Glu Gln Pro Gly Cys Ala Leu Glu Gln Leu Val Leu Tyr Asp Thr		
		420	425 430
	Tyr Trp Thr Glu Glu Val Glu Asp Arg Leu Gln Ala Leu Glu Gly Ser		

435

440

445

Lys Pro Gly Leu Arg Val Ile Ser  
450 455

(2) INFORMATION FOR SEQ ID NO:3:

5

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1374 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both

10

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 1..1368

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

15	ATG AGT CTT GAC ATC CAG TGT GAG CAG CTG AGT GAT GCC CGG TGG ACA	48
	Met Ser Leu Asp Ile Gln Cys Glu Gln Leu Ser Asp Ala Arg Trp Thr	
	1 5 10 15	
	GAG CTC CTT CCC CTG ATC CAA CAA TAC CAA GTG GTC AGG CTG GAT GAC	96
	Glu Leu Leu Pro Leu Ile Gln Gln Tyr Gln Val Val Arg Leu Asp Asp	
20	20 25 30	
	TGT GGC CTC ACT GAA GTG CGG TGC AAA GAC ATC AGG TCA GCG ATC CAG	144
	Cys Gly Leu Thr Glu Val Arg Cys Lys Asp Ile Arg Ser Ala Ile Gln	
	35 40 45	
	GCC AAC CCT GCC CTG ACA GAG CTC AGC CTA CGC ACC AAT GAA CTG GGT	192
25	Ala Asn Pro Ala Leu Thr Glu Leu Ser Leu Arg Thr Asn Glu Leu Gly	
	50 55 60	
	GAT GCT GGT GTG GGT CTG GTG CTC CAG GGC CTG CAG AAT CCC ACT TGT	240
	Asp Ala Gly Val Gly Leu Val Leu Gln Gly Leu Gln Asn Pro Thr Cys	
30	65 70 75 80	
	AAG ATC CAG AAG CTG AGC CTT CAG AAC TGC AGC TTG ACG GAA GCT GGC	288
	Lys Ile Gln Lys Leu Ser Leu Gln Asn Cys Ser Leu Thr Glu Ala Gly	
	85 90 95	
	TGT GGG GTC CTG CCT GAT GTG CTG CGC TCT TTG TCT ACC CTG CGT GAA	336
35	Cys Gly Val Leu Pro Asp Val Leu Arg Ser Leu Ser Thr Leu Arg Glu	
	100 105 110	
	CTA CAT CTC AAT GAC AAC CCT CTG GGG GAT GAA GGC CTG AAG CTG CTC	384
	Leu His Leu Asn Asp Asn Pro Leu Gly Asp Glu Gly Leu Lys Leu Leu	
	115 120 125	
40	TGT GAA GGA CTC CGG GAC CCC CAG TGC CGT CTT GAG AAG CTT CAG TTG	432
	Cys Glu Gly Leu Arg Asp Pro Gln Cys Arg Leu Glu Lys Leu Gln Leu	

		130				135						140							
		GAA	TAC	TGT	AAC	CTC	ACA	GCT	ACC	AGC	TGC	GAG	CCC	CTG	GCC	TCA	GTG		480
		Glu	Tyr	Cys	Asn	Leu	Thr	Ala	Thr	Ser	Cys	Glu	Pro	Leu	Ala	Ser	Val		
		145					150					155					160		
5		CTC	AGG	GTG	AAA	CCT	GAC	TTT	AAA	GAG	CTA	GTA	TTG	AGC	AAC	AAT	GAC		528
		Leu	Arg	Val	Lys	Pro	Asp	Phe	Lys	Glu	Leu	Val	Leu	Ser	Asn	Asn	Asp		
						165					170					175			
		TTC	CAT	GAG	GCT	GGT	ATC	CAC	ACT	CTG	TGC	CAG	GGC	CTG	AAG	GAT	TCT		576
10		Phe	His	Glu	Ala	Gly	Ile	His	Thr	Leu	Cys	Gln	Gly	Leu	Lys	Asp	Ser		
					180					185					190				
		GCC	TGT	CAA	CTG	GAG	TCA	CTC	AAA	CTG	GAG	AAC	TGT	GGT	ATC	ACA	TCA		624
		Ala	Cys	Gln	Leu	Glu	Ser	Leu	Lys	Leu	Glu	Asn	Cys	Gly	Ile	Thr	Ser		
				195					200					205					
15		GCC	AAC	TGC	AAG	GAT	CTG	TGT	GAT	GTT	GTG	GCC	TCC	AAA	GCC	TCA	CTG		672
		Ala	Asn	Cys	Lys	Asp	Leu	Cys	Asp	Val	Val	Ala	Ser	Lys	Ala	Ser	Leu		
			210					215					220						
		CAA	GAA	CTG	GAC	TTG	GGC	AGC	AAC	AAG	CTG	GGC	AAC	ACA	GGC	ATT	GCA		720
20		Gln	Glu	Leu	Asp	Leu	Gly	Ser	Asn	Lys	Leu	Gly	Asn	Thr	Gly	Ile	Ala		
		225					230					235					240		
		GCA	CTG	TGC	TCA	GGA	CTG	CTG	CTT	CCC	AGC	TGC	AGG	CTG	AGG	ACT	CTG		768
		Ala	Leu	Cys	Ser	Gly	Leu	Leu	Leu	Pro	Ser	Cys	Arg	Leu	Arg	Thr	Leu		
						245					250					255			
25		TGG	CTC	TGG	GAC	TGT	GAT	GTC	ACT	GCA	GAA	GGC	TGC	AAG	GAC	CTG	TGC		816
		Trp	Leu	Trp	Asp	Cys	Asp	Val	Thr	Ala	Glu	Gly	Cys	Lys	Asp	Leu	Cys		
					260					265					270				
		CGT	GTC	CTC	AGA	GCC	AAG	CAG	AGC	CTG	AAG	GAA	CTC	AGC	CTA	GCT	GGC		864
		Arg	Val	Leu	Arg	Ala	Lys	Gln	Ser	Leu	Lys	Glu	Leu	Ser	Leu	Ala	Gly		
				275					280					285					
30		AAT	GAG	CTG	AAG	GAT	GAG	GGT	GCC	CAA	CTG	CTG	TGT	GAG	AGC	CTG	TTA		912
		Asn	Glu	Leu	Lys	Asp	Glu	Gly	Ala	Gln	Leu	Leu	Cys	Glu	Ser	Leu	Leu		
			290					295					300						
		GAG	CCT	GGC	TGT	CAG	CTG	GAG	TCA	CTG	TGG	GTA	AAG	ACC	TGT	AGC	CTC		960
35		Glu	Pro	Gly	Cys	Gln	Leu	Glu	Ser	Leu	Trp	Val	Lys	Thr	Cys	Ser	Leu		
		305					310					315					320		
		ACA	GCT	GCC	TCT	TGT	CCC	CAC	TTC	TGC	TCG	GTG	TTG	ACC	AAA	AAC	AGT		1008
		Thr	Ala	Ala	Ser	Cys	Pro	His	Phe	Cys	Ser	Val	Leu	Thr	Lys	Asn	Ser		
						325					330					335			
40		TCT	CTG	TTT	GAG	TTG	CAA	ATG	AGC	AGC	AAC	CCG	CTG	GGA	GAC	TCG	GGA		1056
		Ser	Leu	Phe	Glu	Leu	Gln	Met	Ser	Ser	Asn	Pro	Leu	Gly	Asp	Ser	Gly		
					340						345					350			
		GTC	GTG	GAG	CTT	TGC	AAG	GCC	CTG	GGC	TAT	CCG	GAC	ACA	GTG	CTG	CGT		1104
		Val	Val	Glu	Leu	Cys	Lys	Ala	Leu	Gly	Tyr	Pro	Asp	Thr	Val	Leu	Arg		



	355	360	365	
	GTG CTT TGG CTG GGA GAC TGT GAT GTG ACA GAC AGT GGC TGC AGC AGC			1152
	Val Leu Trp Leu Gly Asp Cys Asp Val Thr Asp Ser Gly Cys Ser Ser			
	370	375	380	
5	CTT GCC ACT GTC CTG CTG GCC AAC CGC AGC TTG AGG GAA CTG GAC CTC			1200
	Leu Ala Thr Val Leu Leu Ala Asn Arg Ser Leu Arg Glu Leu Asp Leu			
	385	390	395	400
	AGT AAC AAC TGC ATG GGG GAC AAC GGT GTC CTA CAA CTG CTG GAG AGC			1248
	Ser Asn Asn Cys Met Gly Asp Asn Gly Val Leu Gln Leu Leu Glu Ser			
10		405	410	415
	CTC AAA CAG CCC AGC TGC ATC CTT CAG CAG CTT GTC CTG TAT GAC ATT			1296
	Leu Lys Gln Pro Ser Cys Ile Leu Gln Gln Leu Val Leu Tyr Asp Ile			
		420	425	430
	TAC TGG ACG GAT GAG GTG GAA GAC CAG CTT CGG GCC CTG GAG GAG GAA			1344
15	Tyr Trp Thr Asp Glu Val Glu Asp Gln Leu Arg Ala Leu Glu Glu Glu			
		435	440	445
	AGG CCA TCC CTG AGG ATC ATT TCC TGATAA			1374
	Arg Pro Ser Leu Arg Ile Ile Ser			
	450	455		

20 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 456 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

	Met Ser Leu Asp Ile Gln Cys Glu Gln Leu Ser Asp Ala Arg Trp Thr	
	1 5 10 15	
30	Glu Leu Leu Pro Leu Ile Gln Gln Tyr Gln Val Val Arg Leu Asp Asp	
	20 25 30	
	Cys Gly Leu Thr Glu Val Arg Cys Lys Asp Ile Arg Ser Ala Ile Gln	
	35 40 45	
	Ala Asn Pro Ala Leu Thr Glu Leu Ser Leu Arg Thr Asn Glu Leu Gly	
	50 55 60	
35	Asp Ala Gly Val Gly Leu Val Leu Gln Gly Leu Gln Asn Pro Thr Cys	
	65 70 75 80	
	Lys Ile Gln Lys Leu Ser Leu Gln Asn Cys Ser Leu Thr Glu Ala Gly	
	85 90 95	
	Cys Gly Val Leu Pro Asp Val Leu Arg Ser Leu Ser Thr Leu Arg Glu	

	100		105		110
	Leu His Leu Asn Asp Asn Pro Leu Gly Asp Glu Gly Leu Lys Leu Leu				
	115		120		125
5	Cys Glu Gly Leu Arg Asp Pro Gln Cys Arg Leu Glu Lys Leu Gln Leu				
	130		135		140
	Glu Tyr Cys Asn Leu Thr Ala Thr Ser Cys Glu Pro Leu Ala Ser Val				
	145		150		155 160
	Leu Arg Val Lys Pro Asp Phe Lys Glu Leu Val Leu Ser Asn Asn Asp				
		165		170	175
10	Phe His Glu Ala Gly Ile His Thr Leu Cys Gln Gly Leu Lys Asp Ser				
		180		185	190
	Ala Cys Gln Leu Glu Ser Leu Lys Leu Glu Asn Cys Gly Ile Thr Ser				
		195		200	205
15	Ala Asn Cys Lys Asp Leu Cys Asp Val Val Ala Ser Lys Ala Ser Leu				
		210		215	220
	Gln Glu Leu Asp Leu Gly Ser Asn Lys Leu Gly Asn Thr Gly Ile Ala				
		225		230	235 240
	Ala Leu Cys Ser Gly Leu Leu Leu Pro Ser Cys Arg Leu Arg Thr Leu				
		245		250	255
20	Trp Leu Trp Asp Cys Asp Val Thr Ala Glu Gly Cys Lys Asp Leu Cys				
		260		265	270
	Arg Val Leu Arg Ala Lys Gln Ser Leu Lys Glu Leu Ser Leu Ala Gly				
		275		280	285
25	Asn Glu Leu Lys Asp Glu Gly Ala Gln Leu Leu Cys Glu Ser Leu Leu				
		290		295	300
	Glu Pro Gly Cys Gln Leu Glu Ser Leu Trp Val Lys Thr Cys Ser Leu				
		305		310	315 320
	Thr Ala Ala Ser Cys Pro His Phe Cys Ser Val Leu Thr Lys Asn Ser				
		325		330	335
30	Ser Leu Phe Glu Leu Gln Met Ser Ser Asn Pro Leu Gly Asp Ser Gly				
		340		345	350
	Val Val Glu Leu Cys Lys Ala Leu Gly Tyr Pro Asp Thr Val Leu Arg				
		355		360	365
35	Val Leu Trp Leu Gly Asp Cys Asp Val Thr Asp Ser Gly Cys Ser Ser				
		370		375	380
	Leu Ala Thr Val Leu Leu Ala Asn Arg Ser Leu Arg Glu Leu Asp Leu				
		385		390	395 400
	Ser Asn Asn Cys Met Gly Asp Asn Gly Val Leu Gln Leu Leu Glu Ser				

	405	410	415
	Leu Lys Gln Pro Ser Cys Ile Leu Gln Gln Leu Val Leu Tyr Asp Ile		
	420	425	430
5	Tyr Trp Thr Asp Glu Val Glu Asp Gln Leu Arg Ala Leu Glu Glu Glu		
	435	440	445
	Arg Pro Ser Leu Arg Ile Ile Ser		
	450	455	

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 1386 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: cDNA

## 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

	ATGAGCCTGG ACATCCAGAG CCTGGACATC CAGTGTGAGG AGCTGAGCGA CGCTAGATGG	60
	GCCGAGCTCC TCCCTCTGCT CCAGCAGTGC CAAGTGGTCA GGCTGGACGA CTGTGGCCTC	120
	ACGGAAGCAC GGTGCAAGGA CATCAGCTCT GCACTTCGAG TCAACCCTGC ACTGGCAGAG	180
	CTCAACCTGC GCAGCAACGA GCTGGGCGAT GTCGGCGTGC ATTGCGTGCT CCAGGGCCTG	240
20	CAGACCCCCT CCTGCAAGAT CCAGAAGCTG AGCCTCCAGA ACTGCTGCCT GACGGGGGCC	300
	GGCTGCGGGG TCCTGTCCAG CACACTACGC ACCCTGCCCCA CCCTGCAGGA GCTGCACCTC	360
	AGCGACAACC TCTTGGGGGA TCGGGGCCTG CAGCTGCTCT GCGAAGGACT CCTGGACCCC	420
	CAGTGCCGCC TGGAAAAGCT GCAGCTGGAG TATTGCAGCC TCTCGGCTGC CAGCTGCGAG	480
	CCCCTGGCCT CCGTGCTCAG GGCCAAGCCG GACTTCAAGG AGCTCACGGT TAGCAACAAC	540
25	GACATCAATG AGGCTGGCGT CCGTGTGCTG TGCCAGGGCC TGAAGGACTC CCCCTGCCAG	600
	CTGGAGGCGC TCAAGCTGGA GAGCTGCGGT GTGACATCAG ACAACTGCCG GGACCTGTGC	660
	GGCATTGTGG CCTCCAAGGC CTCGCTGCGG GAGCTGGCCC TGGGCAGCAA CAAGCTGGGT	720
	GATGTGGGCA TGGCGGAGCT GTGCCAGGG CTGCTCCACC CCAGCTCCAG GCTCAGGACC	780
	CTGTGGATCT GGGAGTGTGG CATCACTGCC AAGGGCTGCG GGGATCTGTG CCGTGTCTCTC	840
30	AGGGCCAAGG AGAGCCTGAA GGAGCTCAGC CTGGCCGGCA ACGAGCTGGG GGATGAGGGT	900
	GCCCCACTGC TGTGTGAGAC CCTGCTGGAA CCTGGCTGCC AGCTGGAGTC GCTGTGGGTG	960

AAGTCCTGCA GCTTCACAGC CGCCTGCTGC CCCCACTTCA GCTCAGTGCT GGCCCAGAAC 1020  
 AGGTTTCTCC TGGAGCTACA GATAAGCAAC AACAGGCTGG AGGATGCGGG CGTGCGGGAG 1080  
 CTGTGCCAGG GCCTGGGCCA GCCTGGCTCT GTGCTGCGGG TGCTCTGGTT GGCCGACTGC 1140  
 GATGTGAGTG ACAGCAGCTG CAGCAGCCTC GCCGCAACCC TGTTGGCCAA CCACAGCCTG 1200  
 5 CGTGAGCTGG ACCTCAGCAA CAACTGCCTG GGGGACGCCG GCATCCTGCA GCTGGTGGAG 1260  
 AGCGTCCGGC AGCCGGGCTG CCTCCTGGAG CAGCTGGTCC TGTACGACAT TTACTGGTCT 1320  
 GAGGAGATGG AGGACCGGCT GCAGGCCCTG GAGAAGGACA AGCCATCCCT GAGGGTCATC 1380  
 TCCTGA 1386

## (2) INFORMATION FOR SEQ ID NO:6:

- 10 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 461 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: not relevant

- 15 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

	Met	Ser	Leu	Asp	Ile	Gln	Ser	Leu	Asp	Ile	Gln	Cys	Glu	Glu	Leu	Ser	
	1				5					10					15		
	Asp	Ala	Arg	Trp	Ala	Glu	Leu	Leu	Pro	Leu	Leu	Gln	Gln	Cys	Gln	Val	
20				20					25					30			
	Val	Arg	Leu	Asp	Asp	Cys	Gly	Leu	Thr	Glu	Ala	Arg	Cys	Lys	Asp	Ile	
			35				40						45				
	Ser	Ser	Ala	Leu	Arg	Val	Asn	Pro	Ala	Leu	Ala	Glu	Leu	Asn	Leu	Arg	
		50				55						60					
25	Ser	Asn	Glu	Leu	Gly	Asp	Val	Gly	Val	His	Cys	Val	Leu	Gln	Gly	Leu	
	65				70					75					80		
	Gln	Thr	Pro	Ser	Cys	Lys	Ile	Gln	Lys	Leu	Ser	Leu	Gln	Asn	Cys	Cys	
				85					90					95			
	Leu	Thr	Gly	Ala	Gly	Cys	Gly	Val	Leu	Ser	Ser	Thr	Leu	Arg	Thr	Leu	
30				100				105					110				
	Pro	Thr	Leu	Gln	Glu	Leu	His	Leu	Ser	Asp	Asn	Leu	Leu	Gly	Asp	Ala	
			115				120						125				
	Gly	Leu	Gln	Leu	Leu	Cys	Glu	Gly	Leu	Leu	Asp	Pro	Gln	Cys	Arg	Leu	

	130	135	140
	Glu Lys Leu Gln Leu Glu Tyr Cys Ser Leu Ser Ala Ala Ser Cys Glu		
	145	150	155 160
5	Pro Leu Ala Ser Val Leu Arg Ala Lys Pro Asp Phe Lys Glu Leu Thr		
		165	170 175
	Val Ser Asn Asn Asp Ile Asn Glu Ala Gly Val Arg Val Leu Cys Gln		
		180	185 190
	Gly Leu Lys Asp Ser Pro Cys Gln Leu Glu Ala Leu Lys Leu Glu Ser		
		195	200 205
10	Cys Gly Val Thr Ser Asp Asn Cys Arg Asp Leu Cys Gly Ile Val Ala		
		210	215 220
	Ser Lys Ala Ser Leu Arg Glu Leu Ala Leu Gly Ser Asn Lys Leu Gly		
		225	230 235 240
15	Asp Val Gly Met Ala Glu Leu Cys Pro Gly Leu Leu His Pro Ser Ser		
		245	250 255
	Arg Leu Arg Thr Leu Trp Ile Trp Glu Cys Gly Ile Thr Ala Lys Gly		
		260	265 270
	Cys Gly Asp Leu Cys Arg Val Leu Arg Ala Lys Glu Ser Leu Lys Glu		
		275	280 285
20	Leu Ser Leu Ala Gly Asn Glu Leu Gly Asp Glu Gly Ala Arg Leu Leu		
		290	295 300
	Cys Glu Thr Leu Leu Glu Pro Gly Cys Gln Leu Glu Ser Leu Trp Val		
		305	310 315 320
25	Lys Ser Cys Ser Phe Thr Ala Ala Cys Cys Pro His Phe Ser Ser Val		
		325	330 335
	Leu Ala Gln Asn Arg Phe Leu Leu Glu Leu Gln Ile Ser Asn Asn Arg		
		340	345 350
	Leu Glu Asp Ala Gly Val Arg Glu Leu Cys Gln Gly Leu Gly Gln Pro		
		355	360 365
30	Gly Ser Val Leu Arg Val Leu Trp Leu Ala Asp Cys Asp Val Ser Asp		
		370	375 380
	Ser Ser Cys Ser Ser Leu Ala Ala Thr Leu Leu Ala Asn His Ser Leu		
		385	390 395 400
35	Arg Glu Leu Asp Leu Ser Asn Asn Cys Leu Gly Asp Ala Gly Ile Leu		
		405	410 415
	Gln Leu Val Glu Ser Val Arg Gln Pro Gly Cys Leu Leu Glu Gln Leu		
		420	425 430
	Val Leu Tyr Asp Ile Tyr Trp Ser Glu Glu Met Glu Asp Arg Leu Gln		

435	440	445
Ala Leu Glu Lys Asp Lys Pro Ser Leu Arg Val Ile Ser		
450	455	460

## (2) INFORMATION FOR SEQ ID NO:7:

- 5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1371 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both
- 10 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

	ATGAACCTGG ACATTCATTG CGAGCAGCTG AGCGACGCCC GGTGGACAGA GTCCTGCCC	60
	CTGCTCCAGC AGTATGAGGT GGTCAAGCTC GACGACTGCG GCCTCACGGA GGAGCACTGC	120
	AAGGACATCG GTTCTGCCCT CCGGGCCAAC CCCTCCCTGA CCGAGCTCTG CCTCCGCACC	180
15	AACGAGCTGG GCGATGCCGG CGTGACCTG GTGCTGCAGG GCCTGCAGAG CCCACCTGC	240
	AAGATCCAGA AGCTCAGCCT GCAGAACTGC TCCCTGACCG AGGCGGGCTG CGGGGTCCTG	300
	CCCAGCACGC TGCCTCCCT GCCCACGCTG CGGGAGCTGC ATCTCAGCGA CAACCCACTG	360
	GGGGACGCCG GCCTGCGGCT GCTCTGTGAG GGGCTCCTGG ACCCCCAGTG CCACCTGGAG	420
	AAGCTGCAGT TGGAGTACTG CCGCCTGACG GCCGCCAGCT GCGAGCCCCCT GGCCTCGGTG	480
20	CTCAGGGCCA CGCGGGCCTT GAAGGAGCTC ACGGTGAGCA ACAACGACAT CGGCGAGGCC	540
	GGCGCCCGGG TGCTGGGCCA GGGTCTGGCC GACTCTGCCT GCCAGCTGGA GACGCTCAGG	600
	CTGGAGAACT GCGGTCTCAC GCCAGCCAAC TGCAAAGACC TGTGCGGAAT TGTGGCCTCC	660
	CAGGCCTCGC TGCGGGAGCT GGCCCTGGGC AGCAACAAGC TGGGTGATGT GGGCATGGCG	720
	GAGCTGTGCC CAGGGCTGCT CCACCCAGC TCCAGGCTCA GGACCCTGTG GATCTGGGAG	780
25	TGTGGCATCA CTGCCAAGGG CTGCGGGGAT CTGTGCCGTG TCCTCAGGGC CAAGGAGAGC	840
	CTGAAGGAGC TCAGCCTGGC CGGCAACGAG CTGGGGGATG AGGGTGCCCG ACTGCTGTGT	900
	GAGACCCTGC TGGAACCTGG CTGCCAGCTG GAGTCGCTGT GGGTGAAGTC CTGCAGCTTC	960
	ACAGCCGCCT GCTGCCCCCA CTTCAAGCTCA GTGCTGGCCC AGAACAGGTT TCTCCTGGAG	1020
	CTACAGATAA GCAACAACAG GCTGGAGGAT GCGGGCGTGC GGGAGCTGTG CCAGGGCCTG	1080

GGCCAGCCTG GCTCTGTGCT GCGGGTGCTC TGGTTGGCCG ACTGCGATGT GAGTGACAGC 1140  
 AGCTGCAGCA GCCTGCGCGC AACCTGTGTT GCCAACCCACA GCCTGCGTGA GCTGGACCTC 1200  
 AGCAACAACCT GCCTGGGGGA CGCCGGCATC CTGCAGCTGG TGGAGAGCGT CCGGCAGCCG 1260  
 GGCTGCCTCC TGGAGCAGCT GGTCTGTAC GACATTTACT GGTCTGAGGA GATGGAGGAC 1320  
 5 CGGCTGCAGG CCCTGGAGAA GGACAAGCCA TCCCTGAGGG TCATCTCCTG A 1371

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 456 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: both

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

15 Met Asn Leu Asp Ile His Cys Glu Gln Leu Ser Asp Ala Arg Trp Thr  
 1 5 10 15  
 Glu Leu Leu Pro Leu Leu Gln Gln Tyr Glu Val Val Arg Leu Asp Asp  
 20 25 30  
 Cys Gly Leu Thr Glu Glu His Cys Lys Asp Ile Gly Ser Ala Leu Arg  
 35 40 45  
 20 Ala Asn Pro Ser Leu Thr Glu Leu Cys Leu Arg Thr Asn Glu Leu Gly  
 50 55 60  
 Asp Ala Gly Val His Leu Val Leu Gln Gly Leu Gln Ser Pro Thr Cys  
 65 70 75 80  
 25 Lys Ile Gln Lys Leu Ser Leu Gln Asn Cys Ser Leu Thr Glu Ala Gly  
 85 90 95  
 Cys Gly Val Leu Pro Ser Thr Leu Arg Ser Leu Pro Thr Leu Arg Glu  
 100 105 110  
 Leu His Leu Ser Asp Asn Pro Leu Gly Asp Ala Gly Leu Arg Leu Leu  
 115 120 125  
 30 Cys Glu Gly Leu Leu Asp Pro Gln Cys His Leu Glu Lys Leu Gln Leu  
 130 135 140  
 Glu Tyr Cys Arg Leu Thr Ala Ala Ser Cys Glu Pro Leu Ala Ser Val  
 145 150 155 160  
 35 Leu Arg Ala Thr Arg Ala Leu Lys Glu Leu Thr Val Ser Asn Asn Asp  
 165 170 175

	Ile	Gly	Glu	Ala	Gly	Ala	Arg	Val	Leu	Gly	Gln	Gly	Leu	Ala	Asp	Ser	
				180					185					190			
	Ala	Cys	Gln	Leu	Glu	Thr	Leu	Arg	Leu	Glu	Asn	Cys	Gly	Leu	Thr	Pro	
			195					200					205				
5	Ala	Asn	Cys	Lys	Asp	Leu	Cys	Gly	Ile	Val	Ala	Ser	Gln	Ala	Ser	Leu	
		210					215					220					
	Arg	Glu	Leu	Ala	Leu	Gly	Ser	Asn	Lys	Leu	Gly	Asp	Val	Gly	Met	Ala	
	225					230					235					240	
10	Glu	Leu	Cys	Pro	Gly	Leu	Leu	His	Pro	Ser	Ser	Arg	Leu	Arg	Thr	Leu	
					245					250					255		
	Trp	Ile	Trp	Glu	Cys	Gly	Ile	Thr	Ala	Lys	Gly	Cys	Gly	Asp	Leu	Cys	
				260					265					270			
	Arg	Val	Leu	Arg	Ala	Lys	Glu	Ser	Leu	Lys	Glu	Leu	Ser	Leu	Ala	Gly	
			275					280					285				
15	Asn	Glu	Leu	Gly	Asp	Glu	Gly	Ala	Arg	Leu	Leu	Cys	Glu	Thr	Leu	Leu	
		290					295					300					
	Glu	Pro	Gly	Cys	Gln	Leu	Glu	Ser	Leu	Trp	Val	Lys	Ser	Cys	Ser	Phe	
	305					310					315					320	
20	Thr	Ala	Ala	Cys	Cys	Pro	His	Phe	Ser	Ser	Val	Leu	Ala	Gln	Asn	Arg	
				325						330					335		
	Phe	Leu	Leu	Glu	Leu	Gln	Ile	Ser	Asn	Asn	Arg	Leu	Glu	Asp	Ala	Gly	
				340					345					350			
	Val	Arg	Glu	Leu	Cys	Gln	Gly	Leu	Gly	Gln	Pro	Gly	Ser	Val	Leu	Arg	
			355					360					365				
25	Val	Leu	Trp	Leu	Ala	Asp	Cys	Asp	Val	Ser	Asp	Ser	Ser	Cys	Ser	Ser	
		370					375					380					
	Leu	Ala	Ala	Thr	Leu	Leu	Ala	Asn	His	Ser	Leu	Arg	Glu	Leu	Asp	Leu	
	385					390					395					400	
30	Ser	Asn	Asn	Cys	Leu	Gly	Asp	Ala	Gly	Ile	Leu	Gln	Leu	Val	Glu	Ser	
				405						410					415		
	Val	Arg	Gln	Pro	Gly	Cys	Leu	Leu	Glu	Gln	Leu	Val	Leu	Tyr	Asp	Ile	
				420					425					430			
	Tyr	Trp	Ser	Glu	Glu	Met	Glu	Asp	Arg	Leu	Gln	Ala	Leu	Glu	Lys	Asp	
		435						440					445				
35	Lys	Pro	Ser	Leu	Arg	Val	Ile	Ser									
		450					455										

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:



- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TAATAGCATA TGAGTCTTGA CATCCAGTGT GAG

33

(2) INFORMATION FOR SEQ ID NO:10:

- 10 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 41 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTATTAGGAT CCTTATCAGG AAATGATCCT CAGGGATGGC C

41

(2) INFORMATION FOR SEQ ID NO:11:

- 20 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

25 TATTATCATA TGAACCTSGA YATYCAYTGY GA

32

(2) INFORMATION FOR SEQ ID NO:12:

- 30 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 37 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATTATAAGC TTGCCCAAAA GGTGTTTTTAC TAAGTAG

37

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 35 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TATTATCATA TGAGCCTGGA CATCCAGAGC CTGGA

35

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

20 TATTAGAATT CAAGCTTATC AGGAGATGAC CCTCAGGGA

39

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTGGCCTCCA AGGCCTCGCT GCGG

24

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: both

5

- (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

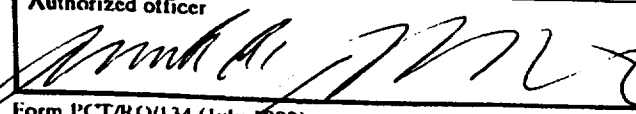
CCGCAGCGAG GCCTTGGAGG CCAC

24

- 58 -

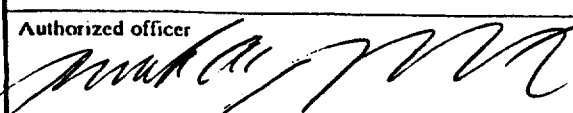
## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>4</u> , line <u>2</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Agricultural Research Service Culture Collection (NRRL)	
Address of depositary institution (including postal code and country) 1815 North University Street Peoria, Illinois 61604 United States of America	
Date of deposit August 12, 1997	Accession Number NRRL Deposit No. B-21810
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
E. coli DH5 $\alpha$ F'IQ(pHumanRI)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<input checked="" type="checkbox"/> For receiving Office use only This sheet was received with the international application Authorized officer 	<input type="checkbox"/> For International Bureau use only This sheet was received by the International Bureau on: Authorized officer

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

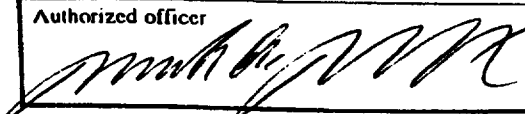
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>10</u> , line <u>10</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Agricultural Research Service Culture Collection (NRRL)	
Address of depositary institution (including postal code and country) 1815 North University Street Peoria, Illinois 61604 United States of America	
Date of deposit August 9, 1996	Accession Number NRRL Deposit No. B-21612
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
E. coli DH5 $\alpha$ F'IQ(pPorcineRI)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<div>For receiving Office use only</div> <div><input checked="" type="checkbox"/> This sheet was received with the international application</div> <div>Authorized officer </div>	<div>For International Bureau use only</div> <div><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div>Authorized officer</div>

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>10</u> , line <u>23</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Agricultural Research Service Culture Collection (NRRL)	
Address of depositary institution (including postal code and country) 1815 North University Street Peoria, Illinois 61604 United States of America	
Date of deposit August 9, 1996	Accession Number NRRL Deposit No. B-21613
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
E. coli DH5 $\alpha$ F'IQ(pRERatRI)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
Form PCT/RO/134 (July 1992)	

<div style="text-align: center;">For receiving Office use only</div> <div><input checked="" type="checkbox"/> This sheet was received with the international application</div> <div>Authorized officer </div>	<div style="text-align: center;">For International Bureau use only</div> <div><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div>Authorized officer</div>
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## WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a polynucleotide encoding a mammalian ribonuclease inhibitor (RI) selected from the group of mammalian RIs consisting of:
  - (a) a porcine RI;
  - 5 (b) a rat RI;
  - (c) a human liver RI; and
  - (d) a chimeric mammalian RI, wherein said polynucleotide comprises a substantial portion of a nucleotide sequence encoding an RI from a first mammalian species and a substantial portion of a nucleotide sequence encoding an RI from a second mammalian species which is different  
10 from the first mammalian species.
2. The nucleic acid molecule of claim 1, wherein said porcine RI is porcine liver RI.
3. The nucleic acid molecule of claim 2, wherein said polynucleotide has a nucleotide sequence selected from the group consisting of:
  - (a) the nucleotide sequence set forth in SEQ ID NO:1, wherein T can also be U;
  - 15 (b) a nucleotide sequence encoding the porcine liver RI polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in NRRL Deposit No. B-21612;
  - (c) a nucleic acid sequence complementary to (a) or (b); and
  - (d) a fragment of (a), (b) or (c) which falls within the portion of SEQ ID NO:1,  
20 wherein said fragment is at least 18 bases in length and wherein said fragment hybridizes under stringent hybridization conditions to a porcine genomic DNA molecule encoding a porcine liver RI.
4. The nucleic acid molecule of claim 1, wherein said rat RI is rat liver RI.
5. The nucleic acid molecule of claim 4, wherein said polynucleotide has a nucleotide sequence selected from the group consisting of:
  - 25 (a) the nucleotide sequence set forth in SEQ ID NO:3, wherein T can also be U;

(b) a nucleotide sequence encoding the rat liver RI polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in NRRL Deposit No. B-21613;  
(c) a nucleic acid sequence complementary to (a) or (b); and  
(d) a fragment of (a), (b) or (c) which falls within the portion of SEQ ID NO:3,  
5 wherein said fragment is at least 18 bases in length and wherein said fragment hybridizes under stringent hybridization conditions to a rat genomic DNA molecule encoding a rat liver RI.

6. The nucleic acid molecule of claim 1, wherein said polynucleotide encoding a human liver RI has a nucleotide sequence at least 99% identical to a reference sequence selected from the group consisting of:

- 10 (a) the nucleotide sequence set forth in SEQ ID NO:5, wherein T can also be U;  
(b) a nucleotide sequence encoding the human liver RI polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in NRRL Deposit No. B-21810; and  
(c) a nucleic acid sequence complementary to (a) or (b).

15 7. The nucleic acid molecule of claim 1, wherein said chimeric mammalian RI is thermostable.

8. The nucleic acid molecule of claim 1, wherein said first mammalian species is non-human and said second mammalian species is human.

9. The nucleic acid molecule of claim 8, wherein said human RI is a human liver RI or  
20 a human placental RI.

10. The nucleic acid molecule of claim 8, wherein said non-human mammalian species is selected from the group consisting of rat, porcine, equine, bovine, ovine, murine, canine, feline, ape, and monkey.

11. The nucleic acid molecule of claim 1, wherein said chimeric mammalian RI is a  
25 chimeric porcine/human RI.



12. The nucleic acid molecule of claim 11, wherein said polynucleotide has a nucleotide sequence at least 90% identical to a reference sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NO:7, wherein T can also be U;

and

5 (b) a nucleic acid sequence complementary to (a).

13. The nucleic acid molecule of claim 12, wherein said polynucleotide has a nucleotide sequence at least 95% identical to said reference sequence.

14. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or SEQ ID NO:7.

10 15. The nucleic acid molecule of claim 1, further comprising at least one nucleotide sequence encoding at least one binding peptide.

16. The nucleic acid molecule of claim 15, wherein said nucleotide sequence encoding said binding peptide is located 5' to the translation start site of said polynucleotide encoding said mammalian RI.

15 17. The nucleic acid molecule of claim 15, wherein said binding peptide is selected from the group consisting of the OmpA signal sequence, a GST tag, a HIS tag, a thioredoxin tag and a hemagglutinin (HA) tag.

18. The nucleic acid molecule of claim 1 or claim 15, wherein said nucleic acid molecule is operably linked to a promoter for expression of said mammalian RI.

20 19. A vector comprising the nucleic acid molecule of claim 1 or claim 15.

20. The vector of claim 19, wherein said vector is an expression vector comprising said nucleic acid molecule operably linked to a promoter.

21. The vector of claim 20, wherein said promoter is an inducible promoter.

22. The vector of claim 21, wherein said inducible promoter is under the control of a repressor.

23. The vector of claim 22, wherein said repressor is *lacI*<sup>q</sup>.

24. A host cell comprising the nucleic acid molecule of claim 1.

5 25. A host cell comprising the vector of claim 19.

26. A method for producing a mammalian RI polypeptide comprising culturing the host cell of claim 24 or claim 25 and isolating said mammalian RI polypeptide.

27. A mammalian RI polypeptide produced by the method of claim 26.

10 28. The mammalian RI polypeptide of claim 27, wherein said polypeptide is a porcine RI polypeptide.

29. The polypeptide of claim 28, wherein said porcine RI polypeptide is a porcine liver RI polypeptide.

30. The polypeptide of claim 29, wherein said porcine liver RI polypeptide has an amino acid sequence as set forth in SEQ ID NO:2.

15 31. The mammalian RI polypeptide of claim 27, wherein said polypeptide is a rat RI polypeptide.

32. The polypeptide of claim 31, wherein said rat RI polypeptide is a rat liver RI polypeptide.

20 33. The polypeptide of claim 32, wherein said rat liver RI polypeptide has an amino acid sequence as set forth in SEQ ID NO:4.

34. The mammalian RI polypeptide of claim 27, wherein said polypeptide is a human liver RI polypeptide.

35. The polypeptide of claim 34, wherein said human liver RI polypeptide has an amino acid sequence as set forth in SEQ ID NO:6.

5 36. The mammalian RI polypeptide of claim 27, wherein said polypeptide is a chimeric mammalian RI polypeptide.

37. The polypeptide of claim 36, wherein said chimeric mammalian RI polypeptide is a chimeric porcine/human RI polypeptide.

38. The polypeptide of claim 37, wherein said chimeric porcine/human RI polypeptide has  
10 an amino acid sequence as set forth in SEQ ID NO:8.

39. The mammalian RI polypeptide of claim 27, wherein said polypeptide is thermostable.

40. A method of obtaining substantially RNase free ribonuclease inhibitor, said method comprising

(a) obtaining a biological sample from a mammalian source which contains  
15 ribonuclease inhibitor;

(b) mixing said sample with a binding partner, with the proviso that said binding partner is not an RNase; and

(c) isolating said ribonuclease inhibitor bound to said binding partner.

41. The method of claim 40, wherein said ribonuclease inhibitor is obtained from a source  
20 selected from the group consisting of human, bovine, rat, porcine, monkey, ape, and mouse.

42. The method of claim 40, wherein said binding partner is an antibody or fragment thereof.

43. The method of claim 40, wherein said biological sample is selected from the group consisting of a cell, tissue, skin, brain, liver, and kidney.

44. The method of claim 40, wherein said binding partner is attached to a solid support.

45. A method of obtaining substantially RNase free recombinant ribonuclease inhibitor,  
5 said method comprising

(a) culturing a recombinant host under conditions sufficient to express said recombinant ribonuclease inhibitor;

(b) mixing said ribonuclease inhibitor with a binding partner, with a proviso that said binding partner is not an RNase; and

10 (c) isolating said ribonuclease inhibitor from said binding partner.

46. The method of claim 45, wherein said ribonuclease inhibitor is derived from a mammal selected from a group consisting of human, bovine, porcine, rat, mouse, monkey, and ape.

47. The method of claim 45, wherein said ribonuclease inhibitor comprises a binding peptide which specifically binds to said binding partner.

15 48. The method of claim 47, wherein said binding peptide is selected from a group consisting of a HIS tag, a thioredoxin tag, a hemagglutinin tag, a GST Tag, and an OmpA signal sequence tag.

49. The method of claim 45, wherein said binding partner is attached to a solid support.

20 50. A method for producing a nucleic acid molecule comprising mixing a template nucleic acid molecule with at least one human liver RI polypeptide of claim 34, or at least one chimeric mammalian RI polypeptide of claim 36, under conditions sufficient to synthesize a nucleic acid molecule from said template nucleic acid molecule.

25 51. The method of claim 50, wherein said chimeric mammalian RI polypeptide is a chimeric porcine/human RI polypeptide.

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52. The method of claim 50, wherein said template nucleic acid molecule is an RNA molecule.

53. The method of claim 52, wherein said RNA molecule is a mRNA or a polyA<sup>+</sup> RNA molecule.

5 54. The method of claim 50, wherein said mixture further comprises at least one thermostable DNA polymerase.

55. The method of claim 54, wherein said thermostable DNA polymerase is selected from the group consisting of *Tne*, *Tma*, *Taq*, *Pfu*, *Tth*, *Tfi*, VENT, DEEPVENT, *Pwo*, *Tfl*, and mutants, variants and derivatives thereof.

10 56. The method of claim 50, wherein said mixture further comprises at least one polypeptide having reverse transcriptase activity.

57. The method of claim 56, wherein said polypeptide having reverse transcriptase activity is substantially reduced in RNase H activity.

15 58. The method of claim 56, wherein said polypeptide having reverse transcriptase activity is selected from the group consisting of M-MLV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase and HIV reverse transcriptase.

20 59. The method of claim 57, wherein said polypeptide having reverse transcriptase activity that is substantially reduced in RNase H activity is selected from the group consisting of M-MLV H<sup>-</sup> reverse transcriptase, RSV H<sup>-</sup> reverse transcriptase, AMV H<sup>-</sup> reverse transcriptase, RAV H<sup>-</sup> reverse transcriptase, MAV H<sup>-</sup> reverse transcriptase and HIV H<sup>-</sup> reverse transcriptase.

60. The method of claim 50, wherein said mixture further comprises at least one polypeptide having RNA polymerase activity.

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61. The method of claim 50, wherein said production is performed at a temperature selected from the group consisting of about 50°C or greater, about 51°C or greater, about 52°C or greater, about 53°C or greater, about 54°C or greater, about 55°C or greater, about 56°C or greater, about 57°C or greater, about 58°C or greater, about 59°C or greater, about 60°C or greater, about 61°C or greater, about 62°C or greater, about 63°C or greater, about 64°C or greater and about 65°C or greater.

62. A nucleic acid molecule produced according to the method of claim 50.

63. The nucleic acid molecule of claim 62, wherein said nucleic acid molecule is a DNA molecule, an RNA molecule or a DNA-RNA hybrid molecule.

10 64. The nucleic acid molecule of claim 63, wherein said DNA molecule is a cDNA molecule.

65. The nucleic acid molecule of claim 62, wherein said nucleic acid molecule is single-stranded.

15 66. The nucleic acid molecule of claim 62, wherein said nucleic acid molecule is double-stranded.

67. A method for *in vitro* production of a polypeptide comprising mixing a mRNA or polyA+ RNA molecule with at least one mammalian RI selected from the group of RIs consisting of a porcine liver RI, a rat liver RI, a human liver RI and a chimeric mammalian RI, under conditions sufficient to synthesize a polypeptide from said mRNA or polyA+ RNA molecule.

20 68. The method of claim 67, wherein said chimeric mammalian RI is a chimeric porcine/human liver RI.

69. A polypeptide produced according to the method of claim 67.

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70. A kit comprising one or more containers, wherein a first container contains at least one mammalian RI selected from the group of RIs consisting of a porcine liver RI, a rat liver RI, a human liver RI and a chimeric mammalian RI.

71. The kit of claim 70, wherein said chimeric mammalian RI is a chimeric porcine/human  
5 liver RI.

72. The kit of claim 70, wherein said RI is thermostable.

73. The kit of claim 70, further comprising a container containing at least one thermostable DNA polymerase.

74. The kit of claim 73, wherein said thermostable DNA polymerase is selected from the  
10 group consisting of *Tne*, *Tma*, *Taq*, *Pfu*, *Tth*, *Tfi*, VENT, DEEPVENT, *Pwo*, *Tfl*, and mutants, variants and derivatives thereof.

75. The kit of claim 70, further comprising a container containing at least one polypeptide having reverse transcriptase activity.

76. The kit of claim 75, wherein said polypeptide having reverse transcriptase activity is  
15 substantially reduced in RNase H activity.

77. The kit of claim 75, wherein said polypeptide having reverse transcriptase activity is selected from the group consisting of M-MLV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase and HIV reverse transcriptase.

20 78. The kit of claim 76, wherein said polypeptide having reverse transcriptase activity that is substantially reduced in RNase H activity is selected from the group consisting of M-MLV H<sup>-</sup> reverse transcriptase, RSV H<sup>-</sup> reverse transcriptase, AMV H<sup>-</sup> reverse transcriptase, RAV H<sup>-</sup> reverse transcriptase, MAV H<sup>-</sup> reverse transcriptase and HIV H<sup>-</sup> reverse transcriptase.

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79. The kit of claim 70, further comprising a container containing at least one polypeptide having RNA polymerase activity.

80. The kit of claim 70, further comprising a container containing at least one cell-free protein translation mixture.



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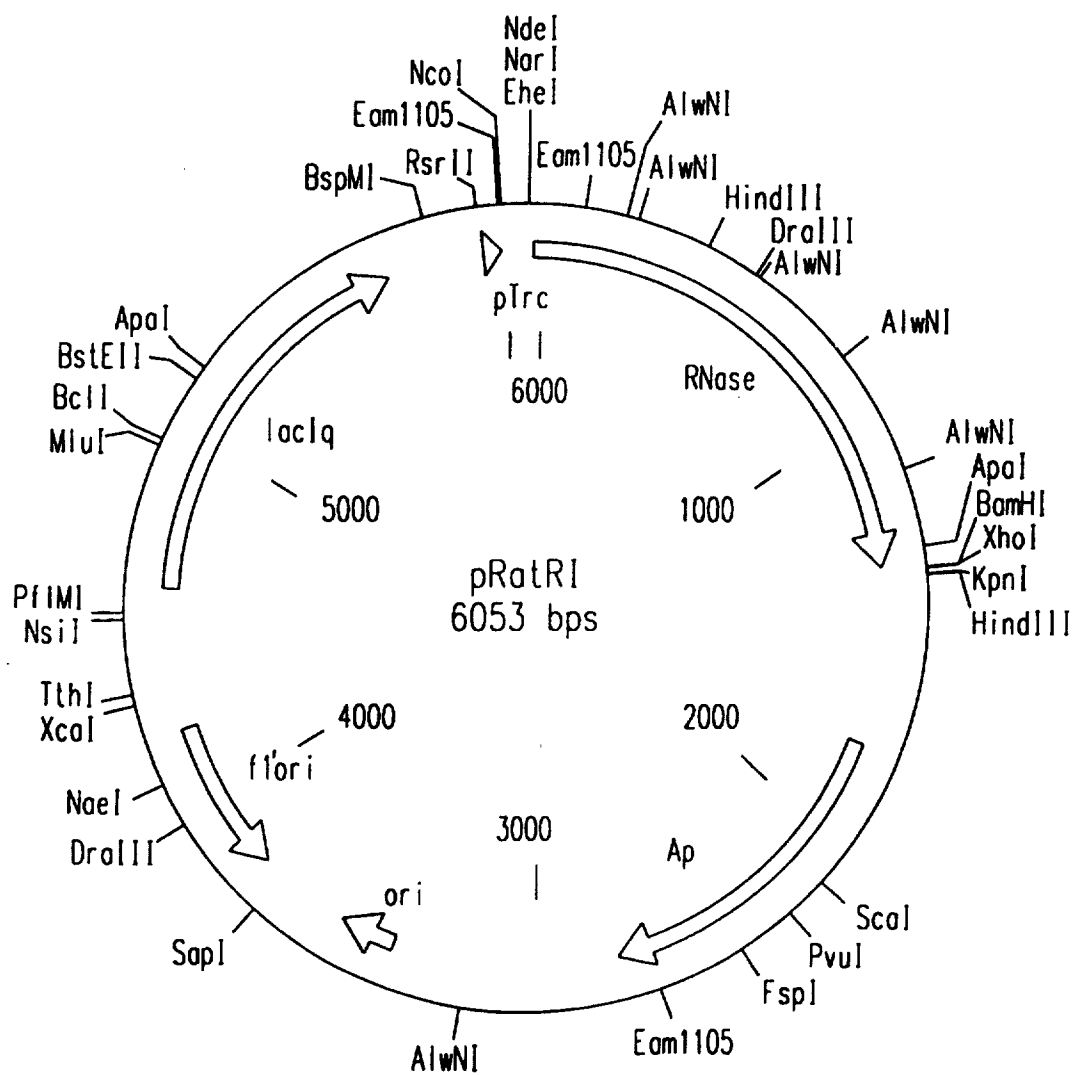


FIG.1

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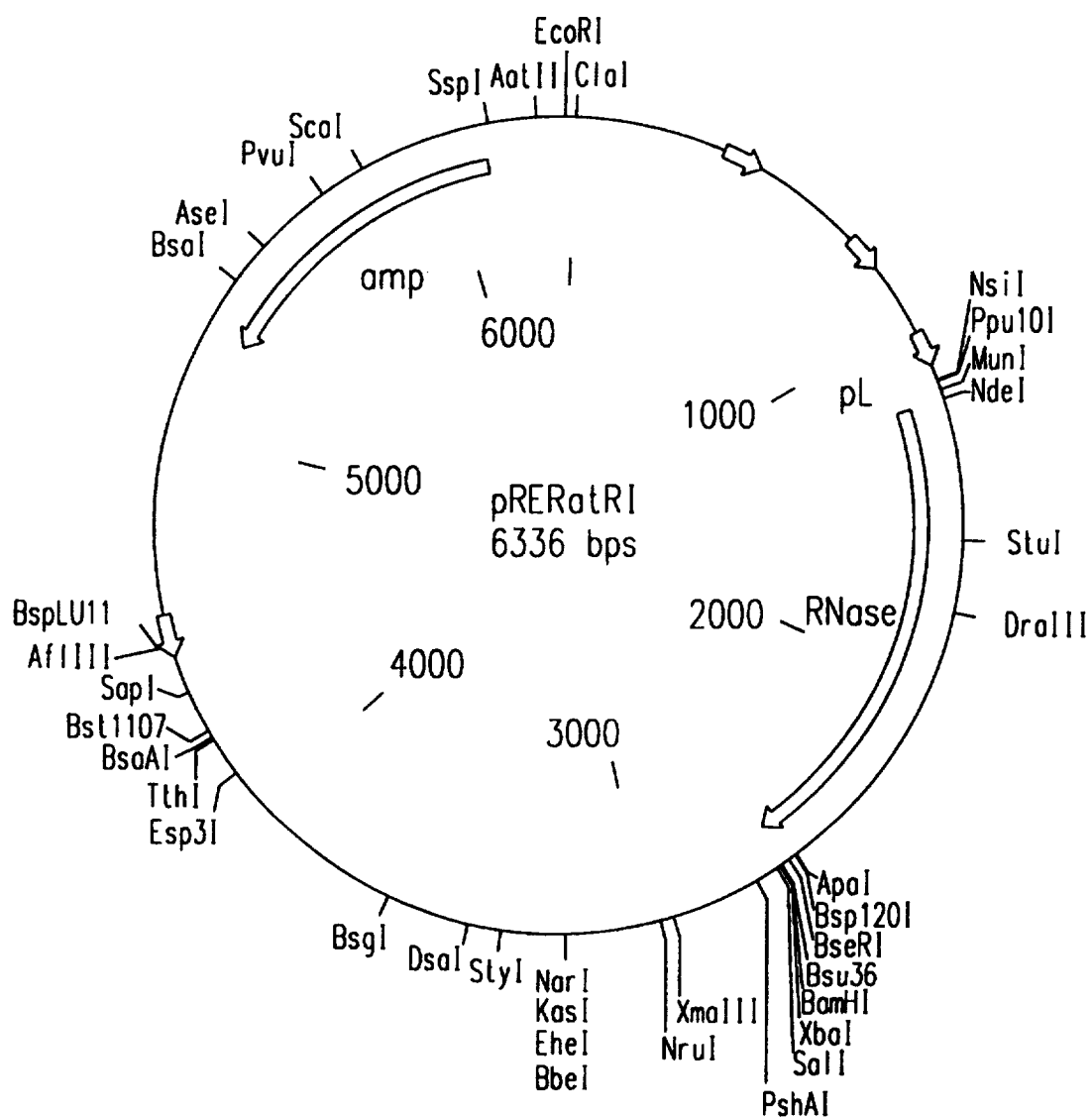


FIG.2

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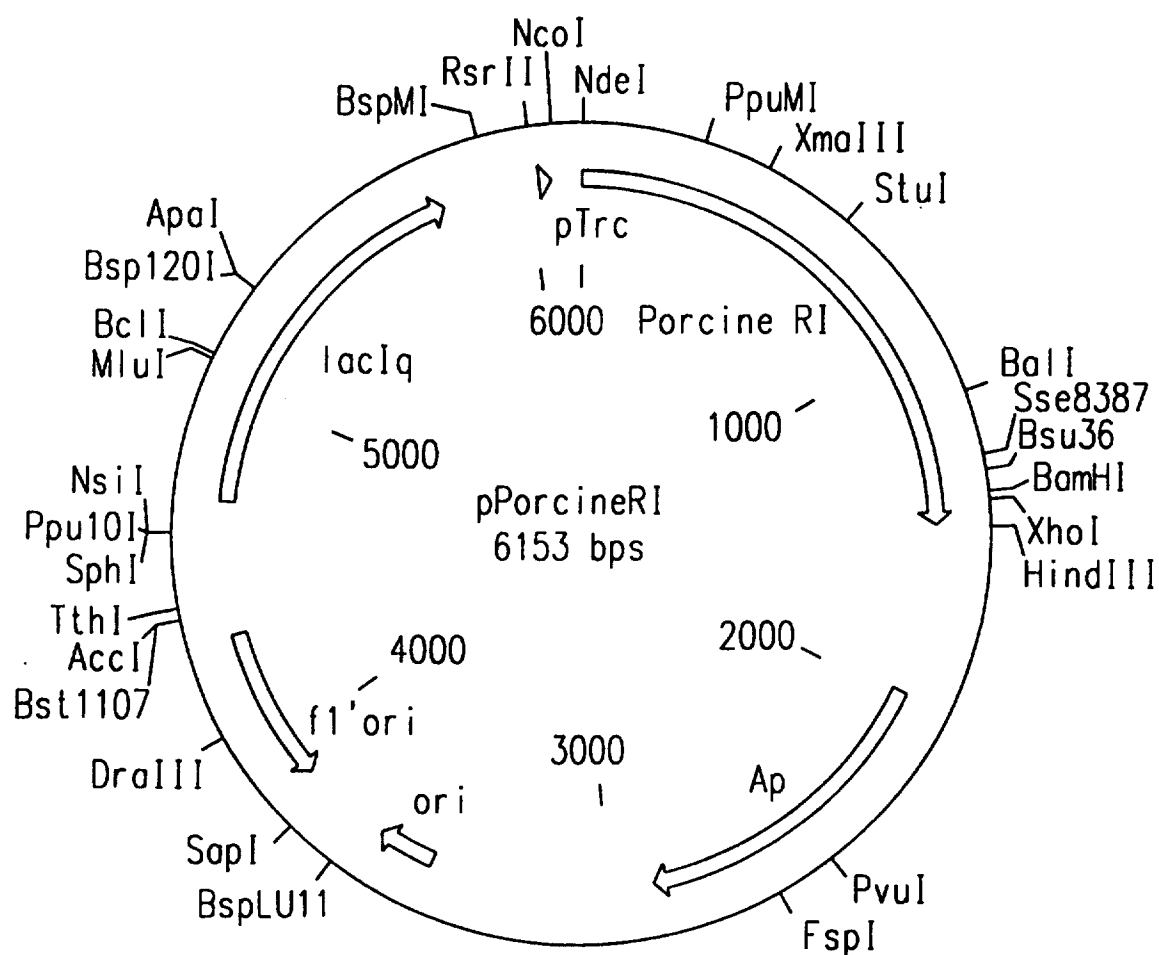


FIG.3

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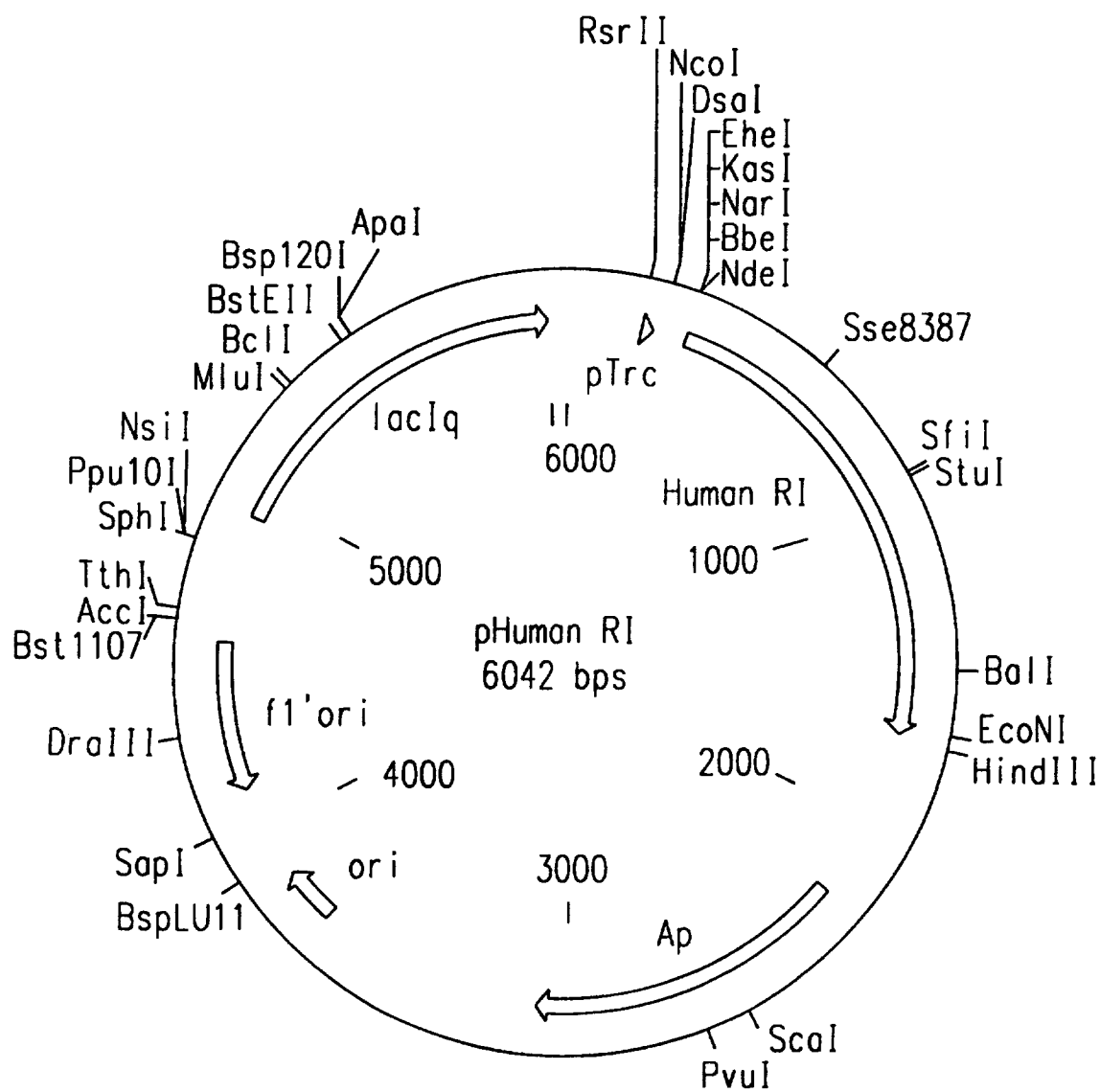


FIG.4

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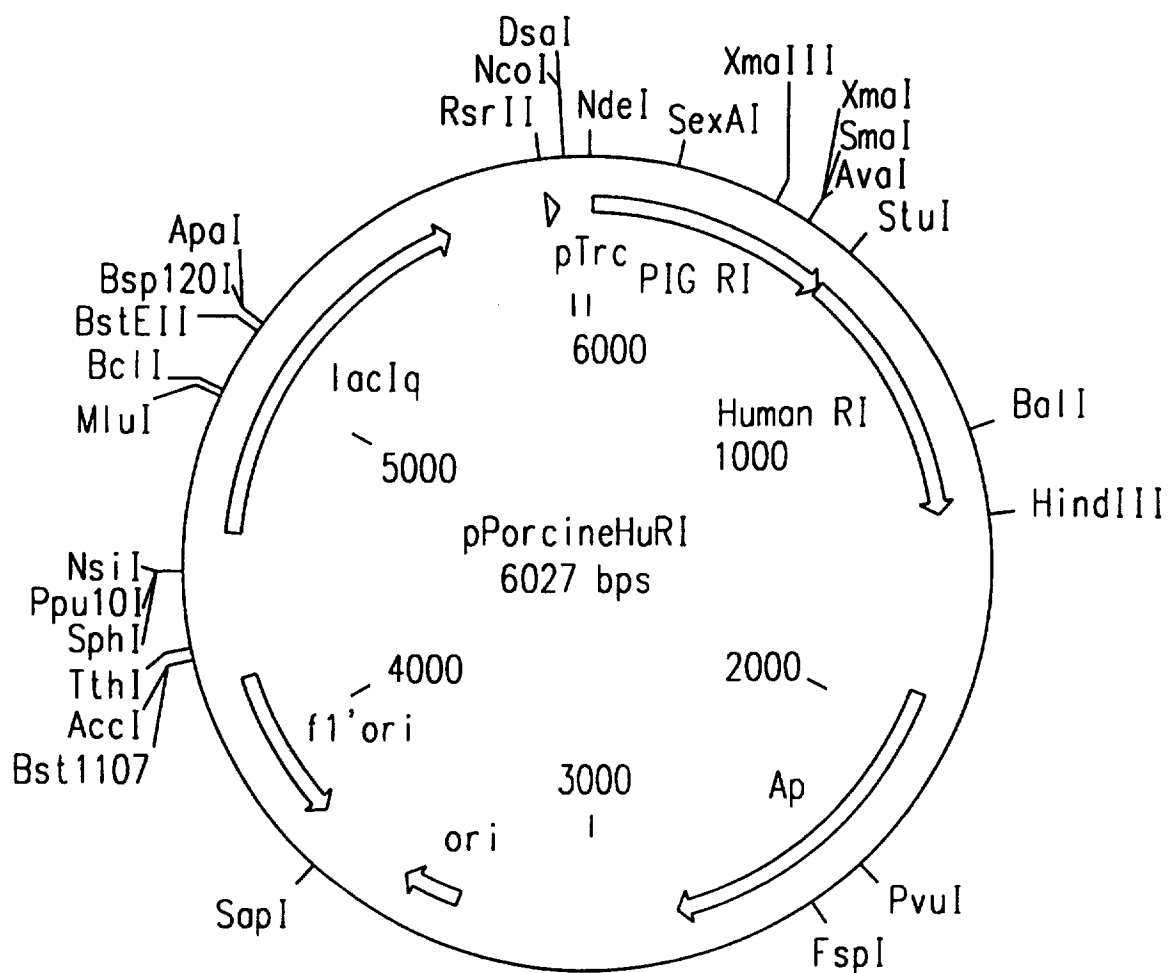


FIG.5

## INTERNATIONAL SEARCH REPORT

 International application No.  
PCT/US97/14254

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/12, 5/10, 5/16; C12Q 1/68; C12P 19/34; C07K 14/47

US CL : 530/350; 536/23.4, 23.5; 435/320.1, 252.3, 325, 6, 91.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.4, 23.5; 435/320.1, 252.3, 325, 6, 91.1

 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: ribonuclease inhibitor, mammal?, porcine, rat, human, liver, fusion

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	VICENTINI et al. Protein chemical and kinetic characterization of recombinant porcine ribonuclease inhibitor expressed in <i>Saccharomyces cerevisiae</i> . Biochemistry. 1990. Vol.29. Pages 8827-8834, see entire document.	NONE1,19-20,24-29,39,70, 72 ----- 2-18,21-23, 30-38,40- 69,71,73-80
X ---- A	NEUMANN et al. Crystallization o porcine liver ribonuclease inhibitor a member of the family of proteins containing leucine-rich repeats. Journal of Molecular Biology. 1993. Vol. 231. Pages 505-508, see entire document.	27-29 ----- 1-26,30-80

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

\* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*B\* earlier document published on or after the international filing date

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\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\*

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\*

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\*

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*Z\*

document member of the same patent family

Date of the actual completion of the international search

01 OCTOBER 1997

Date of mailing of the international search report

2 NOV 1997

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