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Antisense oligonucleotides which can inhibit the formation of capillary tubes by endothelial cells

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(56) Related Art

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(54) Title: ANTISENSE OLIGONUCLEOTIDES WHICH CAN INHIBIT THE FORMATION OF CAPILLARY TUBES BY ENDOTHELIAL CELLS

(54) Titre : OLIGONUCLEOTIDES ANTISENS CAPABLES D'INHIBER LA FORMATION DES TUBES CAPILLAIRES PAR DES CELLULES ENDOTHELIALES

(57) Abstract: The invention relates to pharmaceutical compositions which inhibit the formation of capillary tubes by endothelial cells, comprising at least one oligonucleotide which can inhibit the expression of the IRS-1 protein. According to the invention, the oligonucleotides are embodied as anti-angiogenesis agents. Said pharmaceutical compositions are particularly useful in treating angiogenesis-related pathologies.

(57) Abrégé : La présente invention a pour objet des compositions pharmaceutiques pour inhiber la formation de tubes capillaires par les cellules endothéliales, comprenant au moins un oligonucléotide capable d'inhiber l'expression de la protéine IRS-1. Les oligonucléotides selon l'invention sont indiqués comme agents anti-angiogéniques. Les compositions pharmaceutiques de l'invention sont particulièrement utiles pour le traitement de pathologies liées à l'angiogénèse.

2002345669 23 Dec 2004

ANTISENSE OLIGONUCLEOTIDES CAPABLE OF INHIBITING THE FORMATION OF CAPILLARY TUBES BY ENDOTHELIAL CELLS

The present invention provides antisense oligonucleotides capable of inhibiting the expression of the protein IRS-1 and of inhibiting the formation of capillary tubes by endothelial cells. The oligonucleotides according to the invention are thus indicated as antiangiogenic agents. They are also indicated as anti-cell-multiplication agents, in particular as anti-tumor agents.

The invention also pertains to pharmaceutical compositions containing said oligonucleotides and the use of said oligonucleotides as analysis reagents.

All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

Angiogenesis is a fundamental process by means of which new blood vessels are formed. This process is essential in many normal physiological phenomena such as reproduction, development and even cicatrization. In these normal biological phenomena, angiogenesis is under strict control, i.e., it is triggered during a short period (several days) and then completely inhibited. However, many pathologies are linked to uncontrolled, invasive angiogenesis: arthritis, a pathology due to the damaging of cartilages by invasive neovessels; diabetic retinopathy or the invasion of the retina by neovessels leading to blindness of the patients; neovascularization

of the ocular apparatus presents the major cause of blindness and this neovascularization is involved in about twenty different eye diseases; and moreover the growth and metastasis of tumors which are linked directly to neovascularization and are dependent on angiogenesis. The tumor stimulates the growth of neovessels by its own growth. Moreover, these neovessels are escape routes for tumors which thereby join up with the blood circulation and induce metastases in sites remote from the initial tumor focus, such as the liver, lungs or bones.

Angiogenesis, the formation of neovessels by endothelial cells, involves the migration, growth and differentiation of endothelial cells. The regulation of these biological phenomena is directly linked to genetic expression.

The research studies performed in the framework of the present invention made it possible to identify and prepare nucleic acid sequences involved in the regulation of angiogenesis.

Other studies pertaining to angiogenesis have shown a noteworthy expression and phosphorylation at the level of a tyrosine residue of an intracellular 180-kDa protein by endothelial cells cultured on a surface of type I collagen and stimulated by an angiogenic factor such as bFGF. The noteworthy expression and phosphorylation at the level of the tyrosine residue of the intracellular 180-kDa protein accompanies the formation of capillary tubes by the endothelial cells.

This protein is already known as a substrate of the insulin receptor (called IRS-1). It has been partially identified and investigated by certain diabetes researchers (Quon et al., J. Biol. Chem. (1994), 269 (45), 27920-27924).

These authors studied the role of IRS-1 in (i) the translocation of GLUT 4 stimulated by insulin and (ii) the transport of glucose in rat adipose cells. In order to do this, they constructed a plasmid containing:

- a double chain oligonucleotide obtained from the sense oligonucleotide of the following sequence SEQ ID NO. ID No. 1:5'-TCGATGTGAC GCTACTGATG AGTCCGTGAG GACGAAACTC TGGCCTAG-3'; and
- cDNA coding for human IRS-1,

and transfected rat adipose cells with said plasmid.

The research performed in the framework of the present invention revealed that the expression of the protein IRS-1 is also induced in endothelial cells when said cells are stimulated by the angiogenic factor bFGF.

The invention thus pertains to a pharmaceutical composition active on angiogenesis phenomena comprising as active agent at least one substance selected from among: (i) a nucleic acid molecule of the gene coding for the protein IRS-1, a complementary sequence or a fragment thereof, (ii) a molecule capable of inhibiting the expression of a nucleic acid molecule according to (i).

In the framework of the invention, antisense oligonucleotides of the gene coding for this protein were prepared. These oligonucleotides present remarkable antiangiogenic and antitumor activities. They are therefore particularly useful in the treatment of diseases linked to invasive angiogenesis not controlled by gene therapy methods consisting of administering to an individual a composition containing at least one of these oligonucleotides.

Thus, an oligonucleotide according to the invention is constituted by the following nucleotide sequence of formula SEQ ID NO. 2:

5-TATCCGGAGGGCTCGCCATGCTGCTGCGGAGCAGA-3',

a fragment thereof comprising at least 12 contiguous nucleotides or their derivative.

The invention pertains most particularly to an oligonucleotide constituted by one of the nucleotide sequences of formulas SEQ ID NO. 3 and 4 below:

5'-TATCCGGAGGGCTCGCCATGCTGCT-3'.

5'-TCGCCATGCTGCTGCGGAGCAGA-3',

a fragment of these comprising at least 12 contiguous nucleotides or their derivative.

The term derivative is understood to mean a sequence capable of hybridizing under strict conditions with one of the sequences SEQ ID NO. 2, 3 or 4, or with a fragment of these of at least 12 contiguous nucleotides.

The following sequences can be cited as examples of oligonucleotides according to the invention:

SEQ ID NO. 5: 5'-TATCCGGAGGGCCTGCCATGCTGCT-3',

SEQ ID NO. 6: 5'-TATCCGGAGG GCCTGCCATG CTGC-3',

SEQ ID NO. 7: 5'-TATCCGGAGG GCCTGCCATG CTG-3',

SEQ ID NO. 8: 5'-TATCCGGAGG GCCTGCCATG CT-3',

SEQ ID NO. 9: 5'-TATCCGGAGG GCCTGCCATG C-3',

SEQ ID NO. 10: 5'-TATCCGGAGG GCCTGCCATG-3',

SEQ ID NO. 11: 5'-TATCCGGAGG GCCTGCCAT-3',

SEQ ID NO. 12: 5'-TATCCGGAGG GCCTGCCA-3',

SEQ ID NO. 13: 5'-TATCCGGAGG GCCTGCC-3'.

SEQ ID NO. 14: 5'-TATCCGGAGG GCCTGC-3',

SEQ ID NO. 15: 5'-TATCCGGAGG GCCTG-3',

SEQ ID NO. 16: 5'-TATCCGGAGG GCCT-3',

SEQ ID NO. 17: 5'-TATCCGGAGG GCC-3',

SEQ ID NO. 18: 5'-TATCCGGAGG GC-3',

SEQ ID NO. 19: 5'-CCGGAGG GCCTGCCATG CTGCT-3',

SEQ ID NO. 20: 5'-GAGG GCCTGCCATG CTGCT-3',

SEQ ID NO. 21: 5'-G GCCTGCCATG CTGCT-3',

SEQ ID NO. 22: 5'-CTGCCATG CTGCT-3'.

SEQ ID NO. 23: 5'-TGCCATG CTGCT-3'.

All or part of the phosphodiester bonds of the invention are advantageously protected. This protection is generally implemented via the chemical route using classic methods that are well known by the expert in the field. The phosphodiester bonds can be protected, for example, by a thiol or amine functional group or by a phenyl group.

The 5'- and/or 3'- ends of the oligonucleotides of the invention are also advantageously protected, for example, using the technique described above for protecting the phosphodiester bonds.

The oligonucleotides of the invention can be synthesized using conventional techniques that are well known to the expert in the field, for example, using one of the DNA synthesizers marketed by various companies.

Although their mechanism of action has not been entirely elucidated, the oligonucleotides according to the invention inhibit the expression of the protein IRS-1 within endothelial cells. These oligonucleotides are capable of blocking the formation of neovessels by endothelial cells (i.e., they inhibit angiogenesis) and thus they inhibit the multiplication of tumor cells in mice.

The invention therefore also has as object a pharmaceutical composition for the inhibition of the gene coding for the protein IRS-1 comprising at least one oligonucleotide complementary of a part of said gene or of a transcript of said gene.

The molecule capable of inhibiting the expression of a nucleic acid molecule of the gene coding for the protein IRS-1 is preferably an antisense sequence of the region coding the sequence identified under the number SEQ ID NO. 28 in the attached sequence list.

Said antisense sequence advantageously comprises at least twelve contiguous nucleotides or their derivative.

More preferentially, the active agent capable of inhibiting the expression of a nucleic acid molecule coding for the protein IRS-1 of the composition of the invention is a nucleotide sequence selected from among the set of nucleotide sequences identified as SEQ ID NO. 2 to SEQ ID NO. 23 in the attached sequence list comprising at least twelve contiguous nucleotides or their derivative.

Such a composition advantageously comprises as active agent at least one oligonucleotide as defined above advantageously combined in said composition with an acceptable vehicle.

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The analysis of the research performed in the framework of the invention made it possible to demonstrate that the protein IRS-1 represents a cellular constituent which is essential in the angiogenesis process. In fact, inhibition of the expression of the protein IRS-1 by said antisense oligonucleotides leads to the inhibition of the formation of capillary tubes by endothelial cells.

The oligonucleotides according to the invention and the compositions containing them are thus indicated as antiangiogenic agents. They are also indicated as anti-cell-multiplication agents, particularly as antitumor agents, and consequently are particularly useful for the treatment of tumors. Thus the present invention provides the use of said oligonucleotides for the preparation of a composition intended for the treatment or prevention of pathologies linked to invasive, uncontrolled angiogenesis such as, as nonlimitative example: the treatment of tumour vascularization, eye diseases linked to the neovascularization of the ocular apparatus such as retinopathies, rheumatoid arthritis, Crohn's disease, atherosclerosis, hyperstimulation of the ovary, psoriasis, endometritis associated with neovascularization, restenosis due to balloon angioplasty, tissue superproduction due to cicatrization, peripheral vascular disease, hypertension, vascular inflammation, Raynaud's disease and Raynaud's phenomena, aneurysm, arterial restenosis, thrombophlebitis, lymphangitis, lymphendema, tissue cicatrization and repair, ischemia, angina, myocardial infarction, chronic heart disease, cardiac insufficiencies such as congestive heart failure, age-related macular degeneration and osteoporosis.

The above pharmaceutical compositions are more particularly implemented in a manner such that they can be administered via the subcutaneous, intramuscular, intravenous or transdermal route. For such administration, use is made notably of aqueous suspensions, isotonic saline solutions or sterile,

Injectable solutions containing pharmacologically compatible dispersion agents and/or wetting agents such as, for example, propylene glycol or butylene glycol.

The usual unit dose to be administered contains from 0.001 mg to 50 mg of active principle.

The oligonucleotides of the invention are also useful as research reagents, notably for the *in vitro* study of signalization routes involving the 180-kDa protein, for example on tumor cells or non-tumor cells transfected by said oligonucleotides. They are also useful for the *in vivo* study of signalization routes involving the 180-kDa protein in a large number of physiological and pathological phenomena such as angiogenesis or carcinogenesis essentially from the kinase/phosphatase ratio.

Thus, the pharmaceutical compositions of the invention are particularly useful for the performance of tests for the diagnosis of pathologies linked to angiogenesis phenomena, notably for the diagnosis of retinopathies, rheumatoid arthritis, Crohn's disease, atherosclerosis, hyperstimulation of the ovary, psoriasis, endometritis association with neovascularization, restenosis due to balloon angioplasty, tissue superproduction due to cicatrization, peripheral vascular disease, hypertension, vascular inflammation, Raynaud's disease and Raynaud's phenomena, aneurysm, arterial restenosis, thrombophlebitis, lymphangitis, lymphedema, tissue cicatrization and repair, ischemia, angina, myocardial infarction, chronic heart disease, cardiac insufficiencies such as congestive heart failure or age-linked macular degeneration and osteoporosis.

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to

preclude the presence or addition of further features in various embodiments of the invention.

Other advantages and characteristics of the invention will become clear from the examples below in which the term "oligonucleotide" is used to designate the oligonucleotide of SEQ ID NO.3 and which refer to the attached figures in which:

- figure 1A represents the Western Blot images obtained from supernatant samples stemming from unstimulated cells (track NS) and cells stimulated with bFGF (track S) developed with an anti-IRS-1 antibody,

- figure 1B shows the Western Blot images obtained after staining with silver nitrate obtained from the same supernatant samples stemming from unstimulated cells (track NS) and cells stimulated with bFGF (track S),

- figure 2 shows the Western Blot images obtained from supernatant cells stemming from unstimulated cells (track NS) and cells stimulated with bFGF (track B) when the membrane is incubated with an anti-phosphotyrosine monoclonal antibody and developed with an anti-isotope antibody tagged at the peroxidase as indicated in example 3,

- figures 3A to 3D show the images of the cultures on a type I collagen surface of the different lots of endothelial cells:

- figure 3A shows the culture of untreated endothelial cells,

- figure 3B shows the culture of endothelial cells stimulated with 3 ng/ml of bFGF,

- figure 3C shows the culture of endothelial cells incubated with 100 µg/ml of oligonucleotide of SEQ ID NO. 3 for 4 hours and then stimulated with 3 ng/ml of bFGF,

- figure 3D shows the culture of endothelial cells incubated with 100 µg/ml of oligonucleotide of SEQ ID NO. 3 for 4 hours,

- figures 4A to 4F illustrate the results of tests of the inhibition of corneal neovascularization in rats,

- figure 4A shows the results obtained by subconjunctival injection of an antisense oligonucleotide at a concentration of 60 µm,

- figure 4B shows the results obtained after subconjunctival injection of a sense oligonucleotide at a concentration of 60 µm,

- figure 4C shows the results obtained after topical application of an antisense oligonucleotide at a concentration of 200 µm,

- figure 4D shows the results obtained after topical application of a sense oligonucleotide at a concentration of 200 µm,

- figure 4E illustrates the state of the cornea in the absence of any treatment,

- figure 4F illustrates the state of the cornea when treated with subconjunctival injections of PBS,

- figures 5A to 5J illustrate the results of the inhibition of corneal neovascularization obtained in different groups of rats (described in section 6.6 Results) after de-epithelialization and limbic resection of the corneas of the rats on day 4 (figures 5A to 5E) and on day 9 (figures 5F to 5J). These are slit lamp photographs showing the comparison of the growth of the vessels in the various groups of rats. Enlargement x10.

Example 1: Demonstration of the induction of the expression of IRS-1 (the 180-kDa protein) in endothelial cells resulting from the stimulation of these cells with bFGF.

The 180-kDa protein was demonstrated in the following manner:

The endothelial cells were cultured in a 6-well microtitration plate previously covered with type I collagen as described in (Montesano et al., J. Cell. Biol., 1983, 83, 1648-1652). The culture medium was DMEM (Sigma) enriched with 10% of fetal calf serum, 4 mM glutamine, 500 U/ml penicillin and 100 µg/ml streptomycin. After 3 to 4 days of culture, there resulted a semi-confluent layer of endothelial cells. The culture medium of six wells was aspirated and replaced by fresh culture medium. Three wells were enriched with 3 ng/ml of bFGF. After incubation for 48 hours, the wells were washed three times with a phosphate buffer and the cells were used to extract the messenger RNA (mRNA) according to protocols known by the expert in the field. The mRNAs were

reverse transcribed by a polymerization chain reaction (PCR) using each of four degenerated groups of oligo (dT) (T12MN) primers, M can be G, A or C; and N is G, A, T and C. Each group of primers is imposed by the base in position 3'(N) with a degeneration in the (M) position. Example: the set of primers in which N = G is constituted by:

SEQ ID NO. 24: 5'-TTTTTTTTTTTTGG-3'

SEQ ID NO. 25: 5'-TTTTTTTTTTTTAG-3'

SEQ ID NO. 26: 5'-TTTTTTTTTTTTCG-3'

The cDNAs obtained in this manner were amplified and tagged by means of an arbitrary decamer in the presence of isotopically tagged ATP. The electrophoresis analysis of the cDNAs revealed the presence of an amplified 326-bp cDNA fragment in the sample stemming from the endothelial cells stimulated with bFGF, identified in the attached sequence listing as number SEQ ID NO. 27. However, this same fragment is weakly present or present in the trace state in the sample stemming from the endothelial cells that were not stimulated with bFGF. The sequencing of this fragment and the subsequent interrogation of the databases revealed that this fragment corresponds to a part of an already known gene, coding for the substrate of the insulin receptor (an intracellular 180-kDa protein).

Example 2: Demonstration of the induction of the expression of IRS-1 (the 180-kDa protein).

Endothelial cells cultured on a layer of type I collagen stimulated or not stimulated with bFGF (cf. example 1) were lysed in a cellular lyse buffer containing sodium orthovanadate. These solutions were then clarified by centrifugation at 14,000 g for 15 minutes. Supernatant samples stemming from unstimulated cells and cells stimulated with bFGF containing equivalent amounts of proteins were then taken up with an electrophoresis solution

containing 2% SDS and 15 mM of dithiothreitol, heated at 100°C for 5 minutes then deposited on polyacrylamide gel (gradient from 4 to 15% of acrylamide) under denatured conditions (in the presence of 2% SDS). After migration, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked by incubation at ambient temperature in a 5% milk solution in a PBS buffer. The membrane was then washed three times with a PBS buffer, incubated in a PBS buffer containing 1 µg/ml of anti-IRS-1 monoclonal antibody for 2 hours at ambient temperature and washed three times with a PBS buffer. The proteins were then developed with a secondary anti-isotope antibody coupled to peroxidase. The presence was noted of a protein of molecular weight 180 kDa recognized by the monoclonal anti-IRS-1 antibody in the preparations stemming from the endothelial cells stimulated with bFGF; this protein was weakly present in the preparation stemming from the endothelial cells not simulated with bFGF (figure 1).

Example 3: Demonstration of the induction of phosphorylation at the level of IRS-1 tyrosine (the 180-kDa protein).

Human endothelial cells cultured on a layer of type I collagen stimulated or not stimulated with bFGF were lysed in a cellular lyse buffer containing sodium orthovanadate. These solutions were then clarified by centrifugation at 14,000 g for 15 minutes (cf. example 2). The IRS-1 protein was extracted by means of an anti-IRS-1 monoclonal antibody. This extraction was performed after immunoprecipitation by means of an anti-IRS-1 monoclonal antibody (Sigma). After addition of the anti-IRS-1 antibody coupled to agarose, the suspension was incubated for 2 hours at ambient temperature then centrifuged at 4000 g for 15 minutes. The resultant precipitate was taken up with an electrophoresis solution containing 2% SDS and 15 mM of dithiothreitol, heated at 100°C for 5 minutes, then deposited on polyacrylamide gel (acrylamide gradient of 4 to 15%) under

denaturing conditions (in the presence of 2% SDS). After migration, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked by incubation at ambient temperature in a 5% milk solution in a PBS buffer. The membrane was then washed three times with a PBS buffer, incubated in a PBS buffer containing 1 µg/ml of anti-phosphotyrosine monoclonal antibody for 2 hours at ambient temperature, and then washed three times with a PBS buffer. The proteins were then developed by means of a secondary anti-isotope antibody coupled to peroxidase. It was found that the IRS-1 protein of molecular weight 180 kDa was phosphorylated at the level of the tyrosine residue in the preparations stemming from the endothelial cells stimulated with bFGF; this protein was very weakly phosphorylated at the level of the tyrosine residue in the preparation stemming from the endothelial cells not stimulated with bFGF (figure 2).

Example 4: Evaluation of the in vitro antiangiogenic activity of the oligonucleotide.

Human endothelial cells were cultured on a layer of type I collagen. The culture wells were divided into four lots on the seventh day of culture:

Lot 1: Wells corresponding to the culture of untreated endothelial cells (figure 3A).

Lot 2: Wells corresponding to the culture of endothelial cells stimulated with 3 ng/ml of bFGF (figure 3B).

Lot 3: Wells corresponding to the culture of endothelial cells incubated with 100 µg/ml of oligonucleotide of SEQ ID NO. 3 for 4 hours then stimulated with 3 ng/ml of bFGF (figure 3C).

Lot 4: Wells corresponding to the culture of endothelial cells incubated with 100 µg/ml of oligonucleotide of sequence SEQ ID NO. 3 for 4 hours (figure 3D).

The various wells were examined by means of an inverted phase optical microscope after 3 to 4 days of culture. Upon reading the results, it was found that the human endothelial cells in lot 2 formed capillary tubes following stimulation with bFGF. It was also found that the oligonucleotide inhibits the formation of neovessels by these same cells stimulated with bFGF in lot 3. Finally, it was found that that the oligonucleotide does not modify in a pronounced manner the growth of the endothelial cells. In fact, the numbers of endothelial cells in the lot 1 wells and in the lot 4 wells were comparable.

Example 5: Evaluation of the in vivo activity of the oligonucleotide.

Three lots of naked mice were used. Each lot was constituted by 5 mice.

Lot no. 1: This lot was used as control. Each mouse was inoculated on day 0 with 200 µl of a suspension of B16 melanoma cells (provided by Institut Gustave Roussy, Villejuif) dispersed in PBS at the level of 10^6 cells/ml. These mice did not receive subsequent treatment.

Lot no. 2: Each mouse was inoculated subcutaneously on day 0 with 200 µl of a suspension of B16 melanoma cells dispersed in PBS at the level of 10^6 cells/ml. On day 1, day 2, day 3, day 4, day 5, day 6, day 7, day 8, day 9 and day 10 each mouse received a subcutaneous injection of 200 µl of an oligonucleotide solution diluted in PBS at a concentration of 500 µg/ml. The oligonucleotide injection was performed close to the cell injection site.

Lot no. 3: The mice of this lot were not inoculated with the B16 melanoma cells. However, each of the mice received an injection of 200 µl of an oligonucleotide solution in PBS at a concentration of 500 µg/ml; the injections were performed on day 1, day 2, day 3, day 4, day 5, day 6, day 7, day 8, day 9 and day 10.

The following results were obtained:

In the mice of lot no. 1, the tumor mass developed very rapidly after inoculation. In fact, the tumor mass reached a size of 1.6 to 2.5 cm in diameter after ten days in the mice of said lot no. 1 (untreated mice). The evolution of the tumor mass in the mice of lot no. 2 (mice treated after inoculation by injection of oligonucleotide on day 1, day 2 and day 3), exhibited a clearly lower increase in the volume of the tumor mass. The tumor mass in the mice of lot 2 did not exceed 0.8 cm in diameter on the tenth day. On the fourteenth day, the difference between the tumor mass of the mice of lot no. 2 and those of lot no. 1 was remarkable.

In the mice of lot no. 3 (mice not having received B16 melanoma cells but treated by injection of oligonucleotide for three days), an unexpected general effect was observed on the skin. It was identical to that observed on all of the mice treated with the oligonucleotide (lot 2). The skin had an aged, crumpled appearance. The emergence of hairs was also observed on all of the treated mice. There was a parallelism during the evolution between the regression of the cutaneous signs and the resumption of tumor growth.

Thus, it was found that the oligonucleotide inhibits the development and formation of neovessels by endothelial cells *in vitro*. The oligonucleotide also has a remarkable *in vivo* antitumor activity in the naked mouse.

Example 6: Evaluation of the antiangiogenic oligonucleotide on a corneal neovascularization model in the rat.

Based on the work published by Amano et al. (1998), the applicant employed, modified and analyzed a model of the formation of corneal neovessels in the rat after de-epithelialization and limbectomy (figures 5A to 5J). It is reproducible, allows direct slit-lamp examination and quantification of the neovessels. The details are described below. The model was then used for testing the efficacy of the antiangiogenic agents of the invention.

6.1 Animals and corneal neovascularization model

Male Wister rats (*Rattus norvegicus*), aged five weeks (Charles River France, St-Aubin les Elbeufs, France), free of specific pathogens, were fed and allowed to drink water freely, and maintained in the laboratory animal facility under fixed temperature and humidity conditions, with cycles of 12 hours of light/12 hours of darkness.

The rats were anesthetized with a mixture of ketamine (Kétamine 1000, UVA, Ivry-sur-Seine, France; 128 mg/kg) and chlorpromazine (Largactil 25 mg/ml; Specia Rhône Poulenc, Paris, France; 5 mg/kg), injected via the intramuscular route. A drop of oxybuprocaine (Novésine, Chibret, Clermont-Ferrand, France) was instilled in the right eye. Using an enlargement system (macroscope Wild MPS 51 S, LEICA, Heerbrugg, Switzerland), the corneal epithelium was removed by a microsponge impregnated with 70% ethanol. A 1.5-mm band of conjunctiva, at the limbus, was excised with microsurgical scissors, and the eyelids were closed by a temporary blepharorraphy with a Vicryl 5.0 thread (Dacron, Alcon, Rueil-Malmaison, France). The eye was then rinsed abundantly with 1X PBS, an oxytetracycline cream was applied (Posicycline, Alcon, France) and the blepharorraphy was opened on the fourth day [8, 9].

6.2 Treatment by subconjunctival injections and topical applications of antiangiogenic oligonucleotide

The rats were divided into 6 groups:

Group A: model + subconjunctival injection of a 60-μM antisense oligonucleotide solution in 1X PBS,

Group B: model + topical application of a 200-μM antisense oligonucleotide solution in 1X PBS,

Group C: model + subconjunctival injection of a 60-μM sense oligonucleotide solution in 1X PBS,

Group D: model + topical application of a 200-µM sense oligonucleotide solution in 1X PBS,

Group E: model + subcutaneous injection of 1X PBS,

Group F: model without treatment.

All of the rats were subjected to de-epithelialization as described above; the treatment was performed every 24 hours starting on the fourth day and continuing until the ninth day. Neovascularization was examined at the beginning, in the middle and at the end of the protocol by slit-lamp examination; photographs were taken on day 0 and day 9.

6.3 Visualization and quantification of the neovascularization

The animals were euthanized 10 days after the de-epithelialization by lethal injection of pentobarbital (intraperitoneal injection). In order to fill the microvessels and quantify the corneal neovascularization, the upper part of the animals' bodies were perfused with fluorescein-dextran 2x1,000,000. The eyes were enucleated and immersed in paraformaldehyde/1X PBS 4% for 3 hours, then overnight in 1X PBS. The cornea was then isolated with 1 mm of limbus under surgical microscope and inserted in the flat state between plate and cover by means of 3 to 5 radial incisions. The flat corneas were then examined and photographed using fluorescence microscopy. After the whole corneas were reconstituted, they were scanned and the surfaces were measured by image analysis; a software program (NIH image) was used for the quantification of the neovascularization. For each photo, the total corneal surface was measured three times as was the neovascularized surface; the ratio of the means - neovascularized surface/total corneal surface - was used to obtain the percentage of neovascularization and to measure the inhibition obtained.

6.4 Statistical analysis

The results were expressed as means ± SD. The percentages of neovascularized surface/total surface were compared with the nonparametric test of Mann-Whitney. Values of $P < 0.05$ were considered to be significant.

6.5 Dilution of the oligonucleotide

The oligonucleotide was diluted in 1X PBS at pH 7.2. Based on the data in the literature and the experiments performed with other oligonucleotides, it was decided to use a concentration of 60 µM for the subconjunctival injections and a concentration of 200 µM for the topical applications.

6.6 Results

Using the model of corneal neovessels, treatment was performed with the 5'-TATCCGGAGGGCTCGCCATGCTGCT-3' oligonucleotides identified under SEQ ID NO. 3 in the attached sequence listing modified in phosphorothioate form, daily, from day 4 to day 9, according to the following protocol:

Group A: subconjunctival injection of the antisense oligonucleotide at 60 µM (AS 60),

Group B: topical application of the antisense oligonucleotide at 200 µM (AS 200),

Group C: subconjunctival injection of the sense oligonucleotide at 60 µM (S 60),

Group D: topical application of the sense oligonucleotide at 200 µM (S 200),

Group E: subconjunctival injection of 1X PBS (PBS),

Group F: no treatment (0 Tt).

On the tenth day of the protocol, the rats were perfused with a solution of FITC/dextran and then euthanized. The corneas were collected and fixed in a 4% PAF solution. The corneas were then inserted in the flat state between plate

and cover in a glycerol solution. The fluorescent neovessels were observed and photographed using the fluorescence microscope. The photographs were scanned and the neovascularization percentages were measured for each animal.

The results observed are presented in table 1 below:

Table 1

	Group A AS 60	Group B AS 200	Group C S 60	Group D S 200	Group E PBS	Group F 0 Tt
Mean	0.6157	0.5058	0.9431	0.9392	0.9552	9.9170
SD	0.2194	0.1172	0.0964	0.0308	0.0481	0.0751
Number of measurements	15	15	15	12	9	9
SEM	0.0566	0.0303	0.0249	0.0089	0.0160	0.0250

The statistical analysis of the results using a nonparametric Mann-Whitney test yielded the following results:

The subconjunctival injections of 60-µM of the antisense oligonucleotide (A) reduced neovascularization in relation to the control groups E and F (very significant results, $P < 0.0001$ and $P = 0.0011$); topical application of the antisense oligonucleotide at a concentration of 200 µM (B) reduced neovascularization in relation to the control groups E and F (extremely significant results, $P < 0.0001$).

Compared to the subconjunctival administration of the sense oligonucleotide at 60 µM (C) or the topical application of the sense oligonucleotide at 200 µM (D), injection of the antisense oligonucleotide at 60 µM (A) and topical application of the antisense oligonucleotide at 200 µM (B) reduced neovascularization. These results were extremely significant ($P < 0.0001$) (figures 4A to 4F).

The inhibition of neovascularization was not significantly different depending on whether the antisense oligonucleotide was administered via the subconjunctival route (60 µM) or applied topically (200 µM). It was approximately 35% in relation to the controls (E and F).

The subconjunctival injection of the sense oligonucleotide at 60 µM (C) and the topical application of the sense oligonucleotide at a concentration of 200 µM (D) did not modify the neovascularization in relation to the control groups (E and F). In contrast, there was a small effect of the sense oligonucleotide in topical application (D) compared to the sense oligonucleotide in subconjunctival injections (C) (P = 0.0117).

Moreover, there was seen in the groups treated with the antisense oligonucleotide (A and B), a smaller diameter and density of the neovessels. Their distribution did not differ in relation to the control groups nor was any difference observed in relation to the level of inflammation (figure 4).

6.7 Secondary effects

No noteworthy secondary effects were seen in any of the groups during the two experimental series: after 6 days of treatment at the doses specified above, the skin of the rats was not crumpled, the fur was unchanged and the general condition of the animals was good; they fed normally until the last day and no suspicious mortality was observed. Although neither autopsies nor blood tests were performed, the general status of the animals at the end of the experiments did not suggest hepatic disorders. The only symptom observed was a transitory whitish deposit at the site of the conjunctival injections in 60% of the rats of group A, 60% of the rats of group C and 10% of the rats of group E. This deposit had been resorbed by the end of the experiments in all cases.

This example shows that – contrary to expectations – the subcutaneous injections of antisense oligonucleotide at a concentration of 60 µM did not inhibit neovascularization to a greater extent than the topical application of the antisense oligonucleotide at a concentration of 200 µM.

This can perhaps be explained by the difference in the concentrations employed; but this results suggests also a penetration of the oligonucleotide via the topical route rather than via the limbus. It also suggests the absence of prolonged release of the product from the injection site.

6.8 Conclusion

The application of the antisense oligonucleotide via the topical route or in subconjunctival injections reduces neovascularization in our model of corneal neovessels in the rat.

The purpose of this study was to test the efficacy of the antisense oligonucleotides stemming from the sequence of the gene IRS-1 on a previously developed model of corneal neovascularization in the rat.

This model is readily accessible, reproducible and quantifiable. This study moreover provided an initial evaluation of the concentration of oligonucleotide required *in vivo* to inhibit neovascularization.

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[pages 25 - 26 of French-language document]

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5. Pierga JY, Cammilleri S, Benyahia B, Magdelénat H. Applications of antisense oligonucleotides in cancer research. Bull Cancer 1994; 81: 1023-1042.

9. Hoang-Xuan T, Prisant O. Restoration of corneal epithelium from limbic stem cells. Med Sci 1998; 14: 1375-1377.

12. Berdugo Polak M. Iontophoresis administration of antisense oligonucleotides in the anterior segment of the eye: application to a corneal neovascularization model in the rat. DEA "Biology and Pathology of the Epithelia"; University of Paris VII, Feldmann G; Inserm U450, Director Courtois Y, under the direction of Behar Cohen F. 2000.

Key to figures

Sheet 1/5 = Figure 1
At top: N.S.: cells not stimulated with bFGF
S.: cells stimulated with bFGF
At bottom left: A – Development with anti-IRS-1 antibody
At bottom right B – Development with silver nitrate

Sheet 2/5 = Figure 2
At top: N.S.: cells not stimulated with bFGF
S.: cells stimulated with bFGF
At bottom - Development with anti-phosphotyrosine antibody

Sheet 4/5
Figure 4A. Subconjunctival injections of antisense oligonucleotides (60 µM).
Figure 4B. Subconjunctival injections of sense oligonucleotides (60 µM).
Figure 4C. Topical applications of antisense oligonucleotide (200 µM).
Figure 4D. Topical applications of sense oligonucleotide (200 µM).
Figure 4E. No treatment.
Figure 4F. Subconjunctival injections of PBS.

Sheet 5/5
Figure 5A. Day 4 AS [antisense] 60.
Figure 5B. Day 4 AS [antisense] 200.
Figure 5C. Day 4 S [sense] 200.
Figure 5D. Day 4 PBS.
Figure 5E. Day 4 No treatment.
Figure 5F. Day 9 AS [antisense] 60.
Figure 5G. Day 9 AS [antisense] 200.
Figure 5H. Day 9 S [sense] 200.
Figure 5I. Day 9 PBS.
Figure 5J. Day 9 No treatment.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Use of a nucleic acid molecule, which is complementary to the nucleic acid encoding the IRS-1 protein, or a fragment thereof, and which is capable of inhibiting the expression of a nucleic acid molecule encoding the IRS-1 protein, in the preparation of a medicament for the treatment or prevention of a pathology linked to angiogenesis, by inhibiting neovascularization.

2. Use according to claim 1, wherein said neovascularization inhibition is obtained by the inhibition of the formation of capillary tubes by endothelial cells.

3. Use according to claim 1 or claim 2, wherein said nucleic acid molecule has a sequence selected from the group consisting of SEQ ID NO.2 to SEQ ID NO.23, or a fragment thereof comprising at least twelve contiguous nucleotides, and derivatives thereof.

4. Use according to any one of claims 1 to 3, wherein said nucleic acid molecule is antisense to at least twelve contiguous nucleotides of SEQ ID NO.28, or a derivative thereof.

5. Use according to any one of claims 1 to 4, wherein said nucleic acid molecule is associated with an acceptable vehicle.

6. Use according to any one of claims 1 to 5, wherein said pathology linked to angiogenesis is selected in the group comprising retinopathies, rheumatoid arthritis, Crohn's disease, atherosclerosis, hyperstimulation of the ovary, psoriasis, endometritis associated with neovascularization, restenosis due to balloon angioplasty, tissue superproduction due to cicatrization, peripheral

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vascular disease, hypertension, vascular inflammation, Raynaud's disease and Raynaud's phenomena, aneurysm, arterial restenosis, thrombophlebitis, lymphangitis, lymphedema, tissue cicatrization and repair, ischemia, angina, myocardial infarction, chronic heart disease, cardiac insufficiencies such as congestive heart failure, age-related macular degeneration and osteoporosis.

7. A method for treating or preventing a pathology linked to angiogenesis, by inhibiting neovascularization, comprising the step of administering a nucleic acid molecule, which encodes the IRS-1 protein, or a complementary sequence or a fragment thereof and which is capable of inhibiting the expression of a nucleic acid molecule encoding the IRS-1 protein, to a patient.

8. The method according to claim 7, wherein said neovascularization inhibition is obtained by the inhibition of the formation of capillary tubes by endothelial cells.

9. The method according to claim 7 or claim 8, wherein said nucleic acid molecule has a sequence selected from the group consisting of SEQ ID NO.2 to SEQ ID NO.23, or a fragment thereof comprising at least twelve contiguous nucleotides, and derivatives thereof.

10. The method according to any one of claims 7 to 9, wherein said nucleic acid molecule is antisense to at least twelve contiguous nucleotides of SEQ ID NO.28, or a derivative thereof.

11. The method according to any one of claims 7 to 10, wherein said nucleic acid molecule is associated with an acceptable vehicle.

12. The method according to any one of claims 7 to 11,

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wherein said nucleic acid molecule is administered via the subcutaneous, intramuscular, intravenous or transdermal route, in an amount from about 0.001 mg to about 50 mg.

13. The method according to any one of claims 7 to 12, wherein said pathology linked to angiogenesis is selected in the group comprising retinopathies, rheumatoid arthritis, Crohn's disease, atherosclerosis, hyperstimulation of the ovary, psoriasis, endometritis associated with neovascularization, restenosis due to balloon angioplasty, tissue superproduction due to cicatrization, peripheral vascular disease, hypertension, vascular inflammation, Raynaud's disease and Raynaud's phenomena, aneurysm, arterial restenosis, thrombophlebitis, lymphangitis, lymphedema, tissue cicatrization and repair, ischemia, angina, myocardial infarction, chronic heart disease, cardiac insufficiencies such as congestive heart failure, age-related macular degeneration and osteoporosis.

14. A use according to any one of claims 1 to 6, substantially as herein described with reference to any one of the examples and/or drawings.

15. A method according to any one of claims 7 to 13, substantially as herein described with reference to any one of the examples and/or drawings.

Dated this 4th day of January 2006
GENE SIGNAL
By their Patent Attorneys
GRIFFITH HACK
Fellows Institute of Patent and
Trade Mark Attorneys of Australia

H:\rochb\Keep\2002 3 45669.doc 04/01/06

N, S : cellules non stimulées avec le bFGF
S : cellules stimulées avec le bFGF

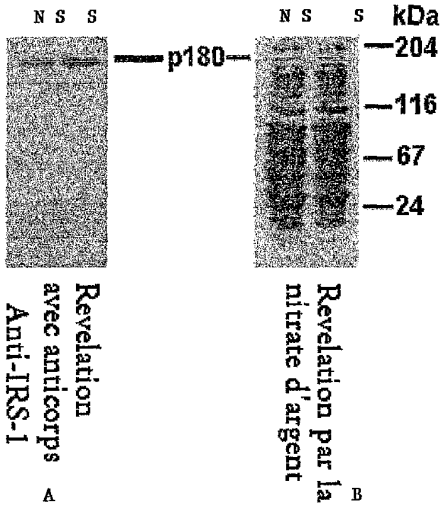
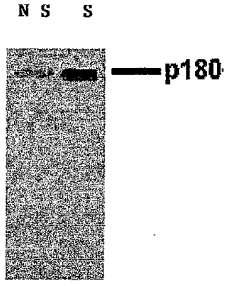


Fig.1

N S: cellules non stimulées avec le bFGF
S : cellules stimulées avec le bFGF



Revelation avec anticorps anti-phosphotyrosine

Fig.2

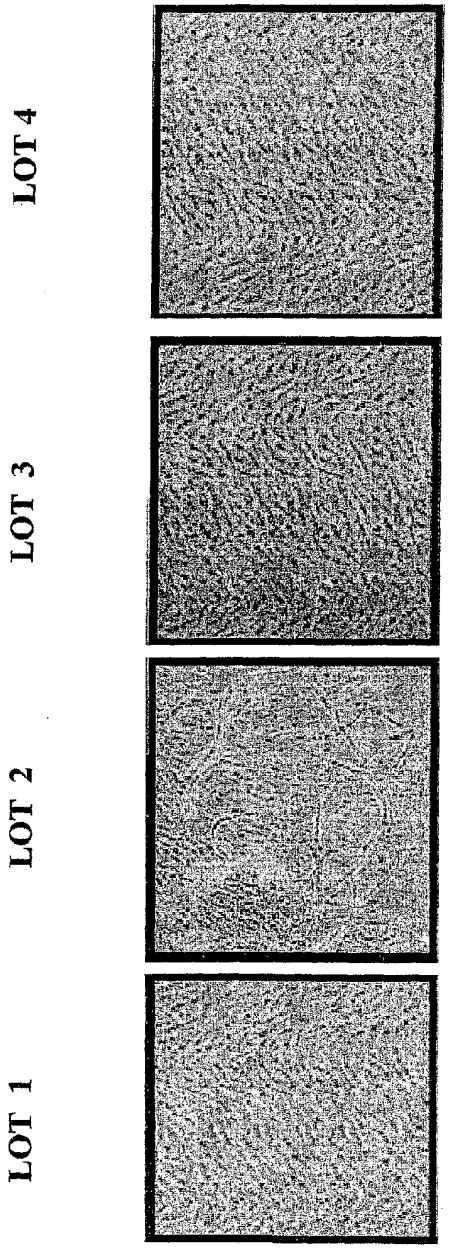


Fig.3

Figure 4A
Injections sous-conjonctivales
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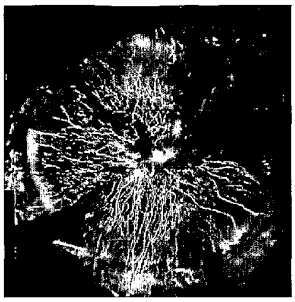

Figure 4B
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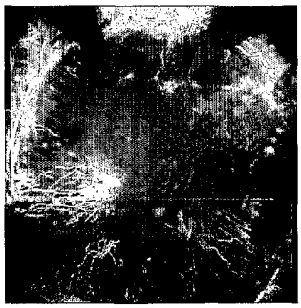

Figure 4C
Applications topiques
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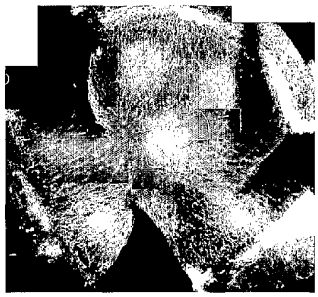

Figure 4D
Applications topiques
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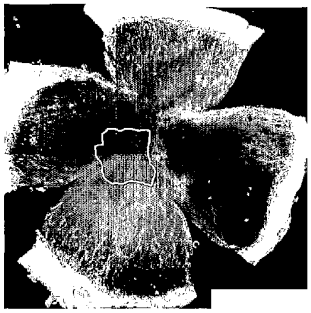


Figure 4E
Absence de traitement

Figure 4F
Injections sous-conjonctivales de PBS

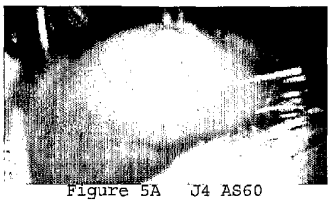


Figure 5A J4 AS60

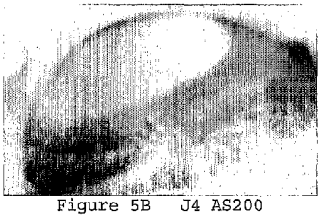


Figure 5B J4 AS200

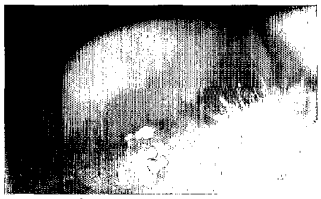


Figure 5C J4 S200

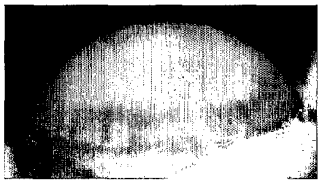


Figure 5D J4 PBS

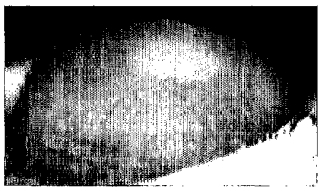


Figure 5E J4 0Tt

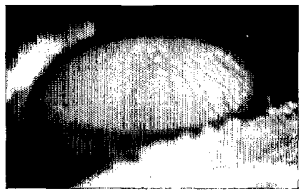
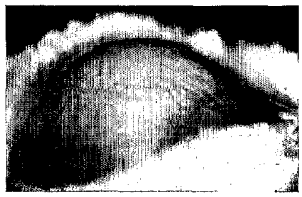


Figure 5F J9 AS60

Figure 5G J9 AS200



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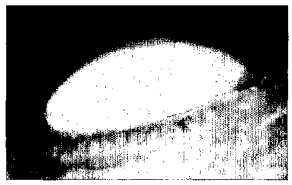

Figure 5I J9 PBS

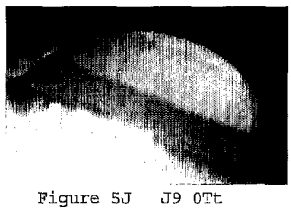

Figure 5J J9 0Tt

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gaggcgaagg aggagggaga accccgtgca acgttgggac ttggcaaccc gcctcccccc    540

gcccaaggat atttaatttg cctcgggaat cgctgcttcc agaggggaac tcaggaggga    600

aggcgcgcgc gcgcgcgcgc tcctggaggg gcaccgcagg gacccccgac tgtcgcctcc    660

ctgtgccgga ctccagccgg ggcgacgaga gatgcatctt cgctccttcc tggtggcggc    720

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gcctccccgc cgggcgtgaa gcgcccgaaa actccggtcg ggctctctcc tgggctcagc    840

agctgcgtcc tccttcagct gcccctcccc ggcgcggggg gcggcgtgga tttcagagtc    900

ggggtttctg ctgcctccag ccctgtttgc atgtgccggg ccgcggcgag gagcctccgc    960

cccccacccg gttgtttttc ggagcctccc tctgctcagc gttggtggtg gcggtggcag   1020

c atg gcg agc cct ccg gag agc gat ggc ttc tcg gac gtg cgc aag gtg   1069
  Met Ala Ser Pro Pro Glu Ser Asp Gly Phe Ser Asp Val Arg Lys Val
  1               5                  10                  15

ggc tac ctg cgc aaa ccc aag agc atg cac aaa cgc ttc ttc gta ctg    1117
Gly Tyr Leu Arg Lys Pro Lys Ser Met His Lys Arg Phe Phe Val Leu
            20                  25                  30

cgc gcg gcc agc gag gct ggg ggc ccg gcg cgc ctc gag tac tac gag    1165
Arg Ala Ala Ser Glu Ala Gly Gly Pro Ala Arg Leu Glu Tyr Tyr Glu
        35                  40                  45

aac gag aag aag tgg cgg cac aag tcg agc gcc ccc aaa cgc tcg atc    1213
Asn Glu Lys Lys Trp Arg His Lys Ser Ser Ala Pro Lys Arg Ser Ile
    50                  55                  60

ccc ctt gag agc tgc ttc aac atc aac aag cgg gct gac tcc aag aac    1261
Pro Leu Glu Ser Cys Phe Asn Ile Asn Lys Arg Ala Asp Ser Lys Asn
65                  70                  75                  80

aag cac ctg gtg gct ctc tac acc cgg gac gag cac ttt gcc atc gcg    1309
Lys His Leu Val Ala Leu Tyr Thr Arg Asp Glu His Phe Ala Ile Ala
                85                  90                  95

gcg gac agc gag gcc gag caa gac agc tgg tac cag gct ctc cta cag    1357
Ala Asp Ser Glu Ala Glu Gln Asp Ser Trp Tyr Gln Ala Leu Leu Gln
            100                 105                 110

ctg cac aac cgt gct aag ggc cac cac gac gga gct gcg gcc ctc ggg    1405
Leu His Asn Arg Ala Lys Gly His His Asp Gly Ala Ala Ala Leu Gly
        115                 120                 125
```

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gcg gga ggt ggt ggg ggc agc tgc agc ggc agc tcc ggc ctt ggt gag     1453
Ala Gly Gly Gly Gly Gly Ser Cys Ser Gly Ser Ser Gly Leu Gly Glu
    130                 135                 140

gct ggg gag gac ttg agc tac ggt gac gtg ccc cca gga ccc gca ttc     1501
Ala Gly Glu Asp Leu Ser Tyr Gly Asp Val Pro Pro Gly Pro Ala Phe
145                 150                 155                 160

aaa gag gtc tgg caa gtg atc ctg aag ccc aag ggc ctg ggt cag aca     1549
Lys Glu Val Trp Gln Val Ile Leu Lys Pro Lys Gly Leu Gly Gln Thr
                165                 170                 175

aag aac ctg att ggt atc tac cgc ctt tgc ctg acc agc aag acc atc     1597
Lys Asn Leu Ile Gly Ile Tyr Arg Leu Cys Leu Thr Ser Lys Thr Ile
            180                 185                 190

agc ttc gtg aag ctg aac tcg gag gca gcg gcc gtg gtg ctg cag ctg     1645
Ser Phe Val Lys Leu Asn Ser Glu Ala Ala Ala Val Val Leu Gln Leu
        195                 200                 205

atg aac atc agg cgc tgt ggc cac tcg gaa aac ttc ttc ttc atc gag     1693
Met Asn Ile Arg Arg Cys Gly His Ser Glu Asn Phe Phe Phe Ile Glu
    210                 215                 220

gtg ggc cgt tct gcc gtg acg ggg ccc ggg gag ttc tgg atg cag gtg     1741
Val Gly Arg Ser Ala Val Thr Gly Pro Gly Glu Phe Trp Met Gln Val
225                 230                 235                 240

gat gac tct gtg gtg gcc cag aac atg cac gag acc atc ctg gag gcc     1789
Asp Asp Ser Val Val Ala Gln Asn Met His Glu Thr Ile Leu Glu Ala
                245                 250                 255

atg cgg gcc atg agt gat gag ttc cgc cct cgc agc aag agc cag tcc     1837
Met Arg Ala Met Ser Asp Glu Phe Arg Pro Arg Ser Lys Ser Gln Ser
            260                 265                 270

tcg tcc aac tgc tct aac ccc atc agc gtc ccc ctg cgc cgg cac cat     1885
Ser Ser Asn Cys Ser Asn Pro Ile Ser Val Pro Leu Arg Arg His His
        275                 280                 285

ctc aac aat ccc ccg ccc agc cag gtg ggg ctg acc cgc cga tca cgc     1933
Leu Asn Asn Pro Pro Pro Ser Gln Val Gly Leu Thr Arg Arg Ser Arg
    290                 295                 300

act gag agc atc acc gcc acc tcc ccg gcc agc atg gtg ggc ggg aag     1981
Thr Glu Ser Ile Thr Ala Thr Ser Pro Ala Ser Met Val Gly Gly Lys
305                 310                 315                 320

cca ggc tcc ttc cgt gtc cgc gcc tcc agt gac ggc gaa ggc acc atg     2029
Pro Gly Ser Phe Arg Val Arg Ala Ser Ser Asp Gly Glu Gly Thr Met
                325                 330                 335

tcc cgc cca gcc tcg gtg gac ggc agc cct gtg agt ccc agc acc aac     2077
Ser Arg Pro Ala Ser Val Asp Gly Ser Pro Val Ser Pro Ser Thr Asn
            340                 345                 350

aga acc cac gcc cac cgg cat cgg ggc agc gcc cgg ctg cac ccc ccg     2125
Arg Thr His Ala His Arg His Arg Gly Ser Ala Arg Leu His Pro Pro
        355                 360                 365

ctc aac cac agc cgc tcc atc ccc atg ccg gct tcc cgc tgc tcg cct     2173
Leu Asn His Ser Arg Ser Ile Pro Met Pro Ala Ser Arg Cys Ser Pro
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    370                 375                 380

tcg gcc acc agc ccg gtc agt ctg tcg tcc agt agc acc agt ggc cat     2221
Ser Ala Thr Ser Pro Val Ser Leu Ser Ser Ser Ser Thr Ser Gly His
385                 390                 395                 400

ggc tcc acc tcg gat tgt ctc ttc cca cgg cga tct agt gct tcg gtg     2269
Gly Ser Thr Ser Asp Cys Leu Phe Pro Arg Arg Ser Ser Ala Ser Val
                405                 410                 415

tct ggt tcc ccc agc gat ggc ggt ttc atc tcc tcg gat gag tat ggc     2317
Ser Gly Ser Pro Ser Asp Gly Gly Phe Ile Ser Ser Asp Glu Tyr Gly
            420                 425                 430

tcc agt ccc tgc gat ttc cgg agt tcc ttc cgc agt gtc act ccg gat     2365
Ser Ser Pro Cys Asp Phe Arg Ser Ser Phe Arg Ser Val Thr Pro Asp
        435                 440                 445

tcc ctg ggc cac acc cca cca gcc cgc ggt gag gag gag cta agc aac     2413
Ser Leu Gly His Thr Pro Pro Ala Arg Gly Glu Glu Leu Ser Asn
    450                 455                 460

tat atc tgc atg ggt ggc aag ggg ccc tcc acc ctg acc gcc ccc aac     2461
Tyr Ile Cys Met Gly Gly Lys Gly Pro Ser Thr Leu Thr Ala Pro Asn
465                 470                 475                 480

ggt cac tac att ttg tct cgg ggt ggc aat ggc cac cgc tgc acc cca     2509
Gly His Tyr Ile Leu Ser Arg Gly Gly Asn Gly His Arg Cys Thr Pro
                485                 490                 495

gga aca ggc ttg ggc acg agt cca gcc ttg gct ggg gat gaa gca gcc     2557
Gly Thr Gly Leu Gly Thr Ser Pro Ala Leu Ala Gly Asp Glu Ala Ala
            500                 505                 510

agt gct gca gat ctg gat aat cgg ttc cga aag aga act cac tcg gca     2605
Ser Ala Ala Asp Leu Asp Asn Arg Phe Arg Lys Arg Thr His Ser Ala
        515                 520                 525

ggc aca tcc cct acc att acc cac cag aag acc ccg tcc cag tcc tca     2653
Gly Thr Ser Pro Thr Ile Thr His Gln Lys Thr Pro Ser Gln Ser Ser
    530                 535                 540

gtg gct tcc att gag gag tac aca gag atg atg cct gcc tac cca cca     2701
Val Ala Ser Ile Glu Glu Tyr Thr Glu Met Met Pro Ala Tyr Pro Pro
545                 550                 555                 560

gga ggt ggc agt gga ggc cga ctg ccg gga cac agg cac tcc gcc ttc     2749
Gly Gly Gly Ser Gly Gly Arg Leu Pro Gly His Arg His Ser Ala Phe
                565                 570                 575

gtg ccc acc cgc tcc tac cca gag gag ggt ctg gaa atg cac ccc ttg     2797
Val Pro Thr Arg Ser Tyr Pro Glu Glu Gly Leu Glu Met His Pro Leu
            580                 585                 590

gag cgt cgg ggg ggg cac cac cgc cca gac agc tcc acc ctc cac acg     2845
Glu Arg Arg Gly Gly His His Arg Pro Asp Ser Ser Thr Leu His Thr
        595                 600                 605

gat gat ggc tac atg ccc atg tcc cca ggg gtg gcc cca gtg ccc agt     2893
Asp Asp Gly Tyr Met Pro Met Ser Pro Gly Val Ala Pro Val Pro Ser
    610                 615                 620

ggc cga aag ggc agt gga gac tat atg ccc atg agc ccc aag agc gta     2941
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Gly Arg Lys Gly Ser Gly Asp Tyr Met Pro Met Ser Pro Lys Ser Val
625                 630                 635                 640

tct gcc cca cag cag atc atc aat ccc atc aga cgc cat ccc cag aga     2989
Ser Ala Pro Gln Gln Ile Ile Asn Pro Ile Arg Arg His Pro Gln Arg
                645                 650                 655

gtg gac ccc aat ggc tac atg atg atg tcc ccc agc ggt ggc tgc tct     3037
Val Asp Pro Asn Gly Tyr Met Met Met Ser Pro Ser Gly Gly Cys Ser
            660                 665                 670

cct gac att gga ggt ggc ccc agc agc agc agc agc agc agc aac gcc     3085
Pro Asp Ile Gly Gly Gly Pro Ser Ser Ser Ser Ser Ser Asn Ala
        675                 680                 685

gtc cct tcc ggg acc agc tat gga aag ctg tgg aca aac ggg gta ggg     3133
Val Pro Ser Gly Thr Ser Tyr Gly Lys Leu Trp Thr Asn Gly Val Gly
    690                 695                 700

ggc cac cac tct cat gtc ttg cct cac ccc aaa ccc cca gtg gag agc     3181
Gly His His Ser His Val Leu Pro His Pro Lys Pro Pro Val Glu Ser
705                 710                 715                 720

agc ggt ggt aag ctc tta cct tgc aca ggt gac tac atg aac atg tca     3229
Ser Gly Gly Lys Leu Leu Pro Cys Thr Gly Asp Tyr Met Asn Met Ser
                725                 730                 735

cca gtg ggg gac tcc aac acc agc agc ccc tcc gac tgc tac tac ggc     3277
Pro Val Gly Asp Ser Asn Thr Ser Ser Pro Ser Asp Cys Tyr Tyr Gly
            740                 745                 750

cct gag gac ccc cag cac aag cca gtc ctc tcc tac tac tca ttg cca     3325
Pro Glu Asp Pro Gln His Lys Pro Val Leu Ser Tyr Tyr Ser Leu Pro
        755                 760                 765

aga tcc ttt aag cac acc cag cgc ccc ggg gag ccg gag gag ggt gcc     3373
Arg Ser Phe Lys His Thr Gln Arg Pro Gly Glu Pro Glu Glu Gly Ala
    770                 775                 780

cgg cat cag cac ctc cgc ctt tcc act agc tct ggt cgc ctt ctc tat     3421
Arg His Gln His Leu Arg Leu Ser Thr Ser Ser Gly Arg Leu Leu Tyr
785                 790                 795                 800

gct gca aca gca gat gat tct tcc tct tcc acc agc agc gac agc ctg     3469
Ala Ala Thr Ala Asp Asp Ser Ser Ser Ser Thr Ser Ser Asp Ser Leu
                805                 810                 815

ggt ggg gga tac tgc ggg gct agg ctg gag ccc agc ctt cca cat ccc     3517
Gly Gly Gly Tyr Cys Gly Ala Arg Leu Glu Pro Ser Leu Pro His Pro
            820                 825                 830

cac cat cag gtt ctg cag ccc cat ctg cct cga aag gtg gac aca gct     3565
His His Gln Val Leu Gln Pro His Leu Pro Arg Lys Val Asp Thr Ala
        835                 840                 845

gct cag acc aat agc cgc ctg gcc cgg ccc acg agg ctg tcc ctg ggg     3613
Ala Gln Thr Asn Ser Arg Leu Ala Arg Pro Thr Arg Leu Ser Leu Gly
    850                 855                 860

gat ccc aag gcc agc acc tta cct cgg gcc cga gag cag cag cag cag     3661
Asp Pro Lys Ala Ser Thr Leu Pro Arg Ala Arg Glu Gln Gln Gln Gln
865                 870                 875                 880
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cag cag ccc ttg ctg cac cct cca gag ccc aag agc ccg ggg gaa tat     3709
Gln Gln Pro Leu Leu His Pro Pro Glu Pro Lys Ser Pro Gly Glu Tyr
                885                 890                 895

gtc aat att gaa ttt ggg agt gat cag tct ggc tac ttg tct ggc ccg     3757
Val Asn Ile Glu Phe Gly Ser Asp Gln Ser Gly Tyr Leu Ser Gly Pro
            900                 905                 910

gtg gct ttc cac agc tca cct tct gtc agg tgt cca tcc cag ctc cag     3805
Val Ala Phe His Ser Ser Pro Ser Val Arg Cys Pro Ser Gln Leu Gln
        915                 920                 925

cca gct ccc aga gag gaa gag act ggc act gag gag tac atg aag atg     3853
Pro Ala Pro Arg Glu Glu Glu Thr Gly Thr Glu Glu Tyr Met Lys Met
    930                 935                 940

gac ctg ggg ccg ggc cgg agg gca gcc tgg cag gag agc act ggg gtc     3901
Asp Leu Gly Pro Gly Arg Arg Ala Ala Trp Gln Glu Ser Thr Gly Val
945                 950                 955                 960

gag atg ggc aga ctg ggc cct gca cct ccc ggg gct gct agc att tgc     3949
Glu Met Gly Arg Leu Gly Pro Ala Pro Pro Gly Ala Ala Ser Ile Cys
                965                 970                 975

agg cct acc cgg gca gtg ccc agc agc cgg ggt gac tac atg acc atg     3997
Arg Pro Thr Arg Ala Val Pro Ser Ser Arg Gly Asp Tyr Met Thr Met
            980                 985                 990

cag atg agt tgt ccc cgt cag agc  tac gtg gac acc tcg  cca gct gcc   4045
Gln Met Ser Cys Pro Arg Gln Ser  Tyr Val Asp Thr Ser  Pro Ala Ala
        995                 1000                1005

cct gta  agc tat gct gac atg  cga aca ggc att gct  gca gag gag      4090
Pro Val  Ser Tyr Ala Asp Met  Arg Thr Gly Ile Ala  Ala Glu Glu
    1010                1015                1020

gtg agc  ctg ccc agg gcc acc  atg gct gct gcc tcc  tca tcc tca      4135
Val Ser  Leu Pro Arg Ala Thr  Met Ala Ala Ala Ser  Ser Ser Ser
    1025                1030                1035

gca gcc  tct gct tcc ccg act  ggg cct caa ggg gca  gca gag ctg      4180
Ala Ala  Ser Ala Ser Pro Thr  Gly Pro Gln Gly Ala  Ala Glu Leu
    1040                1045                1050

gct gcc  cac tcg tcc ctg ctg  ggg ggc cca caa gga  cct ggg ggc      4225
Ala Ala  His Ser Ser Leu Leu  Gly Gly Pro Gln Gly  Pro Gly Gly
    1055                1060                1065

atg agc  gcc ttc acc cgg gtg  aac ctc agt cct aac  cgc aac cag      4270
Met Ser  Ala Phe Thr Arg Val  Asn Leu Ser Pro Asn  Arg Asn Gln
    1070                1075                1080

agt gcc  aaa gtg atc cgt gca  gac cca caa ggg tgc  cgg cgg agg      4315
Ser Ala  Lys Val Ile Arg Ala  Asp Pro Gln Gly Cys  Arg Arg Arg
    1085                1090                1095

cat agc  tcc gag act ttc tcc  tca aca ccc agt gcc  acc cgg gtg      4360
His Ser  Ser Glu Thr Phe Ser  Ser Thr Pro Ser Ala  Thr Arg Val
    1100                1105                1110

ggc aac  aca gtg ccc ttt gga  gcg ggg gca gca gta  ggg ggc ggt      4405
Gly Asn  Thr Val Pro Phe Gly  Ala Gly Ala Ala Val  Gly Gly Gly
    1115                1120                1125
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ggc ggt  agc agc agc agc agc  gag gat gtg aaa cgc  cac agc tct      4450
Gly Gly  Ser Ser Ser Ser Ser  Glu Asp Val Lys Arg  His Ser Ser
    1130                 1135                 1140

gct tcc  ttt gag aat gtg tgg  ctg agg cct ggg gag  ctt ggg gga      4495
Ala Ser  Phe Glu Asn Val Trp  Leu Arg Pro Gly Glu  Leu Gly Gly
    1145                 1150                 1155

gcc ccc  aag gag cca gcc aaa  ctg tgt ggg gct gct  ggg ggt ttg      4540
Ala Pro  Lys Glu Pro Ala Lys  Leu Cys Gly Ala Ala  Gly Gly Leu
    1160                 1165                 1170

gag aat  ggt ctt aac tac ata  gac ctg gat ttg gtc  aag gac ttc      4585
Glu Asn  Gly Leu Asn Tyr Ile  Asp Leu Asp Leu Val  Lys Asp Phe
    1175                 1180                 1185

aaa cag  tgc cct cag gag tgc  acc cct gaa ccg cag  cct ccc cca      4630
Lys Gln  Cys Pro Gln Glu Cys  Thr Pro Glu Pro Gln  Pro Pro Pro
    1190                 1195                 1200

ccc cca  ccc cct cat caa ccc  ctg ggc agc ggt gag  agc agc tcc      4675
Pro Pro  Pro Pro His Gln Pro  Leu Gly Ser Gly Glu  Ser Ser Ser
    1205                 1210                 1215

acc cgc  cgc tca agt gag gat  tta agc gcc tat gcc  agc atc agt      4720
Thr Arg  Arg Ser Ser Glu Asp  Leu Ser Ala Tyr Ala  Ser Ile Ser
    1220                 1225                 1230

ttc cag  aag cag cca gag gac  cgt cag tag ctcaactgga catcacagca     4770
Phe Gln  Lys Gln Pro Glu Asp  Arg Gln
    1235                 1240

gaatgaagac ctaaatgacc tcagcaaatc ctcttctaac tcatgggtac ccagactcta   4830

aatatttcat gattcacaac taggacctca tatcttcctc atcagtagat ggtacgatgc   4890

atccatttca gtttgtttac tttatccaat cctcaggatt tcattgactg aactgcacgt   4950

tctatattgt gccaagcgaa aaaaaaaaat gcactgtgac accagaataa tgagtctgca   5010

taaacttcat cttcaacctt aaggacttag ctggccacag tgagctgatg tgcccaccac   5070

cgtgtcatga gagaatgggt ttactctcaa tgcattttca agatacattt catctgctgc   5130

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tgatccttgt tttaagattg taattcagaa aacataatat aagaatcata gtgccataga   5430

tggttctcaa ttgtatagtt atatttgctg atactatctc ttgtcatata aacctgatgt   5490

tgagctgagt tccttataag aattaatctt aattttgtat tttttcctgt aagacaatag   5550

gccatgttaa ttaaactgaa gaaggatata tttggctggg tgttttcaaa tgtcagctta   5610

aaattggtaa ttgaatggaa gcaaaattat aagaagagga aattaaagtc ttccattgca   5670
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tgtattgtaa acagaaggag atgggtgatt ccttcaattc aaaagctctc tttggaatga 5730

acaatgtggg cgtttgtaaa ttctggaaat gtctttctat tcataataaa ctagatactg 5790

ttgatctttt 5800

<210> 29
<211> 1242
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<400> 29

Met Ala Ser Pro Pro Glu Ser Asp Gly Phe Ser Asp Val Arg Lys Val
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Gly Tyr Leu Arg Lys Pro Lys Ser Met His Lys Arg Phe Phe Val Leu
20 25 30

Arg Ala Ala Ser Glu Ala Gly Gly Pro Ala Arg Leu Glu Tyr Tyr Glu
35 40 45

Asn Glu Lys Lys Trp Arg His Lys Ser Ser Ala Pro Lys Arg Ser Ile
50 55 60

Pro Leu Glu Ser Cys Phe Asn Ile Asn Lys Arg Ala Asp Ser Lys Asn
65 70 75 80

Lys His Leu Val Ala Leu Tyr Thr Arg Asp Glu His Phe Ala Ile Ala
85 90 95

Ala Asp Ser Glu Ala Glu Gln Asp Ser Trp Tyr Gln Ala Leu Leu Gln
100 105 110

Leu His Asn Arg Ala Lys Gly His His Asp Gly Ala Ala Ala Leu Gly
115 120 125

Ala Gly Gly Gly Gly Gly Ser Cys Ser Gly Ser Ser Gly Leu Gly Glu
130 135 140

Ala Gly Glu Asp Leu Ser Tyr Gly Asp Val Pro Pro Gly Pro Ala Phe
145 150 155 160

Lys Glu Val Trp Gln Val Ile Leu Lys Pro Lys Gly Leu Gly Gln Thr
165 170 175

Lys Asn Leu Ile Gly Ile Tyr Arg Leu Cys Leu Thr Ser Lys Thr Ile
180 185 190

Ser Phe Val Lys Leu Asn Ser Glu Ala Ala Ala Val Val Leu Gln Leu
195 200 205

Met Asn Ile Arg Arg Cys Gly His Ser Glu Asn Phe Phe Phe Ile Glu
210 215 220

Val Gly Arg Ser Ala Val Thr Gly Pro Gly Glu Phe Trp Met Gln Val
225 230 235 240

Asp Asp Ser Val Val Ala Gln Asn Met His Glu Thr Ile Leu Glu Ala
245 250 255

Met Arg Ala Met Ser Asp Glu Phe Arg Pro Arg Ser Lys Ser Gln Ser

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           260                 265                 270

Ser Ser Asn Cys Ser Asn Pro Ile Ser Val Pro Leu Arg Arg His His
        275                 280                 285

Leu Asn Asn Pro Pro Pro Ser Gln Val Gly Leu Thr Arg Arg Ser Arg
    290                 295                 300

Thr Glu Ser Ile Thr Ala Thr Ser Pro Ala Ser Met Val Gly Gly Lys
305                 310                 315                 320

Pro Gly Ser Phe Arg Val Arg Ala Ser Ser Asp Gly Glu Gly Thr Met
                325                 330                 335

Ser Arg Pro Ala Ser Val Asp Gly Ser Pro Val Ser Pro Ser Thr Asn
            340                 345                 350

Arg Thr His Ala His Arg His Arg Gly Ser Ala Arg Leu His Pro Pro
        355                 360                 365

Leu Asn His Ser Arg Ser Ile Pro Met Pro Ala Ser Arg Cys Ser Pro
    370                 375                 380

Ser Ala Thr Ser Pro Val Ser Leu Ser Ser Ser Ser Thr Ser Gly His
385                 390                 395                 400

Gly Ser Thr Ser Asp Cys Leu Phe Pro Arg Arg Ser Ser Ala Ser Val
                405                 410                 415

Ser Gly Ser Pro Ser Asp Gly Gly Phe Ile Ser Ser Asp Glu Tyr Gly
            420                 425                 430

Ser Ser Pro Cys Asp Phe Arg Ser Ser Phe Arg Ser Val Thr Pro Asp
        435                 440                 445

Ser Leu Gly His Thr Pro Pro Ala Arg Gly Glu Glu Glu Leu Ser Asn
    450                 455                 460

Tyr Ile Cys Met Gly Gly Lys Gly Pro Ser Thr Leu Thr Ala Pro Asn
465                 470                 475                 480

Gly His Tyr Ile Leu Ser Arg Gly Gly Asn Gly His Arg Cys Thr Pro
                485                 490                 495

Gly Thr Gly Leu Gly Thr Ser Pro Ala Leu Ala Gly Asp Glu Ala Ala
            500                 505                 510

Ser Ala Ala Asp Leu Asp Asn Arg Phe Arg Lys Arg Thr His Ser Ala
        515                 520                 525

Gly Thr Ser Pro Thr Ile Thr His Gln Lys Thr Pro Ser Gln Ser Ser
    530                 535                 540

Val Ala Ser Ile Glu Glu Tyr Thr Glu Met Met Pro Ala Tyr Pro Pro
545                 550                 555                 560

Gly Gly Gly Ser Gly Gly Arg Leu Pro Gly His Arg His Ser Ala Phe
                565                 570                 575

Val Pro Thr Arg Ser Tyr Pro Glu Glu Gly Leu Glu Met His Pro Leu
            580                 585                 590

Glu Arg Arg Gly Gly His His Arg Pro Asp Ser Ser Thr Leu His Thr
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        595                 600                 605

Asp Asp Gly Tyr Met Pro Met Ser Pro Gly Val Ala Pro Val Pro Ser
    610                 615                 620

Gly Arg Lys Gly Ser Gly Asp Tyr Met Pro Met Ser Pro Lys Ser Val
625                 630                 635                 640

Ser Ala Pro Gln Gln Ile Ile Asn Pro Ile Arg Arg His Pro Gln Arg
                645                 650                 655

Val Asp Pro Asn Gly Tyr Met Met Met Ser Pro Ser Gly Gly Cys Ser
            660                 665                 670

Pro Asp Ile Gly Gly Gly Pro Ser Ser Ser Ser Ser Ser Ser Asn Ala
        675                 680                 685

Val Pro Ser Gly Thr Ser Tyr Gly Lys Leu Trp Thr Asn Gly Val Gly
    690                 695                 700

Gly His His Ser His Val Leu Pro His Pro Lys Pro Pro Val Glu Ser
705                 710                 715                 720

Ser Gly Gly Lys Leu Leu Pro Cys Thr Gly Asp Tyr Met Asn Met Ser
                725                 730                 735

Pro Val Gly Asp Ser Asn Thr Ser Ser Pro Ser Asp Cys Tyr Tyr Gly
            740                 745                 750

Pro Glu Asp Pro Gln His Lys Pro Val Leu Ser Tyr Tyr Ser Leu Pro
        755                 760                 765

Arg Ser Phe Lys His Thr Gln Arg Pro Gly Glu Pro Glu Glu Gly Ala
    770                 775                 780

Arg His Gln His Leu Arg Leu Ser Thr Ser Ser Gly Arg Leu Leu Tyr
785                 790                 795                 800

Ala Ala Thr Ala Asp Asp Ser Ser Ser Ser Thr Ser Ser Asp Ser Leu
                805                 810                 815

Gly Gly Gly Tyr Cys Gly Ala Arg Leu Glu Pro Ser Leu Pro His Pro
            820                 825                 830

His His Gln Val Leu Gln Pro His Leu Pro Arg Lys Val Asp Thr Ala
        835                 840                 845

Ala Gln Thr Asn Ser Arg Leu Ala Arg Pro Thr Arg Leu Ser Leu Gly
    850                 855                 860

Asp Pro Lys Ala Ser Thr Leu Pro Arg Ala Arg Glu Gln Gln Gln Gln
865                 870                 875                 880

Gln Gln Pro Leu Leu His Pro Pro Glu Pro Lys Ser Pro Gly Glu Tyr
                885                 890                 895

Val Asn Ile Glu Phe Gly Ser Asp Gln Ser Gly Tyr Leu Ser Gly Pro
            900                 905                 910

Val Ala Phe His Ser Ser Pro Ser Val Arg Cys Pro Ser Gln Leu Gln
        915                 920                 925

Pro Ala Pro Arg Glu Glu Glu Thr Gly Thr Glu Glu Tyr Met Lys Met
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    930                 935                 940

Asp Leu Gly Pro Gly Arg Arg Ala Ala Trp Gln Glu Ser Thr Gly Val
945                 950                 955                 960

Glu Met Gly Arg Leu Gly Pro Ala Pro Pro Gly Ala Ala Ser Ile Cys
                965                 970                 975

Arg Pro Thr Arg Ala Val Pro Ser Ser Arg Gly Asp Tyr Met Thr Met
            980                 985                 990

Gln Met Ser Cys Pro Arg Gln Ser  Tyr Val Asp Thr Ser  Pro Ala Ala
        995                 1000                1005

Pro Val  Ser Tyr Ala Asp Met  Arg Thr Gly Ile Ala  Ala Glu Glu
    1010                1015                1020

Val Ser  Leu Pro Arg Ala Thr  Met Ala Ala Ala Ser  Ser Ser Ser
    1025                1030                1035

Ala Ala  Ser Ala Ser Pro Thr  Gly Pro Gln Gly Ala  Ala Glu Leu
    1040                1045                1050

Ala Ala  His Ser Ser Leu Leu  Gly Gly Pro Gln Gly  Pro Gly Gly
    1055                1060                1065

Met Ser  Ala Phe Thr Arg Val  Asn Leu Ser Pro Asn  Arg Asn Gln
    1070                1075                1080

Ser Ala  Lys Val Ile Arg Ala  Asp Pro Gln Gly Cys  Arg Arg Arg
    1085                1090                1095

His Ser  Ser Glu Thr Phe Ser  Ser Thr Pro Ser Ala  Thr Arg Val
    1100                1105                1110

Gly Asn  Thr Val Pro Phe Gly  Ala Gly Ala Ala Val  Gly Gly Gly
    1115                1120                1125

Gly Gly  Ser Ser Ser Ser Ser  Glu Asp Val Lys Arg  His Ser Ser
    1130                1135                1140

Ala Ser  Phe Glu Asn Val Trp  Leu Arg Pro Gly Glu  Leu Gly Gly
    1145                1150                1155

Ala Pro  Lys Glu Pro Ala Lys  Leu Cys Gly Ala Ala  Gly Gly Leu
    1160                1165                1170

Glu Asn  Gly Leu Asn Tyr Ile  Asp Leu Asp Leu Val  Lys Asp Phe
    1175                1180                1185

Lys Gln  Cys Pro Gln Glu Cys  Thr Pro Glu Pro Gln  Pro Pro Pro
    1190                1195                1200

Pro Pro  Pro Pro His Gln Pro  Leu Gly Ser Gly Glu  Ser Ser Ser
    1205                1210                1215

Thr Arg  Arg Ser Ser Glu Asp  Leu Ser Ala Tyr Ala  Ser Ile Ser
    1220                1225                1230

Phe Gln  Lys Gln Pro Glu Asp  Arg Gln
    1235                1240
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