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(54) Titre : PROTEINES DE FUSION AYANT DES PORTIONS D'IMMUNOGLOBULINES, PREPARATION ET
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(54) Title: FUSION PROTEINS WITH IMMUNOGLOBULIN PORTIONS, THE PREPARATION AND USE THEREOF

(57) **Abrégé/Abstract:**

The invention relates to genetically engineered soluble fusion proteins composed of human proteins not belonging to the immunoglobulin family, or of parts thereof, and of various portions of the constant region of immunoglobulin molecules. The functional properties of the two fusion partners are surprisingly retained in the fusion protein.

Abstract of the disclosure

Fusion proteins with immunoglobulin portions, the preparation and use thereof

The invention relates to genetically engineered soluble fusion proteins composed of human proteins not belonging to the immunoglobulin family, or of parts thereof, and of various portions of the constant region of immunoglobulin molecules. The functional properties of the two fusion partners are surprisingly retained in the fusion protein.

Description

5 Fusion proteins with immunoglobulin portions, the
preparation and use thereof

10 The invention relates to genetically engineered soluble
fusion proteins composed of human proteins not belonging
to the immunoglobulin family, or of parts thereof, and of
various portions of the constant region of immunoglobulin
molecules. The functional properties of the two fusion
partners are, surprisingly, retained in the fusion
protein.

15 EP-A 0 325 262 and EP-A 0 314 317 disclose corresponding
fusion proteins composed of various domains of the CD4
membrane protein of human T cells and of human IgG1
portions. Some of these fusion proteins bind with the
same affinity to the glycoprotein gp120 of human immuno-
deficiency virus as the cell-bound CD4 molecule. The CD4
20 molecule belongs to the immunoglobulin family and,
consequently, has a very similar tertiary structure to
that of immunoglobulin molecules. This also applies to
the α chain of the T-cell antigen receptor, for which
such fusions have also been described (Gascoigne et al.,
25 Proc. Natl. Acad. Sci. USA, vol. 84 (1987), 2937-2940).
Hence, on the basis of the very similar domain structure,
in this case retention of the biological activity of the
two fusion partners in the fusion protein was to be
expected.

30 The human proteins which are, according to the invention,
preferably coupled to the amino terminus of the constant
region of immunoglobulin do not belong to the immuno-
globulin family and are to be assigned to the following
classes: (i) membrane-bound proteins whose extracellular
35 domain is wholly or partly incorporated in the fusion.
These are, in particular, thromboplastin and cytokine

receptors and growth factor receptors, such as the cellular receptors for interleukin-4, interleukin-7, tumor necrosis factor, GM-CSF, G-CSF, erythropoietin; (ii) non-membrane-bound soluble proteins which are wholly or partly incorporated in the fusion. These are, in particular, proteins of therapeutic interest such as, for example, erythropoietin and other cytokines and growth factors.

The fusion proteins can be prepared in known pro- and eukaryotic expression systems, but preferably in mammalian cells (for example CHO, COS and BHK cells).

The fusion proteins according to the invention are, by reason of their immunoglobulin portion, easy to purify by affinity chromatography and have improved pharmacokinetic properties in vivo.

In many cases, the Fc part in fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when the Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations.

There are in existence various proteases whose use for this purpose appears conceivable. Papain and pepsin are employed, for example, to generate F(ab) fragments from immunoglobulins (Immunology, ed. Roitt, I. et al., Gower Medical Publishing, London (1989)), but they do not cleave in a particularly specific manner. Blood coagulation factor Xa by contrast recognises in a protein the relatively rare tetrapeptide sequence Ile-Glu-Gly-Arg and performs a hydrolytic cleavage of the protein after the

arginine residue . Sequences which contain the described tetrapeptide were introduced first by Nagai and Thogersen in a hybrid protein by genetic engineering means (Nagai, K. and Thogersen, H.C., Nature, vol. 309 (1984), 810-812). These authors were able to show that the proteins expressed in E. coli actually are specifically cleaved by factor Xa. However, there is as yet no published example of the possibility of such proteins also being expressed in eukaryotic and, especially, in animal cells and, after their purification, being cleaved by factor Xa. However, expression of the proteins according to the invention in animal cells is preferable because only in a cell system of this type is there expected to be secretion of, for example, normally membrane-bound receptors as fusion partners with retention of their natural structure and thus of their biological activity. Secretion into the cell culture supernatant facilitates the subsequent straightforward purification of the fusion protein.

20 The invention thus relates to genetically engineered soluble fusion proteins composed of human proteins not belonging to the immunoglobulin family, or of parts thereof, and of various portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (IgG, IgM, IgA, IgE). Preferred as immunoglobulin is the constant part of the heavy chain of human IgG, particularly preferably of human IgG1, where fusion takes place at the hinge region. In a particular embodiment, the Fc part can be removed in a simple way by a cleavage sequence which is also incorporated and can be

30 cleaved with factor Xa.

Furthermore, the invention relates to processes for the preparation of these fusion proteins by genetic engineering, and to the use thereof for diagnosis and therapy.

The invention will now be described in relation to the drawings, in which:

Figure 1 shows two oligonucleotide probe molecules
5 used in cloning of thromboplastin cDNA;

Figure 2 shows the nucleotide sequence of clone 2b-Apr5 with the thromboplastin amino acid sequence deduced therefrom;

10 Figure 3 shows two oligonucleotide sequences which are partially homologous with the sequence of the coding strand (A), and with the non-coding strand (B) of thromboplastin cDNA;

Figure 4 shows the restriction map of plasmid pTF1Fc;

15 Figure 5 shows two oligonucleotide sequences which are partially homologous with the sequence of the coding strand (A), and with the non-coding strand (B) of the IL-4 receptor cDNA cloned in the vector pDC302/T22-8;

20 Figure 6 shows the restriction map of plasmid pIL4RFc;

Figure 7 shows two oligonucleotide sequences A and B which are partially homologous with the sequence of the coding strand (A), and with the non-coding strand (B) of the EPO cDNA cloned in the vector pCES; and

25 Figure 8 shows the restriction map of plasmid pEPOFc.

Finally, the invention is explained in further examples.

Example 1: Thromboplastin fusion proteins

Blood coagulation is a process of central importance in the human body. There is appropriately delicate regulation of the coagulation cascade, in which a large number of cellular factors and plasma proteins cooperate. These proteins (and their cofactors) in their entirety are called coagulation factors. The final products of the coagulation cascade are thrombin, which induces the aggregation of blood platelets, and fibrin which stabilizes the platelet thrombus. Thrombin catalyzes the formation of fibrin from fibrinogen and itself is formed by limited proteolysis of prothrombin. Activated factor X (factor Xa) is responsible for this step and, in the presence of factor Va and calcium ions, binds to platelet membranes and cleaves prothrombin.

Two ways exist for factor X to be activated, the extrinsic and the intrinsic pathway. In the intrinsic pathway a series of factors is activated by proteolysis in order for each of them to form active proteases. In the extrinsic pathway, there is increased synthesis of thromboplastin (tissue factor) by damaged cells, and it activates factor X, together with factor VIIa and calcium ions. It was formerly assumed that the activity of thromboplastin is confined to this reaction. However, the thromboplastin/VIIa complex also intervenes to activate the intrinsic pathway at the level of factor IX. Thus, a thromboplastin/VIIa complex is one of the most important physiological activators of blood coagulation.

It is therefore conceivable that thromboplastin, apart from its use as diagnostic aid (see below), can also be employed as constituent of therapeutic agents for treating inborn or acquired blood coagulation deficiencies. Examples of this are chronic hemophilias caused by a deficiency of factors VIII, IX or XI or else acute disturbances of blood coagulation as a consequence of, for example, liver or kidney disease. Use of such a

therapeutic agent after surgical intervention would also be conceivable.

Thromboplastin is an integral membrane protein which does not belong to the immunoglobulin family. Thromboplastin cDNA sequences have been published by a total of four groups (Fisher et al., Thromb. Res., vol. 48 (1987), 89-99; Morrissey et al., Cell, vol. 50 (1987), 129-135; Scarpati et al., Biochemistry, vol. 26 (1987), 5234-5238; Spicer et al., Proc. Natl. Acad. Sci. USA, vol. 84 (1987), 5148-5152). Thromboplastin cDNA contains an open reading frame which codes for a polypeptide of 295 amino-acid residues, of which the 32 N-terminal amino acids act as signal peptide. Mature thromboplastin comprises 263 amino-acid residues and has a three-domain structure: i) amino-terminal extracellular domain (219 amino-acid residues); ii) transmembrane region (23 amino-acid residues); iii) cytoplasmic domain (carboxyl terminus; 21 amino-acid residues). In the extracellular domain there are three potential sites for N-glycosylation (Asn-X-Thr). Thromboplastin is normally glycosylated but glycosylation does not appear essential for the activity of the protein (Paborsky et al., Biochemistry, vol. 29 (1989), 8072-8077).

Thromboplastin is required as additive to plasma samples in diagnostic tests of coagulation. The coagulation status of the tested person can be found by the one-stage prothrombin clotting time determination (for example Quick's test). The thromboplastin required for diagnostic tests is currently obtained from human tissue, and the preparation process is difficult to standardize, the yield is low and considerable amounts of human starting material (placentae) must be supplied. On the other hand, it is to be expected that preparation of native, membrane-bound thromboplastin by genetic engineering will also be difficult owing to complex purification processes. These difficulties can be avoided by the fusion according to the invention to immunoglobulin portions.

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The thromboplastin fusion proteins according to the invention are secreted by mammalian cells (for example CHO, BHK, COS cells) into the culture medium, purified by affinity chromatography on protein A-SepharoseTM and have surprisingly high activity in the one-stage prothrombin clotting time determination.

Cloning of thromboplastin cDNA

The sequence published by Scarpati et al., Biochemistry, vol. 26 (1987), 5234-5238, was used for cloning the thromboplastin cDNA. Two oligonucleotide probe molecules (see Fig. 1) were derived from this. These two probe molecules were used to screen a cDNA bank from human placenta (Grundmann et al., Proc. Natl. Acad. Sci. USA, vol. 83 (1986), 8024-8028).

cDNA clones of various lengths were obtained. One clone, 2b-Apr5, which is used for the subsequent procedure, codes for the same amino-acid sequence as the cDNA described in Scarpati et al. Fig. 2 depicts the total sequence of the clone 2b-Apr5 with the thromboplastin amino-acid sequence deduced therefrom.

Construction of a hybrid plasmid pTF1Fc coding for thromboplastin fusion protein.

The plasmid pCD4E gamma 1 (EP 0 325 262 A2; deposited at the ATCC under the number No. 67610) is used for expression of a fusion protein composed of human CD4 receptor and human IgG1. The DNA sequence coding for the extracellular domain of CD4 is deleted from this plasmid using the restriction enzymes HindIII and BamHI. Only partial cleavage must be carried out with the enzyme HindIII in this case, in order to cut at only one of the two HindIII sites contained in pCD4E gamma 1 (position 2198). The result is an opened vector in which a eukaryotic transcription regulation sequence (promoter) is followed by the open HindIII site. The open BamHI site is

located at the start of the coding regions for a pentapeptide linker, followed by the hinge and the CH2 and CH3 domains of human IgG1. The reading frame in the BamHI recognition sequence GGATCC is such that GAT is translated as aspartic acid. DNA amplification with thermostable DNA polymerase makes it possible to modify a given sequence in such a way that any desired sequences are attached at one or both ends. Two oligonucleotides able to hybridize with sequences in the 5'-untranslated region (A: 5' GATCGATTAAGCTTCGGAACCCGCTCGATCTCGCCGCC 3') or coding region (B: 5' GCATATCTGGATCCCCGTAGAATATTTCTCTGAATTCCCC 3') of thromboplastin cDNA were synthesized. Of these, oligonucleotide A is partially homologous with the sequence of the coding strand, and oligonucleotide B is partially homologous with the non-coding strand; cf. Fig. 3.

Thus, amplification results in a DNA fragment (827 bp) which contains (based on the coding strand) at the 5' end before the start of the coding sequence a HindIII site, and at the 3' end after the codon for the first three amino-acid residues of the transmembrane region a BamHI site. The reading frame in the BamHI cleavage site is such that ligation with the BamHI site in pCD4E gamma 1 results in a gene fusion with a reading frame continuous from the initiation codon of the thromboplastin cDNA to the stop codon of the heavy chain of IgG1. The desired fragment was obtained and, after treatment with HindIII and BamHI, ligated into the vector pCD4E gamma 1, as described above, which had been cut with HindIII (partially) and BamHI. The resulting plasmid was called pTF1Fc (Fig. 4).

Transfection of pTF1Fc into mammalian cells

The fusion protein encoded by the plasmid pTF1Fc is called pTF1Fc hereinafter. pTF1Fc was transiently expressed in COS cells. For this purpose, COS cells were

transfected with pTF1Fc with the aid of DEAE-dextran (EP A 0 325 262). Indirect immunofluorescence investigations revealed that the proportion of transfected cells was about 25 %. 24 h after transfection, the cells were transferred into serum-free medium. This cell supernatant was harvested after a further three days.

Purification of pTF1Fc fusion protein from cell culture supernatants

170 ml of supernatant from transiently transfected COS cells were collected overnight in a batch process in a column containing 0.8 ml of protein A-Sepharose at 4°C, washed with 10 volumes of washing buffer (50 mM tris buffer pH 8.6, 150 mM NaCl) and eluted in 0.5 ml fractions with eluting buffer (93:7 100 mM citric acid: 100 mM sodium citrate). The first 9 fractions were immediately neutralized with 0.1 ml of 2M tris buffer pH 8.6 in each case and then combined, and the resulting protein was transferred by three concentration/dilution cycles in an Amicon microconcentrator (Centricon 30) into TNE buffer (50 mM tris buffer pH 7.4, 50 mM NaCl, 1 mM EDTA). The pTF1Fc obtained in this way is pure by SDS-PAGE electrophoresis (U.K. Lämmli, Nature 227 (1970) 680-685). In the absence of reducing agents it behaves in the SDS-PAGE like a dimer (about 165 KDa).

Biological activity of purified TF1Fc in the prothrombin clotting time determination

TF1Fc fusion protein is active in low concentrations (> 50 ng/ml) in the one-stage prothrombin clotting time determination (Vinazzer, H. Gerinnungsphysiologie und Methoden im Blutgerinnungslabor (1979), Fisher Verlag Stuttgart). The clotting times achieved are comparable with the clotting times obtained with thromboplastin isolated from human placenta.

Example 2: Interleukin-4 receptor fusion proteins

Interleukin-4 (IL-4) is synthesized by T cells and was originally called B-cell growth factor because it is able to stimulate B-cell proliferation. It exerts a large number of effects on these cells. One in particular is the stimulation of synthesis of molecules of immunoglobulin subclasses IgG1 and IgE in activated B cells (Coffmann et al., Immunol. Rev., vol. 102 (1988) 5). In addition, IL-4 also regulates the proliferation and differentiation of T cells and other hemopoietic cells. It thus contributes to the regulation of allergic and other immunological reactions. IL-4 binds with high affinity to a specific receptor. The cDNA which codes for the human IL-4 receptor has been isolated (Idzerda et al., J. Exp. Med., vol. 171 (1990) 861-873). It is evident from analysis of the amino-acid sequence deduced from the cDNA sequence that the IL-4 receptor is composed of a total of 825 amino acids, with the 25 N-terminal amino acids acting as signal peptide. Mature human IL-4 receptor is composed of 800 amino acids and, like thromboplastin, has a three-domain structure: i) amino-terminal extracellular domain (207 amino acids); ii) transmembrane region (24 amino acids) and iii) cytoplasmic domain (569 amino acids). In the extracellular domain there are six potential sites for N-glycosylation (Asn-X-Thr/Ser). IL-4 receptor has homologies with human IL-6 receptor, with the β -subunit of human IL-2 receptor, with mouse erythropoietin receptor and with rat prolactin receptor (Idzerda et al., loc. cit.). Thus, like thromboplastin, it is not a member of the immunoglobulin family but is assigned together with the homologous proteins mentioned to the new family of hematopoietin receptors. Members of this family have four cysteine residues and a conserved sequence (Trp-Ser-X-Trp-Ser) in the extracellular domain located near the transmembrane region in common.

On the basis of the described function of the IL-4/IL-4

receptor system, there is a possible therapeutic use of a recombinant form of the IL-4 receptor for suppressing IL-4-mediated immune reactions (for example transplant rejection reaction, autoimmune diseases, allergic reactions).

The amount of substance required for therapy makes it necessary to prepare such molecules by genetic engineering. Because of the straightforward purification by affinity chromatography and improved pharmacokinetic properties, according to the invention the synthesis of soluble forms of the IL-4 receptor as immunoglobulin fusion protein is particularly advantageous.

The IL-4 receptor fusion proteins are secreted by mammalian cells (for example CHO, BHK, COS cells) into the culture medium, purified by affinity chromatography on protein A-Sepharose and have, surprisingly, identical functional properties to the extracellular domain of the intact membrane-bound IL-4 receptor molecule.

Construction of a hybrid plasmid pIL-4Rfc coding for IL-4 receptor fusion protein.

Cutting of the plasmid pCD4E gamma1 with XhoI and BamHI results in an opened vector in which the open XhoI site is located downstream from the promoter sequence. The open BamHI site is located at the start of the coding regions for a pentapeptide linker, followed by the hinge and the CH2 and CH3 domains of human IgG1. The reading frame in the BamHI recognition sequence GGATCC is such that GAT is translated as aspartic acid. DNA amplification with thermostable DNA polymerase makes it possible to modify a given sequence in such a way that any desired sequences can be attached at one or both ends. Two oligonucleotides able to hybridize with sequences in the 5'-untranslated region

(A: 5' GATCCAGTACTCGAGAGAGAAGCCGGGCGTGGTGGCTCATGC 3') or coding region

(B: 5' CTATGACATGGATCCTGCTCGAAGGGCTCCCTGTAGGAGTTGTG 3')
of the IL-4 receptor cDNA which is cloned in the vector
pDC302/T22-8 (Idzerda et al., loc. cit.) were
synthesized. Of these, oligonucleotide A is partially
5 homologous with the sequence of the coding strand, and
oligonucleotide B is partially homologous with the non-
coding strand; cf. Fig. 5. Amplification using thermo-
stable DNA polymerase results in a DNA fragment (836 bp)
which, based on the coding strand, contains at the 5' end
10 before the start of the coding sequence an XhoI site, and
at the 3' end before the last codon of the extracellular
domain a BamHI site. The reading frame in the BamHI
cleavage site is such that ligation with the BamHI site
in pCD4E gamma 1 results in a gene fusion with a reading
15 frame continuous from the initiation codon of the IL-4
receptor cDNA to the stop codon of the heavy chain of
IgG1. The desired fragment was obtained and, after
treatment with XhoI and BamHI, ligated into the vector
pCD4E gamma 1, described above, which had been cut with
20 XhoI/BamHI. The resulting plasmid was called pIL4RFc
(Fig. 6).

Transfection of pIL4RFc into mammalian cells

The fusion protein encoded by the plasmid pIL4RFc is
called pIL4RFc hereinafter. pIL4RFc was transiently
25 expressed in COS cells. For this purpose, COS cells were
transfected with pIL4RFc with the aid of DEAE-dextran
(EP A 0 325 262). Indirect immunofluorescence investiga-
tions revealed that the proportion of transfected cells
was about 25 %. 24 h after transfection, the cells were
30 transferred into serum-free medium. This cell supernatant
was harvested after a further three days.

Purification of IL4RFc fusion protein from cell culture supernatants

500 ml of supernatant from transiently transfected COS

cells were collected overnight in a batch process in a column containing 1.6 ml of protein A-Sepharose at 4°C, washed with 10 volumes of washing buffer (50 mM tris buffer pH 8.6, 150 mM NaCl) and eluted in 0.5 ml fractions with eluting buffer (93:7 100 mM citric acid: 100 mM sodium citrate). The first 9 fractions were immediately neutralized with 0.1 ml of 2M tris buffer pH 8.6 in each case and then combined, and the resulting protein was transferred by three concentration/dilution cycles in an Amicon microconcentrator (Centricon 30) into TNE buffer (50 mM tris buffer pH 7.4, 50 mM NaCl, 1 mM EDTA). The IL4RFc obtained in this way is pure by SDS-PAGE electrophoresis (U.K. Lämmli, Nature 227 (1970) 680-685). In the absence of reducing agents it behaves in the SDS-PAGE like a dimer (about 150 KDa).

Biological activity of purified IL4RFc

IL4RFc proteins binds ^{125}I -radiolabeled IL-4 with the same affinity ($K_D=0.5$ nM) as membrane-bound intact IL-4 receptor. It inhibits the proliferation of IL-4-dependent cell line CTLLHuIL-4RI clone D (Idzerda et al., loc. cit.) in concentrations of 10-1000 ng/ml. In addition, it is outstandingly suitable for developing IL-4 binding assays because it can be bound via its Fc part to microtiter plates previously coated with, for example, rabbit anti-human IgG, and in this form likewise binds its ligands with high affinity.

Example 3: Erythropoietin fusion proteins

Mature erythropoietin (EPO) is a glycoprotein which is composed of 166 amino acids and is essential for the development of erythrocytes. It stimulates the maturation and the terminal differentiation of erythroid precursor cells. The cDNA for human EPO has been cloned (EP-A-0 267 678) and codes for the 166 amino acids of mature EPO and a signal peptide of 22 amino acids which is essential for secretion. The cDNA can be used to

prepare recombinant functional EPO in genetically manipulated mammalian cells and the EPO can be employed clinically for the therapy of anemic manifestations of various etiologies (for example associated with acute renal failure).

Because of the straightforward purification and the improved pharmacokinetic properties, according to the invention synthesis of EPO as immunoglobulin fusion protein is particularly advantageous.

Construction of a hybrid plasmid pEPOFc coding for erythropoietin fusion protein.

This construction was carried out in analogy to that described in Example 2 (section: "Construction of a hybrid plasmid pIL-4Rfc coding for IL-4 receptor fusion protein"). Two oligonucleotides able to hybridize with sequences in the vicinity of the initiation codon (A: 5' GATCGATCTCGAGATGGGGGTGCACGAATGTCCTGCCTGGCTGTGG 3') and of the stop codon (B: 5' CTGGAATCGGATCCCCTGTCCTGCAGGCCTCCCCTGTGTACAGC 3') of the EPO cDNA cloned in the vector pCES (EP-A 0 267 678) were synthesized. Of these, oligonucleotide A is partially homologous with the sequence of the coding strand, and oligonucleotide B is partially homologous with the non-coding strand; cf. Fig. 7. Amplification with thermostable DNA polymerase results in a DNA fragment (598 bp) which, based on the coding strand, contains at the 5' end in front of the initiation codon an XhoI site and in which at the 3' end the codon for the penultimate C-terminal amino acid residue of the EPO (Asp) is present in a BamHI recognition sequence. The reading frame in the BamHI cleavage site is such that ligation with the BamHI site in pCD4E gamma 1 results in a gene fusion with a reading frame continuous from the initiation codon of EPO cDNA to the stop codon of the heavy chain of IgG1. The desired fragment was obtained and, after treatment with XhoI and BamHI, ligated into

the vector pCD4E gamma 1, described above, which had been cut with XhoI/BamHI. The resulting plasmid was called pEPOFc (Fig. 8).

We claim:

1. A recombinant protein encoded by a polynucleotide, which comprises two DNA subsequences, wherein the first subsequence encodes the extracellular domain of a membrane-bound, human tumor necrosis factor (TNF) receptor protein and the second subsequence encodes all of the domains of the constant region of a human immunoglobulin heavy chain other than the first domain of said constant region, wherein the carboxy terminus of the extracellular domain of the membrane-bound, TNF receptor protein is coupled to the amino terminus of the constant region of the human immunoglobulin heavy chain.
2. A recombinant protein of claim 1, wherein said human immunoglobulin heavy chain is selected from the group consisting of IgG, IgM, IgA, and IgE.
3. A recombinant protein of claim 2, wherein said human immunoglobulin heavy chain is IgG.
4. A recombinant protein of claim 3, wherein IgG is IgG₁.
5. A soluble, recombinant fusion protein consisting of a first and a second fusion partner, wherein the first fusion partner is the extracellular domain of a membrane-bound receptor of human tumor necrosis factor and said second fusion partner is the hinge, C_H2, and C_H3 domains of human IgG₁, wherein the carboxy

terminus of the extracellular domain of the membrane-bound receptor of human tumor necrosis factor is coupled to the amino terminus of the hinge of the human IgG₁.

6. A DNA molecule encoding a recombinant protein as claimed in any one of the claims 1-4.

7. A DNA molecule encoding a soluble fusion protein as claimed in claim 5.

8. A process for preparing a recombinant protein as claimed in any one of claims 1-4, which comprises introducing the DNA coding for said recombinant protein into a mammalian cell expression system, expressing said protein, and, after expression, purifying the produced protein.

9. The process for preparing a recombinant protein as claimed in claim 8, wherein said produced protein is purified by affinity chromatography via the immunoglobulin portion.

10. A process for preparing a soluble fusion protein as claimed in claim 5, which comprises introducing the DNA coding for said fusion protein into a mammalian cell expression system, expressing said protein, and, after expression, purifying the produced protein.

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11. The process for preparing a soluble fusion protein as claimed in claim 10, wherein said produced protein is purified by affinity chromatography via the immunoglobulin portion.

12. A recombinant protein, which is a dimer of the recombinant protein of any one of claims 1-5.

13. A recombinant protein as claimed in claim 12, wherein said dimer is produced in CHO cells.

Fig. 1

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      GTCGCTCGGACGCTCCTGCTCGGCTGGGTCTTCGCCCAGGTGGCCGGCGCTTCAGGCACT
121  -----+-----+-----+-----+-----+-----+ 180
      CAGCGAGCCTGCGAGGACGAGCCGACCCAGAAGCGGGTCCACCGGCCGCGAAGTCCGTGA
      <*****
      Oligonucleotide 1

      ACAAATACTGTGGCAGCATATAATTTAACTTGGAAATCAACTAATTTCAAGACAATTTTG
181  -----+-----+-----+-----+-----+-----+ 240
      TGTTTATGACACCGTCGTATATTAAATTGAACCTTTAGTTGATTAAAGTTCTGTAAAC
      *****|

=====

      Oligonucleotide 2
      |*****>
      AACTACTGTTTCAGTGTTCAAGCAGTGATTCCCTCCCGAACAGTTAACCGGAAGAGTACA
721  -----+-----+-----+-----+-----+-----+ 780
      TTGATGACAAAGTCACAAGTTCGTCACTAAGGGAGGGCTTGTCAATTGGCCTTCTCATGT

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By: Riggs, Benckin & Parr

Fig. 2

10 30 50
 G C C C C C C T C G A G G T C G A C G G T A T C G A T A A G C T T G A T A T C G A A T T C T C T C G G C G A A C C C C
 70 90 110
 C T C G C A C T C C C T C T G G C C G G C C C A G G G C G C C T T C A G C C C A A C C T C C C C A G C C C C A C G G G C
 130 150 170
 G C C A C G G A A C C C G C T C G A T C T C G C C G C C A A C T G G T A G A C A T G G A G A C C C C T G C C T G G C C C
 MetGluThrProAlaTrpPro
 190 210 230
 C G G G T C C C G C G C C C C G A G A C C G C C G T C G C T C G G A C G C T C C T G C T C G G C T G G G T C T T C G C C
 ArgValProArgProGluThrAlaValAlaArgThrLeuLeuLeuGlyTrpValPheAla
 250 270 290
 C A G G T G G C C G G C G C T T C A G G C A C T A C A A T A C T G T G G C A G C A T A T A A T T T A A C T T G G A A A
 GlnValAlaGlyAlaSerGlyThrThrAsnThrValAlaAlaTyrAsnLeuThrTrpLys
 310 330 350
 T C A A C T A A T T T C A A G A C A A T T T T G G A G T G G G A A C C C A A C C C G T C A A T C A A G T C T A C A C T
 SerThrAsnPheLysThrIleLeuGluTrpGluProLysProValAsnGlnValTyrThr
 370 390 410
 G T T C A A A T A A G C A C T A A G T C A G G A G A T T G G A A A A G C A A A T G C T T T T A C A C A A C A G A C A C A
 ValGlnIleSerThrLysSerGlyAspTrpLysSerLysCysPheTyrThrThrAspThr
 430 450 470
 G A G T G T G A C C T C A C C G A C G A G A T T G T G A A G G A T G T G A A G C A G A C G T A C T T G G C A C G G G T C
 GluCysAspLeuThrAspGluIleValLysAspValLysGlnThrTyrLeuAlaArgVal
 490 510 530
 T T C T C C T A C C C G G C A G G G A A T G T G G A G A G C A C C G G T T C T G C T G G G G A G C C T C T G T A T G A G
 PheSerTyrProAlaGlyAsnValGluSerThrGlySerAlaGlyGluProLeuTyrGlu
 550 570 590
 A A C T C C C C A G A G T T C A C A C C T T A C C T G G A G A C A A A C C T C G G A C A G C C A A C A A T T C A G A G T
 AsnSerProGluPheThrProTyrLeuGluThrAsnLeuGlyGlnProThrIleGlnSer

By: Rogers, Breckin & Parr

Fig. 2 (cont.)

610 630 650
 TTTGAACAGGTGGGAACAAAAGTGAATGTGACCGTAGAAGATGAACGGACTTTAGTCAGA
 PheGluGlnValGlyThrLysValAsnValThrValGluAspGluArgThrLeuValArg

 670 690 710
 AGGAACAACACTTTCCTAAGCCTCCGGGATGTTTTTGGCAAGGACTTAATTTATACACTT
 ArgAsnAsnThrPheLeuSerLeuArgAspValPheGlyLysAspLeuIleTyrThrLeu

 730 750 770
 TATTATTGGAAATCTTCAAGTTCAGGAAAGAAAACAGCCAAAACAAACACTAATGAGTTT
 TyrTyrTrpLysSerSerSerSerGlyLysLysThrAlaLysThrAsnThrAsnGluPhe

 790 810 830
 TTGATTGATGTGGATAAAGGAGAAAACACTGTTTCAGTGTTCAAGCAGTGATTCCCTCC
 LeuIleAspValAspLysGlyGluAsnTyrCysPheSerValGlnAlaValIleProSer

 850 870 890
 CGAACAGTTAACCGGAAGAGTACAGACAGCCCGGTAGAGTGTATGGGCCAGGAGAAAGGG
 ArgThrValAsnArgLysSerThrAspSerProValGluCysMetGlyGlnGluLysGly

 910 930 950
 GAATTTCAGAGAAATATTCTACATCATTGGAGCTGTGGTATTTGTGGTCATCATCCTTGTC
 GluPheArgGluIlePheTyrIleIleGlyAlaValValPheValValIleIleLeuVal

 970 990 1010
 ATCATCCTGGCTATATCTCTACACAAGTGTAGAAAGGCAGGAGTGGGGCAGAGCTGGAAG
 IleIleLeuAlaIleSerLeuHisLysCysArgLysAlaGlyValGlyGlnSerTrpLys

 1030 1050 1070
 GAGAACTCCCCACTGAATGTTTCATAAAGGAAGCACTGTTGGAGCTACTGCAAATGCTAT
 GluAsnSerProLeuAsnValSer

 1090 1110 1130
 ATTGCACTGTGACCGAGAACTTTTAAGAGGATAGAATACATGGAAACGCAAATGAGTATT

 1150 1170 1190
 TCGGAGCATGAAGACCCTGGAGTTCAAAAACTCTTGATATGACCTGTTATTACCATTAG

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Fig. 2 (cont.)

1210	1230	1250
CATTCTGGTTTTGACATCAGCATTAGTCACTTTGAAATGTAACGAATGGTACTACAACCA		
1270	1290	1310
ATTCCAAGTTTTAATTTTTAACACCATGGCACCTTTTGCACATAACATGCTTTAGATTAT		
1330	1350	1370
ATATTCCGCACTTAAGGATTAACCAGGTCGTCCAAGCAAAAACAAATGGGAAAATGTCTT		
1390	1410	1430
AAAAAATCCTGGGTGGACTTTTGAAAAGCTTTTTTTTTTTTTTTTTTTTTTTGAGACGGAGTC		
1450	1470	1490
TTGCTCTGTTGCCCAGGCTGGAGTGCAGTAGCACGATCTCGGCTCACTTGCACCCTCCGT		
1510	1530	1550
CTCTCGGGTTCAAGCAATTGTCTGCCTCAGCCTCCCGAGTAGCTGGGATTACAGGTGCGC		
1570	1590	1610
ACTACCAGCCAAGCTAATTTTTGTATTTTTTAGTAGAGATGGGGTTTCACCATCTTGGC		
1630	1650	1670
CAGGCTGGTCTTGAATTCCTGACCTCAGTGATCCACCCACCTTGGCCTCCCAAAGATGCT		
1690	1710	1730
AGTATTATGGGCGTGAACCACCATGCCCAGCCGAAAAGCTTTTGAGGGGCTGACTTCAAT		
1750	1770	1790
CCATGTAGGAAAGTAAAATGGAAGGAAATTGGGTGCATTTCTAGGACTTTTCTAACATAT		
1810	1830	1850
GTCTATAATATAGTGTTTAGGTTCTTTTTTTTTTTCAGGAATACATTTGGAAATTCAAAC		
1870	1890	1910
AATTGGGCAAACTTTGTATTAATGTGTTAAGTGCAGGAGACATTGGTATTCTGGGCAGCT		

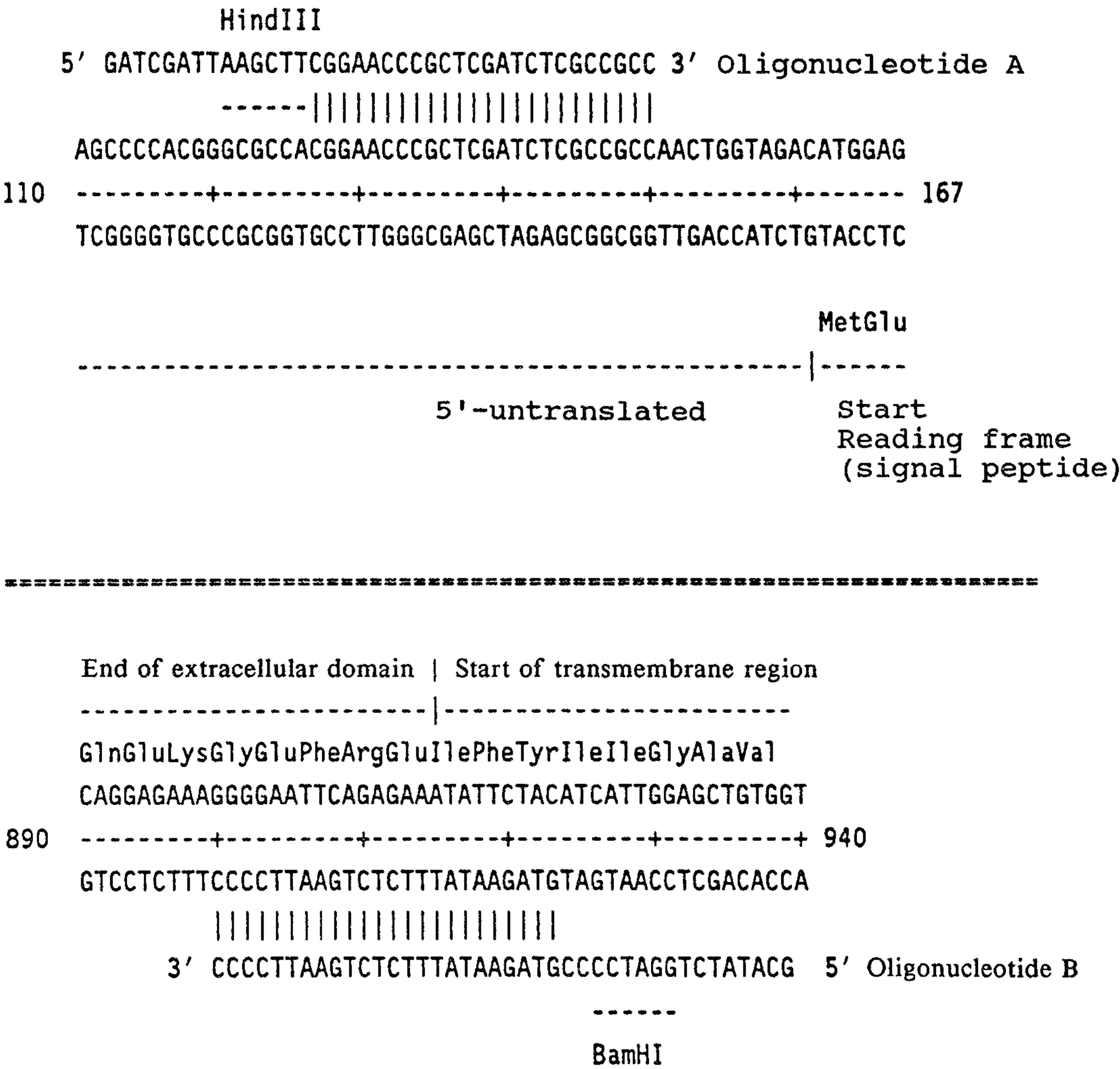
By: Angus Burkin & Pan

Fig. 2 (cont.)

1930	1950	1970
TCCTAATATGCTTTACAATCTGCACTTTAACTGACTTAAGTGGCATTAAACATTTGAGAG		
1990	2010	2030
CTAACTATATTTTTATAAGACTACTATACAACTACAGAGTTTATGATTTAAGGTACTTA		
2050	2070	2090
AAGCTTCTATGGTTGACATTGTATATATAATTTTTTAAAAAGGTTTTTCTATATGGGGAT		
2110	2130	2150
TTTCTATTTATGTAGGTAATATTGTTCTATTTGTATATATTGAGATAATTTATTTAATAT		
2170		
ACTTTAAATAAAGGTGACTGGGAATTGTT		

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Fig. 3



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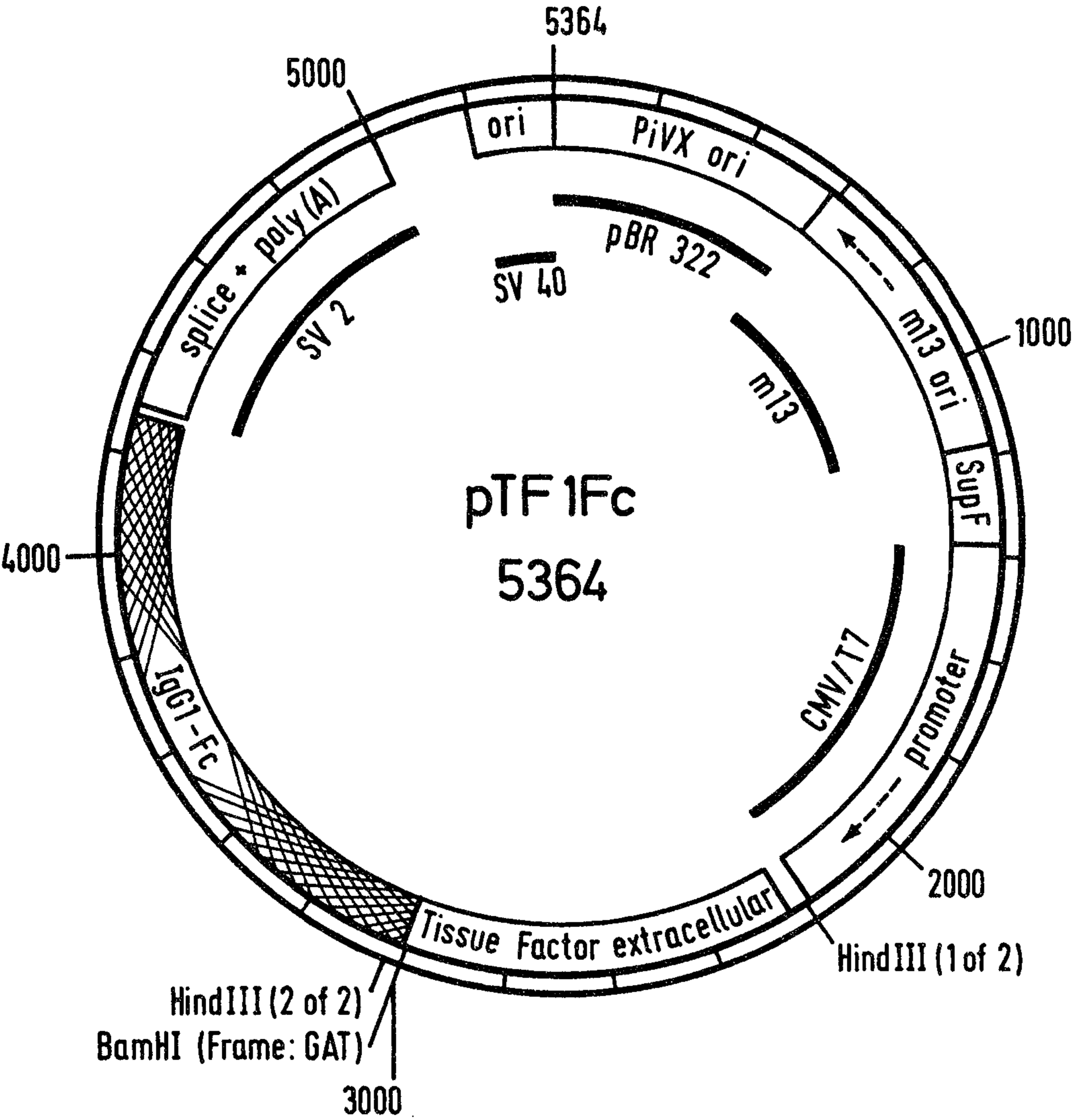


Fig. 4

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Fig. 5

XhoI
5' GATCCAGTACTCGAGAGAGAAGCCGGGCGTGGTGGCTCATGC 3' Oligonucleotide A
-----|||||
AGAGAAGCCGGGCGTGGTGGCTCATGCCTATAATCCCAGCACTTTTGGAGGCTGAGGCGG
61 -----+-----+-----+-----+-----+-----+ 120
TCTCTTCGGCCCGCACCACCGAGTACGGATATTAGGGTCGTGAAAACCTCCGACTCCGCC

-----5'-untranslated-----

GCAGATCACTTGAGATCAGGAGTTCGAGACCAGCCTGGTGCCTTGGCATCTCCCAATGGG
121 -----+-----+-----+-----+-----+-----+ 180
CGTCTAGTGAAGTCTAGTCCTCAAGCTCTGGTCGGACCACGGAACCGTAGAGGGTTACCC

-----5'-untranslated-----|MetGly

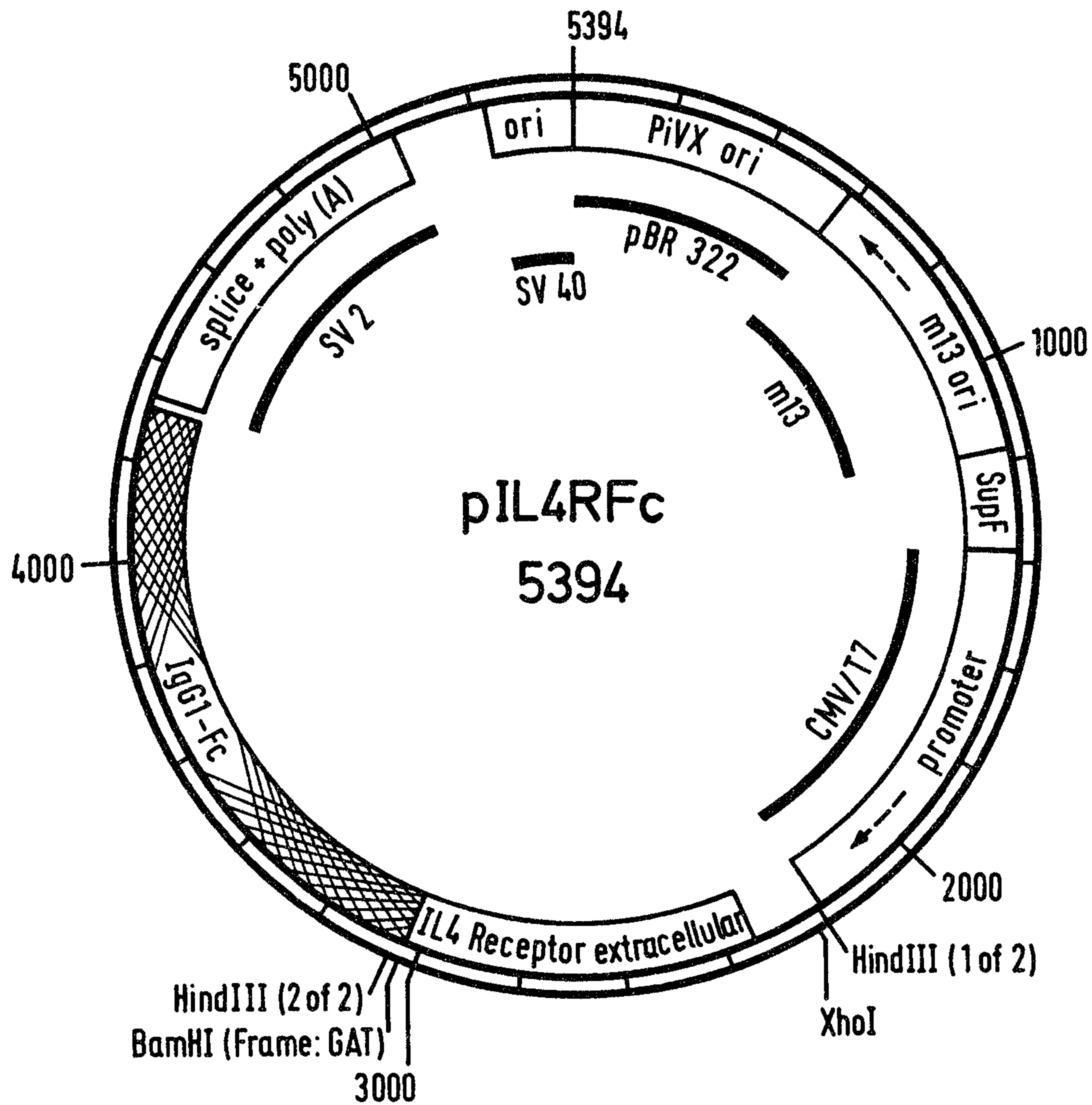
Start
Reading frame (signal peptide)

=====

End of extracellular domain	Start of transmembrane region
HisAsnSerTyrArgGluProPheGluGlnHisLeuLeuLeuGlyValSerValSerCys	
CACAACCTCCTACAGGGAGCCCTTCGAGCAGCACCTCCTGCTGGGCGTCAGCGTTTCCTGC	
839 +-+-----+-----+-----+-----+-----+-----+ 898	
GTGTTGAGGATGTCCCTCGGGAAGCTCGTCGTGGAGGACGACCCGAGTCGCAAAGGACG	
3' GTGTTGAGGATGTCCCTCGGGAAGCTCGTCCTAGGTACAGTATC 5' Oligonucleotide B

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**Fig. 6**

By: Rigus, Benishin & Parr

Fig. 7

XhoI
5' GATCGATCTCGAGATGGGGGTGCACGAATGTCCTGCCTGGCTGTGG 3' Oligonucleotide A
-----|
ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCTGTGG
182 -----+-----+-----+-----+-----+----- 235
TACCCCCACGTGCTTACAGGACGGACCGACACCGAAGAGGACAGGGACGACAGC

MetGlyValHisGluCysProAlaTrpLeuTrpLeuLeuSerLeuLeuSer -

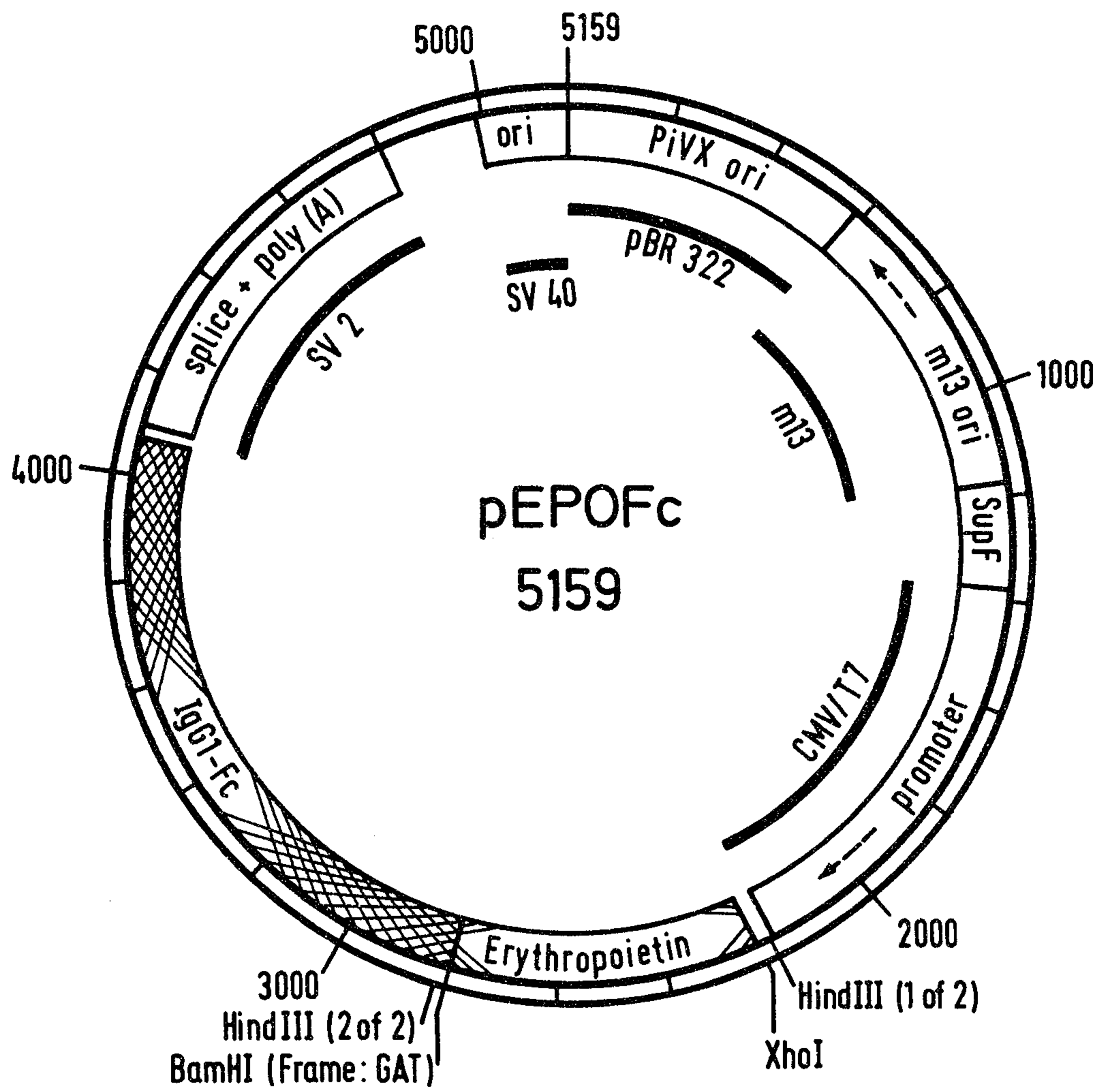
Start reading frame (signal peptide)

=====

End of reading frame-----|
LeuTyrThrGlyGluAlaCysArgThrGlyAspArgEnd
-----|
GCTGTACACAGGGGAGGCCTGCAGGACAGGGGACAGATGACCAGGTGTGTCCACCTGGGC
724 -----+-----+-----+-----+-----+-----+----- 783
CGACATGTGTCCCCTCCGGACGTCCTGTCCCCTGTCTACTGGTCCACACAGGTGGACCCG
|
3' CGACATGTGTCCCCTCCGGACGTCCTGTCCCCTAGGCTAAGGTC 5' Oligonucleotide B

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**Fig. B**

By: Rogers, Bueskin & Parr