659927

SPRUSON & FERGUSON

AUSTRALIA

PATENTS ACT 1990

PATENT REQUEST: STANDARD PATENT

I/We, the Applicant(s)/Nominated Person(s) specified below, request I/We be granted a patent for the invention disclosed in the accompanying standard complete specification.

[70,71] Applicant(s)/Nominated Person(s):

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[54] Invention Title:
TNF-muteins

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Details of Basic Application(s):

[31] Appl'n No(s):	[33] Country:	[32] Application Date:
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In accordance with Regulation 3.25, samples of materials deposited in accordance with the Budapest Treaty in relation to this Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

DATED this THIRTIETH day of MARCH 1993

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NOTICE OF ENTITLEMENT

I, Daniel Meier

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being authorised by the Applicant(s)/Nominated Person(s) in respect of an application entitled:

TNF-Muteins

state the following:-

1. The Applicant(s)/Nominated Person(s) has/have, for the following reasons, gained entitlement from the actual inventor(s):-

   [X] The Applicant is the assignee of the invention from the inventor(s)

   [ ] The inventor(s) have assigned the invention to Hoffmann-La Roche Inc., Nutley, USA, who have reassigned all their rights for Australia to the Applicant

2a.* The Applicant(s)/Nominated Person(s) is/are the applicant(s) of the basic application(s) listed* on the Patent Request/* ~~in the Declaration under Article 8 of the PCT.~~

2b.* The Applicant(s)/Nominated Person(s) is/are entitled to rely on the basic application(s) listed* on the Patent Request/~~* in the Declaration under Article 8 of the PCT as follows:~~

3.* The basic application(s) listed* on the Patent Request/~~* in the Declaration under Article 8 of the PCT~~ is/are the applications first made in a Convention Country in respect of the invention.

4.* The applicant(s)/Nominated Person(s) is/are the depositor(s) of the deposit(s) listed in the Schedule hetero.

DATED this 17th day of February, 1993.

Daniel Meier

Daniel Meier

Australia

Patents Act 1990

SCHEDULE TO NOTICE OF ENTITLEMENT

In respect of an application entitled:

TNF-Muteins

Name of Depositary Institution: DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Organism: M15pREP4 pDS56/RBSII, SphI-TNFαSer29

Deposit Date: November 19, 1990

Accession No.: DSM 6240

Organism: M15pREP4 pDS56/RBSII, SphI-TNFαTrp32

Deposit Date: November 19, 1990

Accession No.: DSM 6241

Organism: M15 (pREP4;DS56/RBSII, SphI-TNFα)

Deposit Date: September 18, 1991

Accession No.: DSM 6713

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Deposit Date:

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Organism:

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(57) Claim

1. A human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof having selective binding affinity for the human p55-Tumor-Necrosis-Factor-Receptor characterized in that the amino acid sequence of human Tumor Necrosis Factor is changed at least at position 86 showing a threonine instead of a serine residue.

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COMPLETE SPECIFICATION

FOR A STANDARD PATENT

ORIGINAL

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Invention Title: TNF-muteins

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

Tumor Necrosis Factor, or more specifically Tumor Necrosis Factor-alpha (TNF-α), is a cytokine, primarily produced by stimulated macrophages, that exhibits not only a striking cytotoxicity against various tumour cells [Carswell et al., Procd. Nat. Acad. Sci., U.S.A. 72, 3666-3670, (1975)] but also plays a multiple role as a mediator of inflammation and the immune response [for an overview see Beutler and Cerami, Ann. Rev. Immunol. 7, 625-655 (1989); Bonavista and Granger (eds.) "Tumor Necrosis Factor: Structure, Mechanism of Action, Role in Disease and Therapy, Karger, Basel (1990)]. The primary structure of human Tumor Necrosis Factor-alpha (hTNF-α) has been deduced from the nucleotide sequence of a cDNA which has been cloned and expressed in E. coli [Pennica et al., Nature 312, 724-729 (1984); Marmenout et al., Europ. J. Biochem. 152, 515-522 (1985); Wang et al., Science 228, 149-154 (1985); Shirai et al., Nature 313, 803-806 (1985)]. A striking homology in amino acid sequence (30%) was found between hTNF-α and human Lymphotoxin, often referred to as human Tumor Necrosis Factor-beta (hTNF-β), a cytokine mainly produced by lymphocytes [Gray et al., Nature 312, 721-724 (1984); Fiers et al., Cold Spring Harbour Symp. 51, 587-595 (1986)].

hTNF-α with modified amino acid sequences, so called TNF-α-muteins, have also been described in the art [for example see Yamagishi et al., Protein Engineering 3, 713-719, (1990) or by Fiers in "Tumor Necrosis Factors: Structure, Function and Mechanism of Action", Aggarwal and Vilcek (eds.), Marcel Dekker, Inc., New York, in press, or by Fiers et al. in Bonavista and Granger, pp. 77-81 (s.a.)]. In addition TNF-α-muteins have also been the object of several patent applications, e.g. International Patent Applications Publ. Nos. WO 86/02381, WO 86/04606, WO 88/06625 and European Patent

Applications Publ. Nos. 155,549; 158,286; 168,214; 251,037 and 340,333, and Deutsche Offenlegungsschrift Nr. 3843534.

Muteins of Lymphotoxin have also been disclosed in the art, e.g. in European Patent Applications Publ. Nos. 250,000; 314,094 and 336,383.

The biological effects of TNF are mediated via specific receptors, namely a receptor with an apparent molecular weight of 55 kD on sodium dodecylsulfate polyacrylamid gel electrophoresis (SDS-PAGE) (p55-TNF-R) and a receptor with an apparent molecular weight of 75 kD on SDS-PAGE (p75-TNF-R). Both forms of TNF-receptors have been cloned, namely p55-TNF-R by Loetscher et al. [Cell 61, 351-359, (1990)] and p75-TNF-R for example by Dembic et al. [Cytokine 2, 53-58, (1990)] (for both receptors see also European Patent Application No. 90116707.2) and it was found more recently that both receptors bind not only TNF-α but also TNF-β with high affinity [Schönfeld et al., J. Biol. Chem. 266, 3863-3869 (1991)].

It is well known in the art that on the basis of its biological activities TNF-α can be a valuable compound for the treatment of various disorders. For example TNF-α, alone or in combination with interferon, can be an effective antitumor agent [Brouckaert et al., Int. J. Cancer 38, 763-769 (1986)]. However, its systemic toxicity is a major limitation to its wider therapeutic use [Taguchi T. and Sohmura Y., Biotherapy 3, 177-186 (1991)].

It has been shown that in mice human TNF-α (hTNF-α), which only binds to the smaller mouse TNF receptor (murine p55-TNF-R) is far less toxic than murine TNF-α (mTNF-α), which binds to both p55-TNF-R and p75-TNF-R. For example, in C57B16 mice, the LD50 is about 10µg/mouse and 500µg/mouse with mTNF-α and hTNF-α, respectively [Brouckaert et al., Agents and Actions 26, 196-198 (1989); Everaerdt, B. et al., Biochem. Biophys. Res. Comm. 163, 378-385 (1989); Lewis, M. et al., Proc. Natl. Acad. Sci. USA 88, 2830

(1991)]. Hence the p75-TNF-R seems to play a special role in systemic toxicity.

hTNF-α and mTNF-α bind almost equally to human p55-TNF-R and to human p75-TNF-R. However, hTNF-α mutants, which have retained the biological activity mediated by hp55-TNF-R but have lost nearly all activity on hp75-TNF-R, are the functional equivalent of hTNF-α in the murine system, and are expected to have reduced systemic toxicity in primates.

Human Tumor Necrosis Factor muteins, showing a significant difference between their binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor (hp75-TNF-R) and to the human p55-Tumor-Necrosis-Factor-Receptor (hp55-TNF-R), have been described in European Patent Application, Publication No. 486 908.

It has now been found that hTNF muteins or pharmaceutically salts thereof, having the amino acid sequence of human Tumor Necrosis Factor, changed at least at position 86 (showing a threonine instead of a serine residue), have retained the binding activity to hp55-TNF-R, but have lost nearly all binding to hp75-TNF-R. Furthermore, such hTNF muteins have been found which have retained biological activity mediated by hp55-TNF-R, while no longer binding to hp75-TNF-R. However, the hTNF muteins of the present invention are not restricted to this type of mutein. Muteins of another type still binding exclusively to hp55-TNF-R but having lost the capacity to elicit a functional cell response are also included.

The present invention, therefore, provides human Tumor Necrosis Factor muteins or pharmaceutically acceptable salts thereof, having selective binding affinity for the human p55-Tumor-Necrosis-Factor-Receptor (hp55-TNF-R) characterized in that the amino acid sequence of human Tumor Necrosis Factor is changed at least at position 86, showing a threonine instead of a serine residue.

The amino acid sequence of human TNF-α as disclosed by Pennica et al. [s.a.] is as follows:

```
 1                                                  10
VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS

                    20                                                  30
VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN

                                                    40
ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY VAL GLU LEU ARG ASP

                    50                                                  60
ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER

                                                    70
GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU

                    80                                                  90
LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS

                                                   100
VAL ASN LEU LEU SER ALA ILE LYS SER PRO CYS GLN ARG GLU THR

                   110                                                 120
PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU

                                                   130
GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP ARG LEU SER ALA GLU

                   140                                                 150
ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL

                              157
TYR PHE GLY ILE ILE ALA LEU
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or as disclosed by Marmenout et al. (s.a.) or Wang et al. (s.a.) or Shirai et al. or more specifically as coded for by the nucleotide sequence of the insert of the plasmid pDS56/RBSII,SphI-TNFα (see Figures 1a and 1b and Example I) coding for mature TNF-α.

The hTNF muteins as defined above may have changed the amino acid sequence of hTNF at one or more additional positions, preferably at one or two additional positions, whereby positions 29, 31, 32, 29 and 32, or 31 and 32 are especially preferred. Any amino acid, preferably any naturally occurring one, can be used at these

additional positions. For substitutions at position 29 serine, glycine or tyrosine are preferred, whereby serine is especially preferred. For substitutions at position 31 glutamic acid or asparagine are preferred. For substitutions at position 32 tyrosine, tryptophan or threonine are preferred, whereby tryptophan and threonine are specifically preferred.

The hTNF muteins of the present invention may contain further amino acid substitutions if such substitutions do not alter their selective binding affinity for the p55-TNF-R. Amino acid substitutions in proteins and polypeptides which do not essentially alter biological activity are known in the art and described, e.g. by H. Neurath and R.L. Hill in "The Proteins", Academic Press, New York (1979), in particular in fig. 6 of page 14. The most frequently observed amino acid substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly and vice versa. The hTNF muteins of the present invention may additionally contain sequences of several amino acids which are coded for by "linker" sequences. These sequences may arise as a result from the expression vectors used for expression of the hTNF muteins as defined above.

The hTNF muteins of the present invention can also contain specific sequences that preferably bind to an affinity carrier material. Examples of such sequences are sequences containing at least two adjacent histidine residues (see in this respect European Patent Application, Publication No. 282 042). Such sequences bind selectively to nitrilotriacetic acid nickel chelate resins (Hochuli and Döbeli, Biol. Chem. Hoppe-Seyler 368, 748 (1987); European Patent Application, Publication No. 253 303). hTNF muteins which contain such a specific sequence can be linked either to the C-terminus or the N-terminus, or to both termini, of the hTNF-mutein amino acid sequences.

The hTNF muteins of the present invention can also be combined with different immunoglobulin heavy chain or light chain polypeptides. This leads to chimaeric hTNF mutein immunoglobulin

polypeptides which could have increased half-life in vivo. Increased half-life in vivo has been shown, e.g., for chimeric polypeptides consisting of the first two domains of the constant regions of the heavy chain or the light chain of a mammalian immunoglobulin (see Traunecker et al., Nature 331, 84-86 [1988] and European Patent Application, Publication No. 394 827).

The hTNF muteins can also be coupled to polymers, e.g. polyethylene glycol or polypropylene glycol having a molecular weight of 500 to 20.000 daltons. This leads to protected hTNF mutein compositions which could be substantially non-immunogenic. Several modes of coupling the polymer with the polypeptide are available and described, e.g., in U.S. Patent No. 4.179.337.

Especially preferred hTNF muteins of the present invention are Thr^{86}-TNF-α, Ser^{29}-Thr^{86}-TNF-α, Glu^{31}-Thr^{86}-TNF-α, Trp^{32}-Thr^{86}-TNF-α, Ser^{29}-Trp^{32}-Thr^{86}-TNF-α or Asn^{31}-Thr^{32}-Thr^{86}-TNF-α.

The hTNF muteins of the present invention can be produced by methods known in the art and described e.g. in Sambrook et al. [Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbour Laboratory, Cold Spring Harbour Laboratory Press, USA (1989)] or in the following paragraphs. Whether such hTNF muteins still show selective binding affinity for the p55-TNF-R can be determined as described in the following Examples. Alternatively, the hTNF muteins of the present invention can also be chemically synthesized using standard methods known in the art, preferably solid state methods, such as the methods of Merrifield (J. Am. Chem. Soc., 85, 2149-2154 [1963]). Furthermore salts of such muteins are also an object of the present invention. Such salts can be produced by methods known in the art.

It is believed that the strategy of dissecting beneficial and unwanted TNF-α activities by using compounds specifically binding to one or the other TNF-receptor, such as the hTNF muteins of the

present invention, can be used in general in other disease states where TNF plays a role.

DNA-sequences comprising a DNA-sequence coding for hTNF-muteins as hereinbefore described are also an object of the present invention. Such DNA-sequences can be constructed starting from genomic- or cDNA-sequences coding for hTNF as disclosed in the art [s.a.] using known methods of in vitro mutagenesis [see e.g. Sambrook et al., 1989]. Such mutagenesis can be carried out ad-random in order to obtain a large number of mutants which can then be tested for their desired properties in appropriate assay systems or, in order to mutate defined positions in a given DNA-sequence, by so called site directed mutagenesis [see e.g. Sambrook et al., 1989, 15.51-15.113] or by mutagenesis using the polymerase chain reaction [see e.g. White et al., Trends in Genetics 5, 185-189 (1989)].

One chemical mutagen which is often used for mutagenesis ad-random is sodium bisulfite which converts a cytosine residue into an uracil residue and hence leads to a transition of "C" to "T" (standard abbreviations for nucleotides) [for the method see e.g. Shortle and Nathans, Procd. Nat. Acad. Sci. U.S.A. 75, 2170-2174 (1978) or Pine and Huang, Meth. Enzym. 154, 415-430 (1987)]. This mutagen acts solely on single stranded DNA whereas the expression of the mutated target DNA sequence is achieved with a double stranded plasmid vector. One possibility to avoid the necessity of recloning in mutagenesis and expression vectors is the use of so called "phasmids". These are vectors which, in addition to a plasmid origin of replication, carry also an origin of replication derived from a filamentous phage. Examples of such phasmids are the pMa- and pMc-phasmids as described by Stanssen et al. [Nucleic Acids Res. 17, 4441-4454, (1989)]. Using this expression system one can construct so called "gap-duplex"-structures [see also Kramer et al., Nucl. Acids. Res. 12, 9441-9456 (1984)] where only the TNF-coding sequence (s.a.) is in a single stranded configuration and therefore accessible for the specific chemical mutagen. "gap-duplexes" to be used in ad-random mutagenesis can be constructed as described for site-specific

mutagenesis by Stanssen et al. [s.a.] with the exception that the (-)strand contains the same active antibiotic resistance gene as the (+)strand. By making use of different restriction sites in the DNA-sequence encoding hTNFα, variation of the width of the gap is possible. Examples of such restriction sites are the ClaI-SalI sites (470 nucleotides), BstX1-BstX1 sites (237 nucleotides) or StyI-StyI sites (68 nucleotides). Such gap-duplex-constructs can then be treated with increasing concentrations (up to 4M) of bisulfite, followed by several dialysis steps, as described by Shortle and Nathans (s.a.). A suitable procaryotic host cell can then be transformed by such phasmid constructs according to methods known in the art and described e.g. by Sambrook et al. (s.a.). A suitable procaryotic host cell means in this context a host cell deficient in a specific repair function so that an uracil residue is maintained in the DNA during replication and which host cell is capable of expressing the corresponding mutated TNF. Such specific host strains are known in the art, for example for E. coli strains, e.g. E. coli BW 313 [Kunkel, T.A., Procd. Natl. Acad. Sci. USA 82, 488-492 (1985)]. The resulting clones can then be screened for those expressing a desired hTNF mutein by appropriate assay systems. For example each colony can be inoculated in a microtiterplate in a suitable medium containing the relevant antibiotic. The cells may be lysed by addition of lysozyme, followed by sequential freeze-thaw cycles. After precipitation of nucleic acids and centrifugation, the supernatant of each colony can directly be used in appropriate assays as described, e.g., in Example IIa and IIb or Example VIII measuring binding to the p75-TNF-R and the p55-TNF-R on the surface of living cells or in purified form.

If desired, the specific sites of mutation can be determined, for example by restriction fragment analysis [see e.g. Sambrook et al. (s.a.)]. By determination of the DNA-sequence of such fragments the exact position of the mutation can be determined and if such mutation leads to an amino acid replacement the new amino acid can be derived from the determined DNA-sequence. DNA-sequencing can be performed according to methods known in the art, e.g. by using T7

polymerase on supercoiled DNA with a commercially available sequencing kit (Pharmacia, Uppsala, Sweden).

As already mentioned above, another possibility of mutating a given DNA-sequence is by "site directed mutagenesis". A widely used strategy for such kind of mutagenesis as originally outlined by Hutchinson and Edgell [J. Virol. 8, 181 (1971)] involves the annealing of a synthetic oligonucleotide carrying the desired nucleotide substitution to a target region of a single-stranded DNA-sequence wherein the mutation should be introduced [for review see Smith, Annual. Rev. Genet. 19, 423 (1985) and for improved methods see references 2-6 in Stanssen et al. (1989)].

One such preferred method is the one of Stanssen et al. (1989) using "gapped duplex DNA" as originally described by Kramer et al. (1984) [see above and Kramer and Fritz, Methods in Enzymology, (1987), Academic Press, Inc., USA] but using antibiotic resistance genes instead of M13 functional genes for selection of the mutation containing strand in addition with the phasmid-technology as also described by Stanssen et al. (1989) [s.a.]. An advantage of this method lies also in the capability of performing successive cycles of mutagenesis without the need to transfer the gene to a new mutagenesis vector: second round mutagenesis differs only in the selection to another antibiotic marker (Stranssen et al., s.a.). As a control site-specific back mutagenesis of the mutant to the wild-type TNF can be used. In addition, the use of an oligonucleotide, creating or destroying a restriction site in the TNF gene, allows to control the mutant not only by hybridization to the oligonucleotide used for site directed mutagenesis but also by the presence or absence of the restriction site. In order to create a set of hTNF muteins wherein at a defined position of their amino acid sequence the wild-type amino acid is replaced by any naturally occurring amino acid a set of oligonucleotides is used with all possible codons at the defined position.

As already mentioned above, another possibility of mutating a given DNA-sequence is the mutagenesis by using the polymerase chain reaction (PCR). The principles of this method are outlined e.g. by White et al. (1989), whereas improved methods are described e.g. in Innis et al. [PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc. (1990)].

PCR is an in vitro method for producing large amounts of a specific DNA fragment of defined length and sequence from small amounts of a template DNA. Thereby, PCR is based on the enzymatic amplification of the DNA fragment which is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are oriented with their 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment between the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. Since the primers are physically incorporated into the amplified product and mismatches between the 5' end of the primer and the template do not significantly affect the efficiency of the amplification, it is possible to alter the amplified sequence thereby introducing the desired mutation into the amplified DNA. By utilizing the thermostable Taq DNA polymerase isolated from the thermophilic bacteria Thermus aquaticus, it has been possible to avoid denaturation of the polymerase which necessitated the addition of enzyme after each heat denaturation step. This development has led to the automation of PCR by a variety of simple temperature-cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension. The increased specificity improves the overall yield of amplified products by minimizing the competition by non-target fragments for enzyme and primers.

Design and synthesis of oligonucleotides can be effected as known in the art and described e.g. in Sambrook et al. (1989) or in one of the references cited above with respect to site directed mutagenesis.

As soon as a DNA-sequence coding for a hTNF-mutein of the present invention has been created, expression can be effected by the phasmid technology as described above or by use of any suitable pro- or eukaryotic expression system well known in the art [see e.g. Sambrook et al., s.a.].

Expression is effected preferably in prokaryotic cells, e.g., in E. coli, Bacillus subtilis and so on, whereby E. coli, specifically E. coli K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474 (1974)], HB 101 [ATCC No. 33694], WK6 (Stranssens et al. s.a.) or E. coli SG13009 [Gottesman et al., J. Bacteriol. 148, 265-273 (1981)] are preferred. Expression of the hTNF muteins of the present invention can also be effected in lower or higher eukaryotic cells, like for example yeast cells (like Saccharomyces, Pichia etc.), filamentous fungi (like Aspergillus etc.) or cell lines (like chinese hamster ovary cell lines etc.), whereby expression in yeast cells is preferred [see Sreekrishna et al., Biochem. 28, 4117-4125, (1989); Hitzeman et al., Nature 293, 717-722 (1981); European Patent Application Publication No. 263 311]. Expression of the hTNF muteins of the present invention may occur in such systems either intracellularly, or, after suitable adaption of the gene, extracellularly (see Leemans et al., Gene 85, 99-108, 1989).

Suitable vectors used for expression in E. coli are mentioned e.g. by Sambrook et al. [s.a.] or by Fiers et al. in "Procd. 8th Int. Biotechnology Symposium" [Soc. Franc. de Microbiol., Paris, (Durand et al., eds.), pp. 680-697 (1988)] or and more specifically vectors of the pDS family [Bujard et al., Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc. Vol. 155, 416-433 (1987); Stüber et al., Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152 (1990)] like for example pDS56/RBSII,SphI-TNFα(Thr86) (see Example I) or pDS56/RBSII,SphI-

TNFα(Trp32Thr86) (see Example III) or pDS56/RBSII,SphI-TNFα(Ser29Thr86) or pDS56/RBSII,SphI-TNFα(Ser29Trp32Thr86) or pDS56/RBSII,SphI-TNFα(Asn31Thr32Thr86) or pDS56/RBSII,SphI-TNFα(Glu31Thr86) (see Example IV). Since with these specific pDS56/RBSII-plasmids due to their specific regulatable promoter/operator elements and ribosomal binding sites a high level of expression can be achieved, the plasmids can be maintained in E. coli cells only when the activity of the promoter/operator element is repressed by the binding of a lac repressor to the operator. The activity of the promoter can be restored at the desired cell density by addition of IPTG, which inactivates the repressor and clears the promoter. Since most of the E. coli strains do not provide enough repressor molecules to completely repress the function of the promoter sequences present in these high copy number plasmids, such E. coli strains, like E. coli M15 or SG13009, have to be transformed at first with a plasmid, like pREP 4 (see Figures 2a and b), coding for the lac repressor, before being transformed with the specific pDS56/RBSII-plasmids of the invention which can then be stably maintained in the E. coli cells. Beside coding for the lac repressor, pREP4 contains also a region of the plasmid pACYC184 [Chang and Cohen, J. Bacteriol. 134, 1141-1156 (1978)], which contains all information required for replication and stable transmission to daughter cells [for additional information see also "System for high level production in E. coli and rapid purification of recombinant proteins: application to epitope mapping, preparation of antibodies and structure function analysis" by Stüber et al. in Immunological Methods, Vol. IV, pp 121-152, Lefkovits and Pernis (eds.), Academic Press, New York (1990)].

Transformation of the host cells by vectors as described above may be carried out by any conventional procedure [see for example Sambrook et al. (s.a.)]. Where the host cell is a prokaryote, such as E. coli for example, competent cells which are capable of DNA uptake are prepared from cells harvested after exponential growth phase and subsequently treated according to the known $CaCl_2$-method. Transformation can also be performed after forming a protoplast of

the host cell or by other methods known in the art and described, e.g., in Sambrook et al. (s.a.). Therefore a vector, especially for expression in a prokaryotic or lower eukaryotic host cell, comprising a DNA-sequence coding for an hTNF mutein as described above, and a host cell, especially a prokaryotic host cell, e.g. E. coli, or a lower eukaryotic host cell, transformed by such a vector are also an object of the present invention.

Usually, the host organisms which contain a desired expression vector are grown under conditions which are optimal for their growth. In case of a procaryotic host at the end of the exponential growth, when the increase in cell number per unit time decreases, the expression of the desired hTNF mutein is induced, i.e. the DNA coding for the desired hTNF mutein is transcribed and the transcribed mRNA is translated. The induction can be carried out by adding an inducer or a derepressor to the growth medium or by altering a physical parameter, e.g. a change in temperature. In the expression vectors used in the preferred embodiments of the present invention, the expression is controlled by the lac repressor. By adding isopropyl-β-D-thiogalactopyranoside (IPTG), the expression control sequence is derepressed and the synthesis of the desired hTNF mutein is thereby induced.

The hTNF muteins of the present invention produced by transformed host cells as stated above can be recovered from the culture medium or after opening the cells and/or extraction by any appropriate method known in protein and peptide chemistry such as, for example, precipitation with ammonium sulfate, dialysis, ultrafiltration, gelfiltration or ion-exchange chromatography, gel electrophoresis, isoelectric focusing, affinity chromatography, like immunoaffinity chromatography, HPLC or the like. Specifically preferred methods are precipitation with ammonium sulfate and/or polyethylenimine, dialysis, affinity chromatography, e.g. on phenyl-agarose, specifically phenyl-sepharose, or ion-exchange chromatography, specifically on a MONO-Q- and/or MONO-S-matrix (Pharmacia, Uppsala, Sweden) or more specifically are those as

described by Tavernier et al. [J. Mol. Biol. 211, 493-501 (1990)] and those disclosed in Example V.

It is therefore also an object of the present invention to provide a process for the preparation of hTNF muteins as specified above which process comprises cultivating a transformed host cell as described above in a suitable medium and isolating a mutein from the culture supernatant or the host cell itself, and if desired converting said mutein into a pharmaceutically acceptable salt. The compounds whenever prepared according to such a process are also an object of the present invention.

The hTNF muteins of the present invention are characterized by showing a selective binding affinity for the human p55-TNF-R. Such property can be determined by any assay known in the art measuring binding affinities. For example the binding of TNF itself and of the muteins of the present invention can be measured using cells in cell culture which express the two types of TNF-receptors to a different degree, like for example Hep-2 cells which exclusivly express the human p55-TNF-R and U937 or HL60 cells which express in addition also the human p75-TNF-R [see Brockhaus et al., Procd. Nat. Acad. Sci. U.S.A. 87, 3127-3131, (1990); Hohmann et al., J. Biol. Chem. 264, 14927-14934, (1989); Loetscher et al. (1990); Dembic et al. (1990)]. Of course binding affinities can also be determined directly by using purified native or recombinant p55-TNF-R and p75-TNF-R as specifically described in the Examples, or by using the corresponding soluble analogs of such receptors.

The term selective binding affinity for the human p55-Tumor-Necrosis-Factor-Receptor" refers in the context of the present invention to a difference in binding affinities to the two types of TNF-receptors which is with respect to the used assay system significant enough to say that a mutein of the present invention binds selectively to the p55TNF-Receptors similar to wild-type TNF but has essentially lost functionally relevant binding to hp75-TNF-R. More specifically this term means in the context of the assay-system of the Examples

that a K_D-value of a specific hTNF mutein of the present invention is at least a factor of 10 or more, especially preferred at least a factor of 10^2, larger than for wild-type TNF-α determined by using the in vitro binding assay with recombinant soluble hp75-TNF-R whereas its K_D-value determined by using the in vitro binding assay to recombinant soluble hp55-TNF-R for the same hTNF mutein differs not by more than a factor of 2 from that of wild-type TNF-α. It is however understood that these specific K_D-values are given for illustration and should not be considered as limiting in any manner.

The hTNF muteins of the present invention can be characterized by their anti-tumour activity by methods known in the art.

The hTNF muteins may be administered alone or with one or more additional compounds of the present invention in pharmaceutically acceptable oral, injectable or topical compositions and modes. Administration will be in a dosage such that the amount of the composition in the patient is effective to modify the biological function associated with hTNF mutein function.

Pharmaceutical compositions containing hTNF muteins in association with a compatible pharmaceutically acceptable carrier material are therefore a further object of the present invention. Any conventional carrier material can be utilized. The carrier material can be an organic or inorganic one suitable for enteral, percutaneous or parenteral administration. Suitable carriers include water, gelatin, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, polyalkylene-glycols, petroleum jelly and the like. Furthermore, the pharmaceutical preparations may contain other pharmaceutically active agents. Additional additives such as flavouring agents, preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

The pharmaceutical preparations can be made up in any conventional form including: a) a solid form of oral administration

such as tablets, capsules, pills, powders, granules and the like; b) a liquid form for oral administration such as solutions, syrups, suspensions, elixirs and the like; c) preparations for parenteral administration such as sterile solutions, suspensions or emulsions; and d) preparations for topical administrations such as solutions, suspensions, ointments, creams, gels, micronized powders, aerosols and the like. The pharmaceutical preparations may be sterilized and/or may contain adjuvants such as preservatives, stabilizers, wetting agents, emulsifiers, salts for varying the osmotic pressure and/or buffers.

Parenteral dosage forms may be infusions or injectable solutions which can be injected intravenously or intramuscularly. These preparations can also contain other medicinally active substances. Additional additives such as preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

Accordingly it is also an object of the present invention to provide a process for the preparation of a pharmaceutical composition which process is characterized in that a compound obtained by a process of the present invention and if desired, additional pharmaceutically active substances are mixed with a non-toxic, inert, therapeutically compatible carrier material and the mixture is brought into a galenical application form.

Furthermore the use of a compound prepared according to a process of the present invention for the preparation of a pharmaceutical composition as described above is also an object of the present invention.

Finally, antibodies can be raised against the hTNF muteins of the present invention. These antibodies can be used in a well-known manner for diagnostic or therapeutic purposes as well as for purification purposes. Such antibodies can be produced by injecting a mammalian or avian animal with a sufficient amount of a vaccine

formulation comprising a hTNF mutein of the present invention and a compatible pharmaceutical carrier to elicit the production of antibodies against said hTNF mutein. The appropriate amount of the hTNF mutein which would be required would be known to one of skill in the art or could be determined by routine experimentation. As used in connectin with this invention, the term "pharmaceutical carrier" can mean either the standard compositions which are suitable for human administration or the typical adjuvants employed in animal vaccinations.

TNF is a potent pleiotropic cytokine. Its many different activities such as, for example, the activity of growth factor for immune cells, mediator in inflammation, or inductor of specific genes in endothelium, may be seen in the context of host defense to infection and injury. TNF also exhibits high systemic toxicity; the deleterious effects of bacteriaemia and septic shock or of bacterial meningitis are mediated to a large extent by endogenous cytokines among which TNF has an early and important role. Furthermore, many cells and cell lines are sensitive to a direct cytotoxic activity of TNF. Various systemic effects and cellular toxicity presumably combine in the antitumor activity of TNF seen in animal studies.

These facts form the rational basis for the development of novel therapeutic strategies using the hTNF muteins of the present invention, where in particular the potential to dissect the many different hTNF activities shall be fully exploited to separate unwanted toxic from desired activities. One example is to use the hTNF muteins of the present inventions as antitumor agents at the high doses which are made possible by the presumably lower systemic toxicity and thus to overcome the dose-limiting toxicity which presumably severely restricts the use of wild-type hTNF in cancer patients. However, the potential use of the hTNF muteins of the present invention is not restricted to cancer therapy. Any disease where TNF as host defense factor in bacterial infection [for example Kindler, V. et al., CELL 56, 731-740 (1989); Nakano, Y. et al., J. Immunol. 144, 1935, (1990)] or as mediator in

inflammation plays a beneficial role might benefit from a 55kDa TNF receptor type specific drug such as the hTNF muteins of the present invention. TNF has also been shown to play a role in cachexia [eg. Beutler, B. and Cerami, (sa)] and TNF muteins of the present invention with low systemic toxicity might be used for anti-obesity therapy. Even disease states characterised by the toxic activities exerted by excessive TNF release such as septic shock or bacterial meningitis can benefit from 55kDA TNF receptor specific agonists such as the muteins of the present invention above or in combination with TNF antagonists

A concise summary of the emerging role of TNF for novel therapies, where p55-TNF-Receptor type specific agonists of lower systemic toxicity and selectively triggering only some of the many different TNF activities may be expected to have significant advantages when compared to wild-type TNF, has been published [Tumor Necrosis Factors, The Molecules and their Emerging Role in Medicine, B. Beutler, ed., Raven Press, 1992, ISBN 0-88167-852-X]. It includes the activities of TNF in modulating endothelial cell homostatic properties and neutrophil adhesion, tissue ischemia and reperfusion injury, on osteoblasts and osteoclasts in bone resorption, as growth factor on many cells in general and in hematopoiesis, as well as in metabolic and nutritional effects. The induction of specific genes providing cellular protection mechanisms such as induction of Mn-superoxide dismutase known to be under the control of p55-TNFR [Lewis et al, Proc. Natl. Acad. Sci. USA 88, 2830 (1991); Tartaglia et al, Proc. Natl. Acad. Sci. USA 88, 9292 (1991)] or the direct cytotoxicity of TNF in some cells all provide a rational base for novel therapeutic strategies using receptor type specific TNF agonists. TNF as growth/differentiation factor in the generation of lymphokine-activated killer (LAK) cells appears to contribute to the antitumor activities of TNF.

An important aspect is that all these activities may be enhanced or modulated in combination with other recombinant cytokines such as for example interferon-gamma.

After the invention has been described in general hereinbefore, the following Examples are intended to illustrate details of the invention,

without thereby limiting it in any manner, in connection with the following Figures:

The following abbreviations and symbols used are:
B, E, H, S, Xb and X which indicate cleavage sites for restriction enzymes BglI, EcoRI, HindIII, SalI, XbaI and XhoI, respectively.

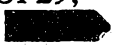
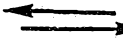
 represents the regulatable promoter/operator element N25OPSN25OP29,  represents the synthetic ribosomal binding site RBSII,SphI,  represents genes for TNFα (TNFα), β-lactamase (bla), chloramphenicol acetyltransferase (cat), lac repressor (lacI) and neomycin phosphotransferase (neo),  represents transcriptional terminators t_0 of phage lambda (t_0) and T1 of the E. coli rrnB operon (T1)  represents the replication regions of plasmids pBR322 and pREP4 (repl.),  represents the coding region under control of N250PSN250P29 and RBSII,SphI.

Figure 1a is a schematic drawing of the plasmid pDS56/RBSII,SphI-TNFα.

Figure 1b displays the complete nucleotide sequence of plasmid pDS56/RBSII,SphI-TNFα. In this sequence, the recognition sequences of the restriction enzymes depicted in Figure 1a are indicated. The amino acid sequence shown represents in the three letter code the sequence of the mature TNFα (157 amino acids).

Figure 2a is a schematic drawing of the plasmid pREP4.

Figure 2b displays the complete nucleotide sequence of plasmid pREP4. In this sequence, the recognition sequences of the restriction enzymes depicted in Figure 2a are indicated.

Figure 3 outlines the preparation of an EcoRI-HindIII fragment encoding the TNFα muteins Thr^{86}-TNFα.

Figure 4 displays the nucleotide sequence of Fragment 1 of plasmid pDS56/RBSII,SphI-INFα(Trp32)

Figure 5 displays the nucleotide sequence of Fragment 1 of plasmid "pDS56/RBSII,SphI-TNFα(Ser29)

Figure 6 displays the nucleotide sequence of Fragment 1 of plasmid pDS56/RBSII,SphI-TNFα(Ser29Trp32)

Figure 7 Competitive binding of wild-type human TNFα and Thr^{86}, Trp^{32}-Thr^{86} and Ser^{29}-Trp^{32}-Thr^{86} muteins to recombinant human p-75 and p-55 TNF-R's.

Microtiter plates coated with recombinant human p-75TNF-R-IgGγ3 fusion protein (panel A) and recombinant human p-55TNF-R-IgGγ3 fusion protein (panel B) were incubated with radiolabelled human TNFα in the presence of different concentrations of wild-type TNFα (closed circles), Thr^{86} mutein (open circles), Trp^{32}-Thr^{86} mutein (open squares) and Ser^{29}-Trp^{32}-Thr^{86} mutein (open triangles). After three hours at room temperature bound radioactivity was counted in a γ-counter.

Figure 8 Competitive binding of wild-type human TNFα and Ser^{29}-Thr^{86}, Glu^{31}-Thr^{86} and Asn^{31}-Thr^{32}-Thr^{86} muteins to recombinant human p-75 and p-55TNF-R's.

Microtiter plates coated with recombinant human p-75TNF-R-IgGγ3 fusion protein (panel A) and recombinant human p-55TNF-R-IgGγ3 fusion protein (panel B) were incubated with radiolabelled human TNFα in the presence of different concentrations of wild-type TNFα (closed circles), Ser^{29}-Thr^{86} mutein (open circles), Asn^{31}-Thr^{32}-Thr^{86} mutein (open squares) and Glu^{31}-Thr^{86} mutein (open triangles). After three hours at room temperature bound radioactivity was counted in a γ-counter.

Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively.

Example I

Preparation of Thr^{86}-TNFα

Plasmid pDS56/RBSII,SphI-TNFα

The human TNFα expression plasmid pDS56/RBSII,SphI-TNFα (see Figure 1) was the source of the TNFα gene for preparation of the various TNFα muteins of this invention. The transformed E. coli strain M15 [pREP4;pDS56/RBSII,SphI-TNFα] has been deposited under the Budapest Treaty for patent purposes at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, BRD, at September 8th, 1991, under the accession number DSM 6713.

Mutagenesis of the TNFα gene using PCR

Two PCR reactions were performed with plasmid pDS56/RBSII,SphI-TNFα (Figure 1) as the template DNA using a Perkin-Elmer Cetus GeneAmp™ DNA Amplification Reagent Kit with AmpliTaq™ Recombinant Taq DNA Polymerase [see Figure 3].

Reaction I was performed with primers 17/F (5'-GGCGTATCACGAGGCCCTTTCG-3'; primer 17/F comprises nucleotides 3949-3970 of plasmid pDS56/RBSII,SphI-TNFα) and 29/M22 (5-GTAGGTGACGGCGATGCGGCTGATGGT-3'; primer 29/M22 comprises nucleotides which are complementary to nucleotides 378-352 of plasmid pDS56/RBSII,SphI-TNFα, the mutated base is underlined).

Reaction II was performed with primers 29/MR1 (5'-CAGACCAAGGTCAACCTCCTC-3'; primer 29/MR1 comprises nucleotides 379-399 of plasmid pDS56/RBSII,SphI-TNFα) and 17/O (5'-CATTACTGGATCTATCAACAGG-3'; primer 17/O comprises nucleotides which are complementary to nucleotides 748-727 of plasmid pDS56/RBSII,SphI-TNFα).

In a typical experiment, 10 µl template DNA (10 ng), 5 µl each of the two primers (100 pmoles each), 16 µl dNTP's mix (1.25 mM of dATP, dGTP, dCTP, and dTTP), 10 µl 10x reaction buffer (100 mM Tris-HCl pH8.3, 500 mM KCL, 15 mM $MgCl_2$ and 0.1 % gelatin), 1 µl (5 units)

AmpliTaq™ DNA polymerase and 53 µl H_2O were mixed in an Eppendorf tube and overlaid with 80 ml mineral oil (Perkin-Elmer Cetus). The tubes were transferred to a DNA thermal cycler (TRIO-Thermoblock, Biometra) and kept for 1 min at 94°C, before 35 cycles of melting the DNA (1 min at 94°C), annealing the primers (1 min at 50°C), and extending the primers (3 min at 72°C) were performed. After additional 2 min at 72°C, the reactions were cooled to room temperature and extracted with chloroform. The DNA present in the aqueous phase was precipitated with ethanol and subjected to electrophoresis in a 6 % polyacrylamide gel [Sambrook et al., 1989]. After staining of the DNA with ethidium bromide, fragments I and II (see Figure 3) were isolated from the gel and purified [Sambrook et al., 1989].

Preparation of a DNA fragment encoding Thr^{86}-TNFα

Fragments I and II were enzymatically phosphorylated, before they were ligated with each other [Sambrook et al., 1989]. After heat-inactivation of the ligase and digestion with restriction enzymes EcoRI and HindIII, the DNA was subjected to electrophoresis in a 6 % polyacrylamide gel. After staining of the DNA with ethidium bromide, the EcoRI-HindIII fragment A [see Figure 3] was isolated from the gel and purified [s.a].

Preparation of a plasmid encoding Thr^{86}-TNFα

The EcoRI-HindIII fragment A was inserted according to standard methods [Sambrook et al., 1989] into the EcoRI-HindIII opened plasmid pDS56/RBSII,SphI-TNFα generating the plasmid pDS56/RBSII,SphI-TNFα(Thr86). Plasmid DNA was prepared [Birnboim et al., 1979] and the identity of the coding region for the TNFα mutein was confirmed by sequencing the double-stranded DNA [Sambrook et al., 1989].

Production of Thr^{86}-TNFα

Plasmid pDS56/RBSII,SphI-TNFα(Thr86) was transformed into E. coli M15 cells containing already plasmid pREP4 by standard methods [s.a.]. Transformed cells were grown at 37°C in LB medium [Sambrook et al., 1989] containing 100 mg/l ampicillin and 25 mg/l kanamycin. At an

optical density at 600 nm of about 0.7 to 1.0 IPTG was added to a final concentration of 2 mM. After additional 2.5 to 5 h at 37°C the cells were harvested by centrifugation.

Example II

Preparation of Glu^{31}-TNFα and $Asn^{31}Thr^{32}$-TNFα

Principles

The TNFα muteins Glu^{31}-TNFα and $Asn^{31}Thr^{32}$-TNFα were prepared following the procedure described in detail in Example I for the preparation of Thr^{86}-TNFα. Therefore, in the description of the preparation of the TNFα muteins listed above only the primers used in PCR reactions I and II are specified. Furthermore, the names of the expression plasmids encoding the various TNFα muteins are given.

Preparation of Glu^{31}-TNFα

PCR reaction I was performed with primers 17/F (5'-GGCGTATCACGAGGCCCTTTCG-3'; primer 17/F comprises nucleotides 3949-3970 of plasmid pDS56/RBSII,SphI-TNFα) and 21/M5 (5-ATTGGCCCGCTCGTTCAGCCACTGGAGCTGCCCCTC-3'; primer 21/M5 comprises nucleotides which are complementary to nucleotides 219-184 of plasmid pDS56/RBSII,SphI-TNFα, mutated bases are underlined). PCR reaction II was performed with primers 21/MR (5'-GCCCTCCTGGCCAATGGCGTGG-3'; primer 21/MR comprises nucleotides 220-241 of plasmid pDS56/RBSII,SphI-TNFα) and 17/O (5'-CATTACTGGATCTATCAACAGG-3'; primer 17/O comprises nucleotides which are complementary to nucleotides 748-727 of plasmid pDS56/RBSII,SphI-TNFα).

The resulting expression plasmid pDS56/RBSII,SphI-TNFα(Glu31) was used for production of Glu^{31}-TNFα and in the construction of plasmid pDS56/RBSII,SphI-TNFα(Glu31Thr86) (see Example IV).

Preparation of $Asn^{31}Thr^{32}$-TNFα

PCR reaction I was performed with primers 17/F (5'-GGCGTATCACGAGGCCCTTTCG-3'; primer 17/F comprises nucleotides 3949-3970 of plasmid pDS56/RBSII,SphI-TNFα) and 21/M6 (5-ATTGGCAGTGTTGTTCAGCCACTGGAGCTGCCCCTC-3'; primer 21/M6 comprises nucleotides which are complementary to nucleotides 219-184 of plasmid pDS56/RBSII,SphI-TNFα, mutated bases are underlined). PCR reaction II was performed with primers 21/MR (5'-GCCCTCCTGGCCAATGGCGTGG-3'; primer 21/MR comprises nucleotides 220-241 of plasmid pDS56/RBSII,SphI-TNFα) and 17/O (5'-CATTACTGGATCTATCAACAGG-3'; primer 17/O comprises nucleotides which are complementary to nucleotides 748-727 of plasmid pDS56/RBSII,SphI-TNFα).

The resulting expression plasmid pDS56/RBSII,SphI-TNFα(Asn31Thr32) was used for production of $Asn^{31}Thr^{32}$-TNFα and in the construction of plasmid pDS56/RBSII,SphI-TNFα(Asn31Thr32Thr86) (see Example IV).

Example III

Preparation of $Trp^{32}Thr^{86}$-TNFα

Principles

For preparation of $Trp^{32}Thr^{86}$-TNFα the expression plasmid pDS56/RBSII,SphI-TNFα(Trp32Thr86) was constructed, which was subsequently used for the production of $Trp^{32}Thr^{86}$-TNFα in E. coli.

Construction of plasmid pDS56/RBSII,SphI-TNFα(Trp32Thr86)

All the expression plasmids described in Examples I and II contain the same two sites for the restriction enzyme BglI as plasmid pDS56/RBSII,SphI-TNFα (see Figure 1). One of these sites is located in the β-lactamase gene whereas the other site is located in the TNFα gene. This latter site separates the coding region for TNFα into two parts: one part is coding for amino acids 1 to 36 of TNFα, the other part encodes amino acids 37 to 157 of TNFα (see Figure 1b).

For construction of plasmid pDS56/RBSII,SphI-TNFα(Trp32Thr86) DNA fragments 1 and 2 were prepared according to standard methods [Sambrook et al., 1989]. Fragment 1 (for sequences see Fig. 4) was the small BglI fragment of plasmid pDS56/RBSII,SphI-TNFα(Trp32) with the regulatable promoter and the coding region for Trp^{32}-TNFα up to amino acid 36. Fragment 2 was the large BglI fragment of plasmid pDS56/RBSII,SphI-TNFα(Thr86) with the coding region for Thr^{86}-TNFα starting at amino acid 37 and the replication region of the plasmid. Fragment 1 and the enzymatically dephosphorylated fragment 2 were ligated with each other [Sambrook et al., 1989] resulting in plasmid pDS56/RBSII,SphI-TNFα(Trp32Thr86).

M15(pREP4;pDS56/RBSII,SphI-TNFα(Trp32)) cells have been deposited under the Budapest Treaty for patent purposes at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, BRD at November 19th, 1990 under accession number DSM 6241.

Production of $Trp^{32}Thr^{86}$-TNFα

Plasmid pDS56/RBSII,SphI-TNFα(Trp32Thr86) was transformed into E. coli M15 cells containing already plasmid pREP4 by standard methods [s.a.]. Transformed cells were grown at 37°C in LB medium [s.a.] containing 100 mg/l ampicillin and 25 mg/l kanamycin. At an optical density at 600 nm of about 0.7 to 1.0 IPTG was added to a final concentration of 2 mM. After additional 2.5 to 5 h at 37°C the cells were harvested by centrifugation.

Example IV

Preparation of $Ser^{29}Thr^{86}$-TNFα, $Ser^{29}Trp^{32}Thr^{86}$-TNFα, $Glu^{31}Thr^{86}$-TNFα and $Asn^{31}Thr^{32}Thr^{86}$-TNFα

Principles

The TNFα muteins $Ser^{29}Thr^{86}$-TNFα, $Ser^{29}Trp^{32}Thr^{86}$-TNFα, $Glu^{31}Thr^{86}$-TNFα and $Asn^{31}Thr^{32}Thr^{86}$-TNFα were prepared following the procedure described in detail in Example III for the preparation of

$Trp^{32}Thr^{86}$-TNFα. Therefore, in the description of the preparation of the TNFα muteins listed above only the DNA fragments corresponding to fragment 1 of Example III are specified. Furthermore, the names of the expression plasmids encoding the various TNFα muteins are given.

Preparation of $Ser^{29}Thr^{86}$-TNFα

Fragment 1 (for sequences see Fig. 5)was the small BglI fragment of plasmid pDS56/RBSII,SphI-TNFα(Ser29) with the regulatable promoter and the coding region for Ser^{29}-TNFα up to amino acid 36. Fragment 1 and the enzymatically dephosphorylated fragment 2 (see Example III) were ligated with each other [Sambrook et al., 1989] resulting in plasmid pDS56/RBSII,SphI-TNFα(Ser29Thr86), which was subsequently used for the production of $Ser^{29}Thr^{86}$-TNFα in E. coli.

M15 (pREP4;pDS56/RBSII,SphI-TNFα(Ser29)) cells have been deposited under the Budapest Treaty for patent purposes at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, BRD at November 19th, 1990 under accession number DSM 6240.

Preparation of $Ser^{29}Trp^{32}Thr^{86}$-TNFα

Fragment 1 (for sequences see Fig. 6) was the small BglI fragment of plasmid pDS56/RBSII,SphI-TNFα(Ser29Trp32) with the regulatable promoter and the coding region for $Ser^{29}Trp^{32}$-TNFα up to amino acid 36. Fragment 1 and the enzymatically dephosphorylated fragment 2 (see Example III) were ligated with each other [Sambrook et al., 1989] resulting in plasmid pDS56/RBSII,SphI-TNFα(Ser29Trp32Thr86), which was subsequently used for the production of $Ser^{29}Trp^{32}Thr^{86}$-TNFα in E. coli.

Preparation of $Glu^{31}Thr^{86}$-TNFα

Fragment 1 was the small BglI fragment of plasmid pDS56/RBSII,SphI-TNFα(Glu31) with the regulatable promoter and the coding region for Glu^{31}-TNFα up to amino acid 36. Fragment 1 and the enzymatically dephosphorylated fragment 2 (see Example III) were

ligated with each other [Sambrook et al., 1989] resulting in plasmid pDS56/RBSII,SphI-TNFα(Glu31Thr86), which was subsequently used for the production of $Glu^{31}Thr^{86}$-TNFα in E. coli.

Preparation of $Asn^{31}Thr^{32}Thr^{86}$-TNFα

Fragment 1 was the small BglI fragment of plasmid pDS56/RBSII,SphI-TNFα(Asn31Thr32) with the regulatable promoter and the coding region for $Asn^{31}Thr^{32}$-TNFα up to amino acid 36. Fragment 1 and the enzymatically dephosphorylated fragment 2 (see Example III) were ligated with each other [Sambrook et al., 1989] resulting in plasmid pDS56/RBSII,SphI-TNFα(Asn31Thr32Thr86), which was subsequently used for the production of $Asn^{31}Thr^{32}Thr^{86}$-TNFα in E. coli.

Example V

Purification of Human TNFα Muteins

One liter overnight cultures of E. coli cells transformed and induced as described above were collected by centrifugation and resuspended in 20 ml 50 mM Tris, pH 7.2, 200 mM KCl, 50 mM $MgCl_2$, 5% glycerol. The cells were disrupted in a French press at a pressure of 20'000 psi. After clarification by centrifugation (70'000 x g, 30 min, 4°C) solid ammonium sulfate was added to a final concentration of 30%. The solution was stirred at room temperature for one hour and then centrifuged at 10'000 x g for 20 min at 4°C. The supernatant was filtered through a 0.45µm filter and adjusted to 70% in ammonium sulfate. The precipitated proteins were collected by centrifugation, dissolved in 20 ml 20 mM Tris, pH 9.0, and dialyzed against the same buffer overnight at 4°C. 1 ml aliquots of the dialyzed samples were applied to a MonoQ column (HR 5/5, LKB-Pharmacia) equilibrated in 20 mM Tris pH 9.0 and eluted with a linear NaCl gradient (0 to 400 mM in 20 mM Tris pH 9.0) at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected and analyzed for the presence of TNFα muteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Positive fractions were pooled, dialyzed against 20 mM 2-morpholinoethanesulfonic acid (MES) pH 6.0 and

applied to a MonoS column (HR 5/5, LKB-Pharmacia) equilibrated in 20 mM MES pH 6.0. Proteins were eluted with a linear NaCl gradient (0 to 400 mM in 20 mM MES pH 6.0) at a flow rate of 0.5 ml/min. The various TNFα muteins eluted as electrophoretically pure proteins between 250 mM and 350 mM NaCl. After dialysis against phosphate buffered saline (PBS) the protein concentration was determined by the BCA Protein Assay (Pierce Chemical Company) using wild-type human TNFα as a standard.

Example VI

Competitive Binding of Human TNFα and Muteins to Recombinant Human p75-TNF-R and p55-TNF-R

For the competitive binding assay microtiter plates were coated with recombinant human p75-TNF-R-human IgGγ3 and p55-TNF-R-human IgGγ3 fusion proteins dissolved in PBS at 0.3 µg/ml and 0.1 µg/ml, respectively, (100 µl/well, overnight at 4°C) [Loetscher, H. et al., J. Biol. Chem. 266, 18324 - 18329 (1991); Lesslauer, W. et al., Eur. J. Immunol. 21, 2883 - 2886 (1991)]. After blocking with blocking buffer (50 mM Tris pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 1% defatted milk powder) the microtiter plate was washed with PBS and incubated with 10 ng/ml human ^{125}I-TNFα (labelled by the Iodogen method (Pierce Chemical Company) to a specific activity of about 30 µCi/µg) in the presence of different concentrations of the muteins. The volume was 100 µl/well and each concentration was assayed in triplicate. After three hours at room temperature the wells were thoroughly washed with PBS and counted in a g-counter.

The claims defining the invention are as follows:

~~Claims~~

1. A human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof having selective binding affinity for the human p55-Tumor-Necrosis-Factor-Receptor characterized in that the amino acid sequence of human Tumor Necrosis Factor is changed at least at position 86 showing a threonine instead of a serine residue.

2. A mutein as claimed in claim 1 or a pharmaceutically acceptable salt thereof, wherein said amino acid sequence is changed at one or more additional positions, preferably at one or two additional positions.

3. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof, wherein said amino acid sequence is changed at positions 29 and 86, 31 and 86, 32 and 86, 29, 32 and 86, and 31, 32 and 86.

4. A mutein as claimed in any one of claims 1 to 3 or a pharmaceutically acceptable salt thereof which is Thr^{86}-TNFα, Ser^{29}-Thr^{86}-TNFα, Glu^{31}-Thr^{86}-TNFα, Trp^{32}-Thr^{86}-TNFα, Ser^{29}-Trp^{32}-Thr^{86}-TNFα or Asn^{31}-Thr^{32}-Thr^{86}-TNFα.

5. A DNA-sequence comprising a DNA-sequence coding for a mutein as claimed in any one of claims 1-4.

6. A vector, especially for expression in a prokaryotic or lower eukaryotic host cell, comprising a DNA-sequence as claimed in claim 5.

7. A host cell, especially a prokaryotic or lower eukaryotic host cell transformed with a vector as claimed in claim 6.

8. A host cell as claimed in claim 7 which is E. coli.

9. A compound as claimed in any one of claims 1-4 for the treatment of illnesses.

10. A process for the preparation of a compound as claimed in any one of claims 1-4 which process comprises cultivating a host cell as claimed in claim 7 or claim 8 in a suitable medium and isolating the mutein from the culture supernatant or the host cell itself, and if

desired converting said mutein into a pharmaceutically acceptable salt.

11. A human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof, substantially as hereinbefore described with reference to any one of the Examples.

12. A compound as claimed in any one of claims 1 to 4 whenever prepared according to the process as claimed in claim 10.

13. A pharmaceutical composition which contains one or more compounds as claimed in any one of claims 1 to 4, 11 or 12, if desired, in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

14. A method for the treatment or prophylaxis of an illness which would benefit from a human Tumor Necrosis Factor mutein, which method comprises administering a patient in need of such treatment or prophylaxis, a compound as claimed in any one of claims 1 to 4, 11 or 12 or a pharmaceutical composition as claimed in claim 13.

15. The method according to claim 14 whereas as in the illness it is caused by a tumor.

16. A process for the preparation of a human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof, substantially as hereinbefore described with reference to any one of the Examples.

Dated 20 March, 1995
F.Hoffmann-La Roche AG
Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

TNF-Muteins

Abstract

The present invention is directed to a human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof having selective binding affinity for the human p55-Tumor-Necrosis-Factor-Receptor characterized in that the amino acid sequence of human Tumor Necrosis Factor is changed at least at position 86 showing a threonine instead of a serine residue, a DNA sequence coding for such a mutein, a vector comprising such a DNA sequence, a host cell transformed by such a vector, a process for the production of such muteins by such host cells, pharmaceutical compositions containing such a mutein and the use of such a mutein for the treatment of illnesses.

Fig. 1a

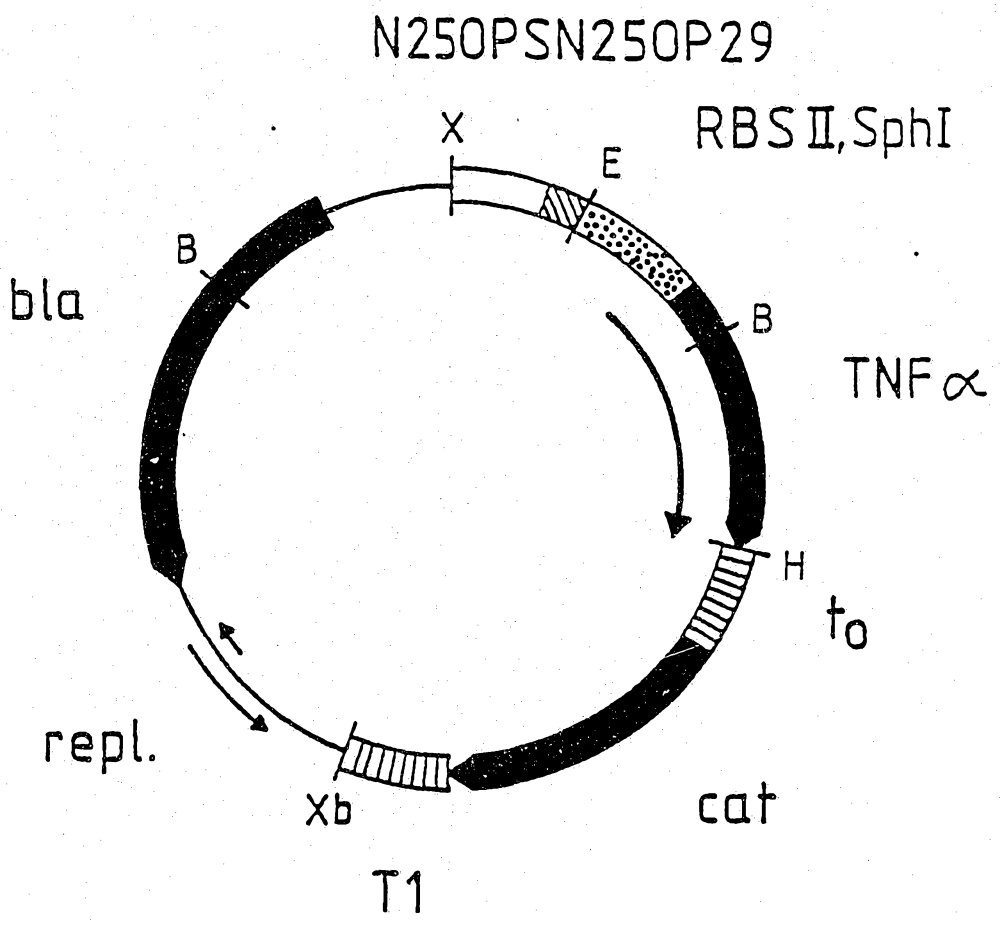


Fig. 1b

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EcoRI
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101 AGGAGAAATT AAGCATGGTC AGATCATCTT CTCGAACCCC GAGTGACAAG
Val ArgSerSerS erArgThrPr oSerAspLys
1 11

151 CCTGTAGCCC ATGTTGTCGC GAACCCTCAA GCTGAGGGGC AGCTCCAGTG
ProValAlaH isValValAl aAsnProGln AlaGluGlyG lnLeuGlnTr
21

BglI
201 GCTGAACCGC CGGGCCAATG CCCTCCTGGC CAATGGCGTG GAGCTGAGAG
pLeuAsnArg ArgAlaAsnA laLeuLeuAl aAsnGlyVal GluLeuArgA
31 41

251 ATAACCAGCT GGTGGTGCCA TCAGAGGGCC TGTACCTCAT CTACTCCCAG
spAsnGlnLe uValValPro SerGluGlyL euTyrLeuIl eTyrSerGln
51 61

301 GTCCTCTTCA AGGGCCAAGG CTGCCCCTCC ACCCATGTGC TCCTCACCCA
ValLeuPheL ysGlyGlnGl yCysProSer ThrHisValL euLeuThrHi
71

351 CACCATCAGC CGCATCGCCG TCTCCTACCA GACCAAGGTC AACCTCCTCT
sThrIleSer ArgIleAlaV alSerTyrGl nThrLysVal AsnLeuLeuS
81 91

401 CTGCCATCAA GAGCCCCTGC CAGAGGGAGA CCCCAGAGGG GGCTGAGGCC
erAlaIleLy sSerProCys GlnArgGluT hrProGluGl yAlaGluAla
101 111

451 AAGCCCTGGT ATGAGCCCAT CTATCTGGGA GGGGTCTTCC AGCTGGAGAA
LysProTrpT yrGluProIl eTyrLeuGly GlyValPheG lnLeuGluLy
121

501 GGGTGACCGA CTCAGCGCTG AGATCAATCG GCCCGACTAT CTCGACTTTG
sGlyAspArg LeuSerAlaG luIleAsnAr gProAspTyr LeuAspPheA
131 141

551 CCGAGTCTGG GCAGGTCTAC TTTGGGATCA TTGCCCTGTG AGGAGGACGA
laGluSerGl yGlnValTyr PheGlyIleI leAlaLeu
151 157

601 ACATCCAACC TTCCCAAACG CCTCCCCTGC CCCAATCCCT TTATTACCCC

651 CTCCTTCAGA CACCCTCAAC CTCTTCTGGC TCAAAAAGAG AATTGGGGGC

HindIII
701 TTAGGGTCGG AACCCAAGCT TGGACTCCTG TTGATAGATC CAGTAATGAC

751 CTCAGAACTC CATCTGGATT TGTTCAGAAC GCTCGGTTGC CGCCGGGCGT

Fig. 1b (cont.)

```
 801  TTTTTATTGG TGAGAATCCA AGCTAGCTTG GCGAGATTTT CAGGAGCTAA
 851  GGAAGCTAAA ATGGAGAAAA AAATCACTGG ATATACCACC GTTGATATAT
 901  CCCAATGGCA TCGTAAAGAA CATTTTGAGG CATTTCAGTC AGTTGCTCAA
 951  TGTACCTATA ACCAGACCGT TCAGCTGGAT ATTACGGCCT TTTTAAAGAC
1001  CGTAAAGAAA AATAAGCACA AGTTTTATCC GGCCTTTATT CACATTCTTG
1051  CCCGCCTGAT GAATGCTCAT CCGGAATTTC GTATGGCAAT GAAAGACGGT
1101  GAGCTGGTGA TATGGGATAG TGTTCACCCT TGTTACACCG TTTTCCATGA
1151  GCAAACTGAA ACGTTTTCAT CGCTCTGGAG TGAATACCAC GACGATTTCC
1201  GGCAGTTTCT ACACATATAT TCGCAAGATG TGGCGTGTTA CGGTGAAAAC
1251  CTGGCCTATT TCCCTAAAGG GTTTATTGAG AATATGTTTT TCGTCTCAGC
1301  CAATCCCTGG GTGAGTTTCA CCAGTTTTGA TTTAAACGTG GCCAATATGG
1351  ACAACTTCTT CGCCCCCGTT TTCACCATGG GCAAATATTA TACGCAAGGC
1401  GACAAGGTGC TGATGCCGCT GGCGATTCAG GTTCATCATG CCGTCTGTGA
1451  TGGCTTCCAT GTCGGCAGAA TGCTTAATGA ATTACAACAG TACTGCGATG
1501  AGTGGCAGGG CGGGGCGTAA TTTTTTTAAG GCAGTTATTG GTGCCCTTAA
1551  ACGCCTGGGG TAATGACTCT CTAGCTTGAG GCATCAAATA AAACGAAAGG
1601  CTCAGTCGAA AGACTGGGCC TTTCGTTTTA TCTGTTGTTT GTCGGTGAAC
                                        XbaI
1651  GCTCTCCTGA GTAGGACAAA TCCGCCGCTC TAGAGCTGCC TCGCGCGTTT
1701  CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA
1751  CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG
1801  TCAGCGGGTG TTGGCGGGTG TCGGGGCGCA GCCATGACCC AGTCACGTAG
1851  CGATAGCGGA GTGTATACTG GCTTAACTAT GCGGCATCAG AGCAGATTGT
1901  ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA TGCGTAAGGA
1951  GAAAATACCG CATCAGGCGC TCTTCCGCTT CCTCGCTCAC TGACTCGCTG
2001  CGCTCGGTCT GTCGGCTGCG GCGAGCGGTA TCAGCTCACT CAAAGGCGGT
2051  AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG
2101  CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG
```

Fig. 1b (cont.)

2151 TTTTTCCATA GGCTCCGCCC CCCTGACGAG CATCACAAAA ATCGACGCTC

2201 AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCGTTTC

2251 CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCT GCCGCTTACC

2301 GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCAATG

2351 CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG

2401 GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT

2451 AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC

2501 AGCAGCCACT GGTAACAGGA TTAGCAGAGC GAGGTATGTA GGCGGTGCTA

2551 CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG AAGGACAGTA

2601 TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG

2651 TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG

2701 TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT

2751 TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA

2801 AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT

2851 TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT

2901 TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA TCTCAGCGAT

2951 CTGTCTATTT CGTTCATCCA TAGCTCCTG ACTCCCCGTC GTGTAGATAA

3001 CTACGATACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG

3051 CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA ACCAGCCAGC

BglI

3101 CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC AACTTTATCC GCCTCCATCC

3151 AGTCTATTAA TTGTTGCCGG GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT

3201 AGTTTGCGCA ACGTTGTTGC CATTGCTACA GGCATCGTGG TGTCACGCTC

3251 GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG

3301 TTACATGATC CCCCATGTTG TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT

3351 CCGATCGTTG TCAGAAGTAA GTTGGCCGCA GTGTTATCAC TCATGGTTAT

3401 GGCAGCACTG CATAATTCTC TTACTGTCAT GCCATCCGTA AGATGCTTTT

3451 CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATGCGG

Fig. 1b (cont.)

3501 CGACCGAGTT GCTCTTGCCC GGCGTCAATA CGGGATAATA CCGCGCCACA

3551 TAGCAGAACT TTAAAAGTGC TCATCATTGG AAAACGTTCT TCGGGGCGAA

3601 AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT

3651 CGTGCACCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA GCGTTTCTGG

3701 GTGAGCAAAA ACAGGAAGGC AAAATGCCGC AAAAAAGGGA ATAAGGGCGA

3751 CACGGAAATG TTGAATACTC ATACTCTTCC TTTTTCAATA TTATTGAAGC

3801 ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA

3851 GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC

3901 CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG

3951 CGTATCACGA GGCCCTTTCG TCTTCAC

Fig. 2a

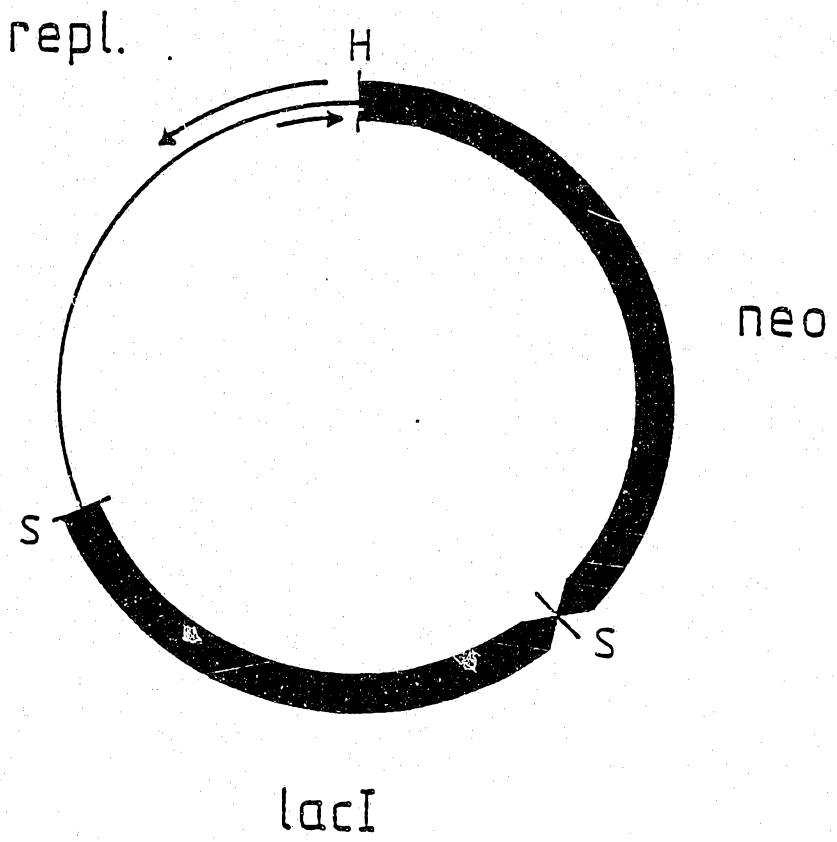

Fig. 2b

HindIII

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 101  TGTCAGCTAC TGGGCTATCT GGACAAGGGA AAACGCAAGC GCAAAGAGAA
 151  AGCAGGTAGC TTGCAGTGGG CTTACATGGC GATAGCTAGA CTGGGCGGTT
 201  TTATGGACAG CAAGCGAACC GGAATTGCCA GCTGGGGCGC CCTCTGGTAA
 251  GGTTGGGAAG TGCGCAAAG TAAACTGGAT GGCTTTCTTG CCGCCAAGGA
 301  TCTGATGGCG CAGGGGATCA AGATCTGATC AAGAGACAGG ATGACGGTCG
 351  TTTCGCATGC TTGAACAAGA TGGATTGCAC GCAGGTTCTC CGGCCGCTTG
 401  GGTGGAGAGG CTATTCGGCT ATGACTGGGC ACAACAGACA ATCGGCTGCT
 451  CTGATGCCGC CGTGTTCCGG CTGTCAGCGC AGGGGCGCCC GGTTCTTTTT
 501  GTCAAGACCG ACCTGTCCGG TGCCCTGAAT GAACTGCAGG ACGAGGCAGC
 551  GCGGCTATCG TGGCTGGCCA CGACGGGCGT TCCTTGCGCA GCTGTGCTCG
 601  ACGTTGTCAC TGAAGCGGGA AGGGACTGGC TGCTATTGGG CGAAGTGCCG
 651  GGGCAGGATC TCCTGTCATC TCACCTTGCT CCTGCCGAGA AAGTATCCAT
 701  CATGGCTGAT GCAATGCGGC GGCTGCATAC GCTTGATCCG GCTACCTGCC
 751  CATTCGACCA CCAAGCGAAA CATCGCATCG AGCGAGCACG TACTCGGATG
 801  GAAGCCGGTC TTGTCGATCA GGATGATCTG GACGAAGAGC ATCAGGGGCT
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1101  CCCGATTCGC AGCGCATCGC CTTCTATCGC CTTCTTGACG AGTTCTTCTG
1151  AGCGGGACTC TGGGGTTCGA AATGACCGAC CAAGCGACGC CCAACCTGCC
1201  ATCACGAGAT TTCGATTCCA CCGCCGCCTT CTATGAAAGG TTGGGCTTCG
1251  GAATCGTTTT CCGGGACGCC GGCTGGATGA TCCTCCAGCG CGGGGATCTC
1301  ATGCTGGAGT TCTTCGCCCA CCCCGGGCTC GATCCCCTCG CCAGTTGGTT
```

Fig. 2b (cont.)

```
1351  CAGCTGCTGC CTGAGGCTGG ACGACCTCGC GGAGTTCTAC CGGCAGTGCA
1401  AATCCGTCGG CATCCAGGAA ACCAGCAGCG GCTATCCGCG CATCCATGCC
                                                      SalI
1451  CCCGAACTGC AGGAGTGGGG AGGCACGATG GCCGCTTTGG TCGACAATTC
1501  GCGCTAACTT ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG
1551  GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA
1601  GGCGGTTTGC GTATTGGGCG CCAGGGTGGT TTTTCTTTTC ACCAGTGAGA
1651  CGGGCAACAG CTGATTGCCC TTCACCGCCT GGCCCTGAGA GAGTTGCAGC
1701  AAGCGGTCCA CGCTGGTTTG CCCCAGCAGG CGAAAATCCT GTTTGATGGT
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2201  TTCCACAGCA ATGGCATCCT GGTCATCCAG CGGATAGTTA ATGATCAGCC
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2301  ACGCCGCTTC GTTCTACCAT CGACACCACC ACGCTGGCAC CCAGTTGATC
2351  GGCGCGAGAT TTAATCGCCG CGACAATTTG CGACGGCGCG TGCAGGGCCA
2401  GACTGGAGGT GGCAACGCCA ATCAGCAACG ACTGTTTGCC CGCCAGTTGT
2451  TGTGCCACGC GGTTGGGAAT GTAATTCAGC TCCGCCATCG CCGCTTCCAC
2501  TTTTTCCCGC GTTTTCGCAG AAACGTGGCT GGCCTGGTTC ACCACGCGGG
2551  AAACGGTCTG ATAAGAGACA CCGGCATACT CTGCGACATC GTATAACGTT
2601  ACTGGTTTCA CATTCACCAC CCTGAATTGA CTCTCTTCCG GGCGCTATCA
2651  TGCCATACCG CGAAAGGTTT TGCGCCATTC GATGGTGTCA ACGTAAATGC
```

Fig. 2b (cont.)

SalI

2701 ATGCCGCTTC GCCTTCGCGC GCGAATTGTC GACCCTGTCC CTCCTGTTCA

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2851 TGAAGTGCTT CATGTGGCAG GAGAAAAAAG GCTGCACCGG TGCGTCAGCA

2901 GAATATGTGA TACAGGATAT ATTCCGCTTC CTCGCTCACT GACTCGCTAC

2951 GCTCGGTCGT TCGACTGCGG CGAGCGGAAA TGGCTTACGA ACGGGGCGGA

3001 GATTTCCTGG AAGATGCCAG GAAGATACTT AACAGGGAAG TGAGAGGGCC

3051 GCGGCAAAGC CGTTTTTCCA TAGGCTCCGC CCCCCTGACA AGCATCACGA

3101 AATCTGACGC TCAAATCAGT GGTGGCGAAA CCCGACAGGA CTATAAAGAT

3151 ACCAGGCGTT TCCCCTGGCG GCTCCCTCGT GCGCTCTCCT GTTCCTGCCT

3201 TTCGGTTTAC CGGTGTCATT CCGCTGTTAT GGCCGCGTTT GTCTCATTCC

3251 ACGCCTGACA CTCAGTTCCG GGTAGGCAGT TCGCTCCAAG CTGGACTGTA

3301 TGCACGAACC CCCCGTTCAG TCCGACCGCT GCGCCTTATC CGGTAACTAT

3351 CGTCTTGAGT CCAACCCGGA AAGACATGCA AAAGCACCAC TGGCAGCAGC

3401 CACTGGTAAT TGATTTAGAG GAGTTAGTCT TGAAGTCATG CGCCGGTTAA

3451 GGCTAAACTG AAAGGACAAG TTTTGGTGAC TGCGCTCCTC CAAGCCAGTT

3501 ACCTCGGTTC AAAGAGTTGG TAGCTCAGAG AACCTTCGAA AAACCGCCCT

3551 GCAAGGCGGT TTTTTCGTTT TCAGAGCAAG AGATTACGCG CAGACCAAAA

3601 CGATCTCAAG AAGATCATCT TATTAATCAG ATAAAATATT TCTAGATTTC

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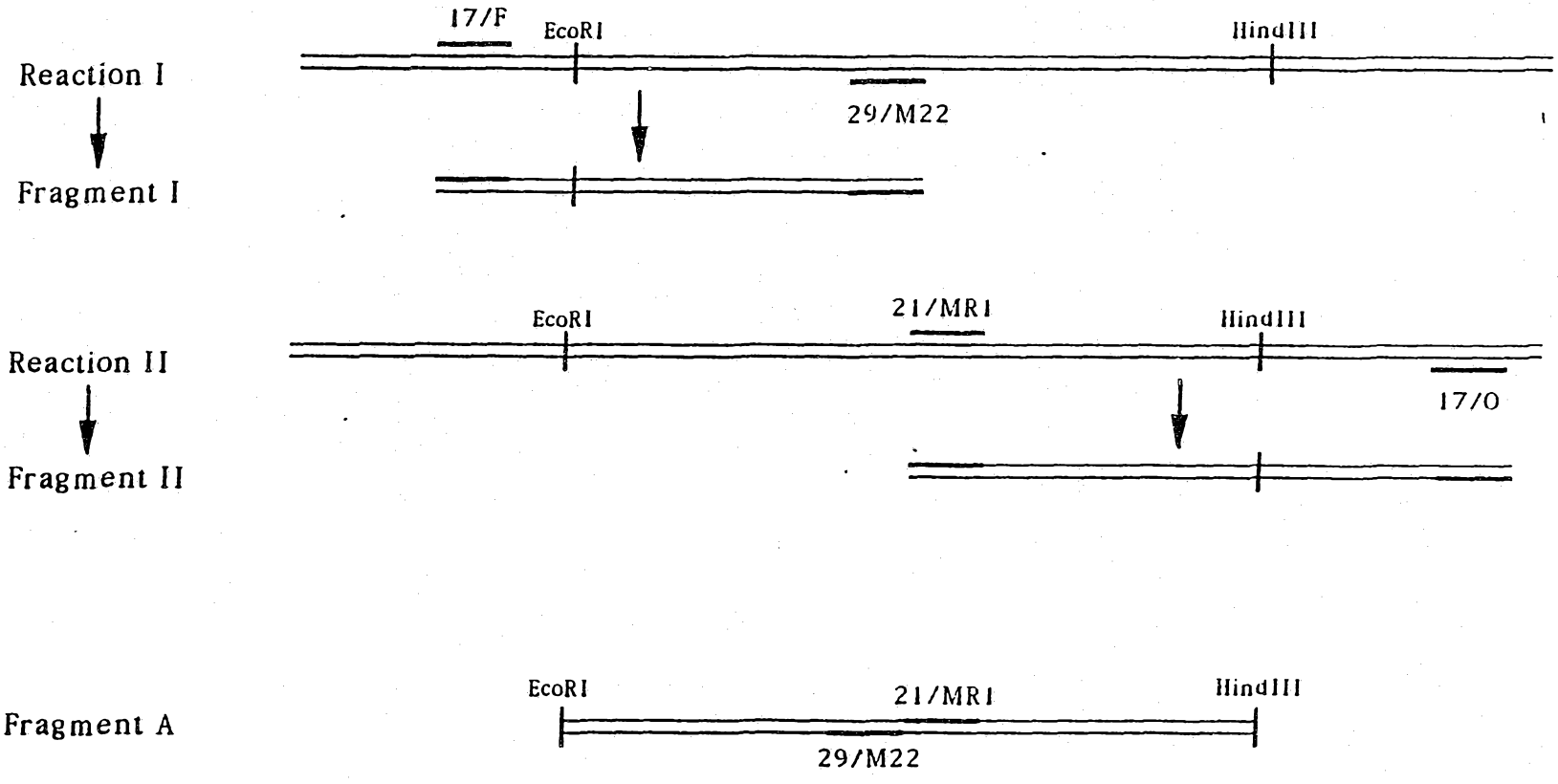
Fig. 3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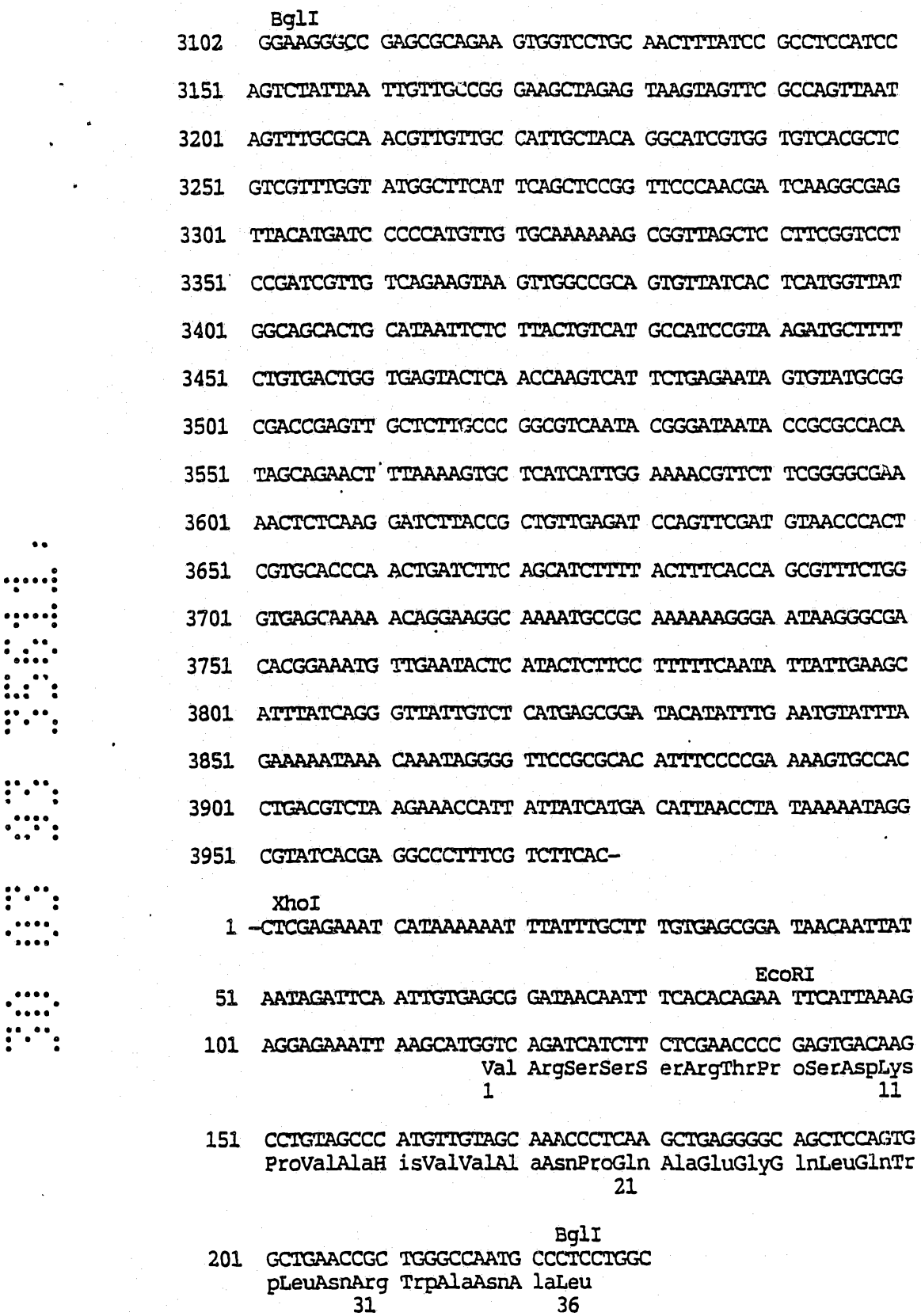
EcoRI
HindIII
Reaction I
29/M22
Fragment I
EcoRI
21/MRI
HindIII
Reaction II
17/O
Fragment II
EcoRI
21/MRI
HindIII
Fragment A
29/M22

Fig. 4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3201 AGTTTGCGCA ACGTTGTTGC CATTGCTACA GGCATCGTGG TGTCACGCTC
3251 GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG
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3501 CGACCGAGTT GCTCTTGCCC GGCGTCAATA CGGGATAATA CCGCGCCACA
3551 TAGCAGAACT TTAAAAGTGC TCATCATTGG AAAACGTTCT TCGGGGCGAA
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3851 GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC
3901 CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG
3951 CGTATCACGA GGCCCTTTCG TCTTCAC-
XhoI
1 -CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
EcoRI
51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
101 AGGAGAAAAT AAGCATGGTC AGATCATCTT CTCGAACCCC GAGTGACAAG
Val ArgSerSerS erArgThrPr oSerAspLys
1 11
151 CCTGTAGCCC ATGTTGTAGC AAACCCTCAA GCTGAGGGGC AGCTCCAGTG
ProValAlaH isValValAl aAsnProGln AlaGluGlyG lnLeuGlnTr
21
BglI
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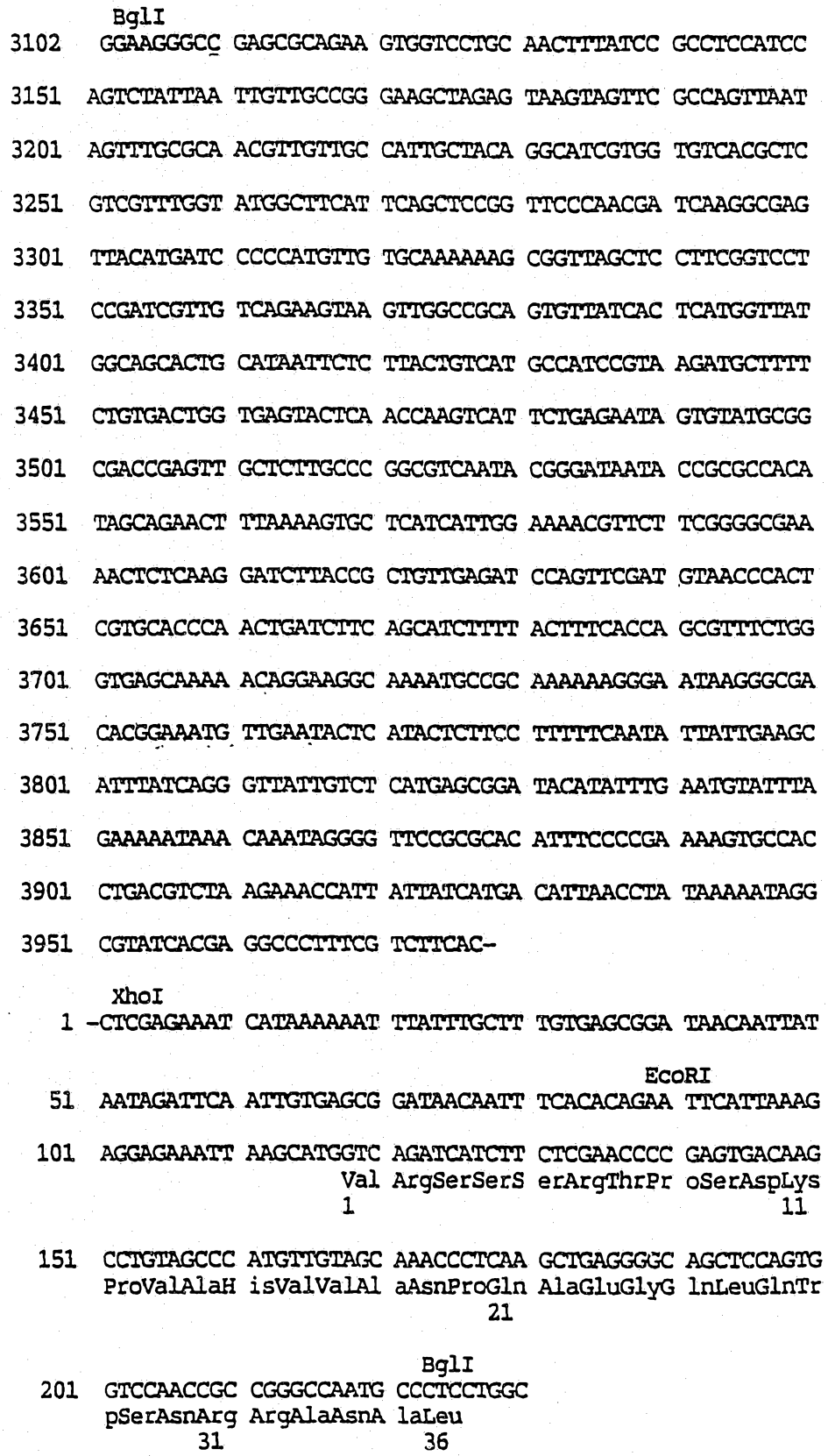
31 36

Fig. 5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3951 CGTATCACGA GGCCCTTTCG TCTTCAC-
XhoI
1 -CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
EcoRI
51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
101 AGGAGAAAAT AAGCATGGTC AGATCATCTT CTCGAACCCC GAGTGACAAG
Val ArgSerSerS erArgThrPr oSerAspLys
1 11
151 CCTGTAGCCC ATGTTGTAGC AAACCCTCAA GCTGAGGGGC AGCTCCAGTG
ProValAlaH isValValAl aAsnProGln AlaGluGlyG lnLeuGlnTr
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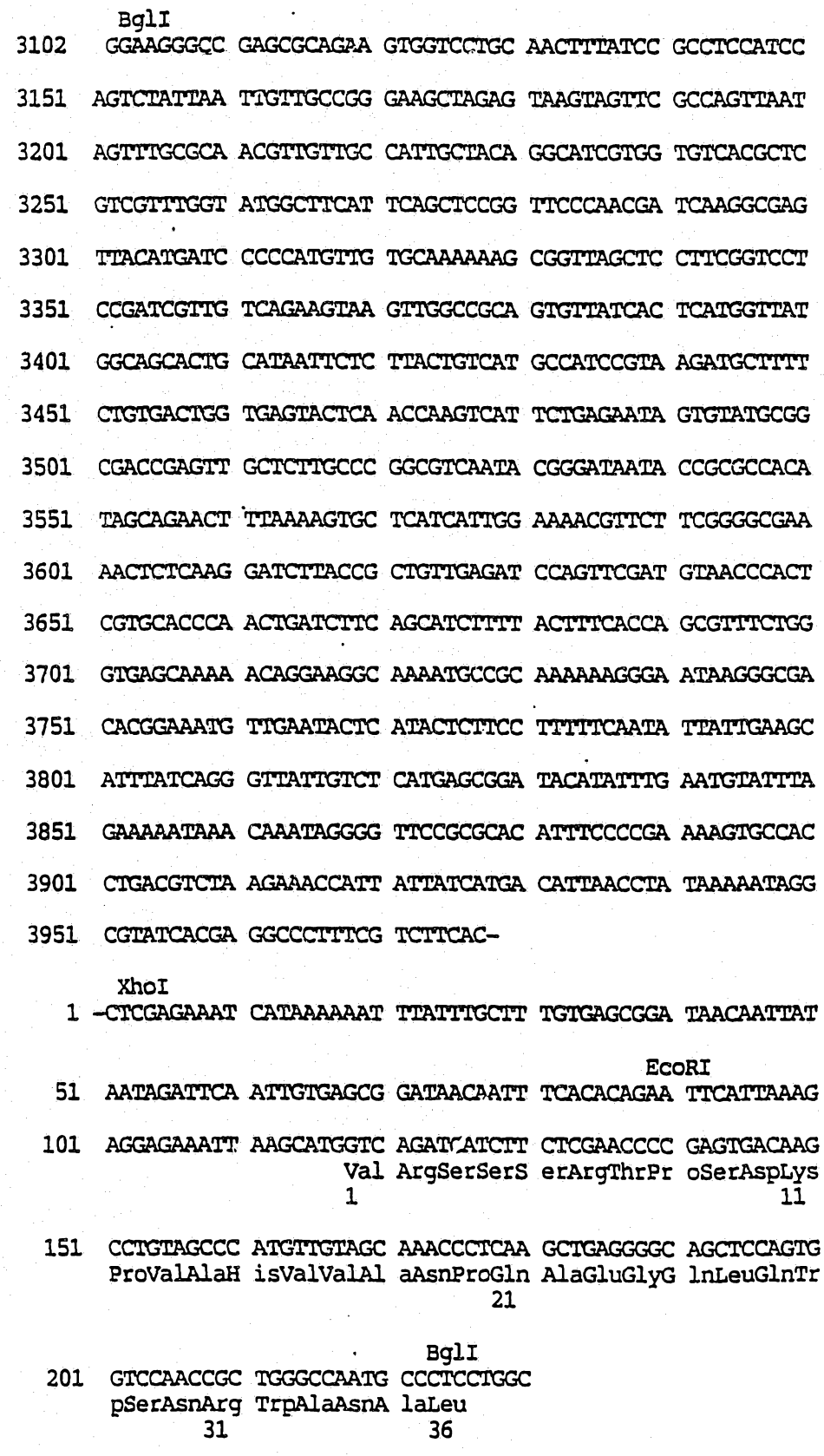
BglI
201 GTCCAACCGC CGGGCCAATG CCCTCCTGGC
pSerAsnArg ArgAlaAsnA laLeu
31 36

Fig. 6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3251 GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG
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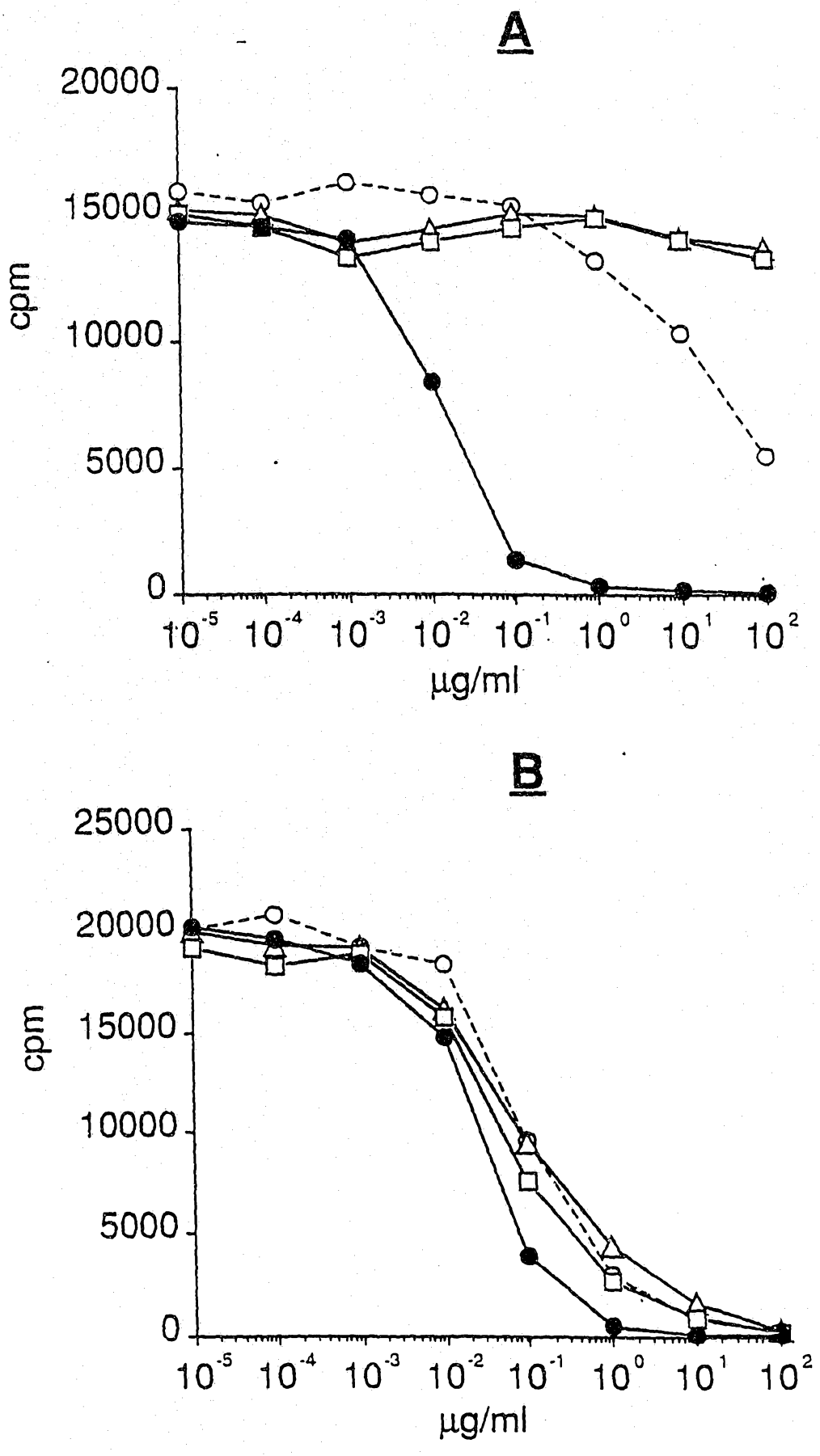
3801 ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA
3851 GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC
3901 CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG
3951 CGTATCACGA GGCCCTTTCG TCTTCAC-
XhoI
1 -CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
EcoRI
51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
101 AGGAGAAAAT AAGCATGGTC AGATCATCTT CTCGAACCCC GAGTGACAAG
Val ArgSerSerS erArgThrPr oSerAspLys
1 11
151 CCTGTAGCCC ATGTTGTAGC AAACCCTCAA GCTGAGGGGC AGCTCCAGTG
ProValAlaH isValValAl aAsnProGln AlaGluGlyG lnLeuGlnTr
21
BglI
201 GTCCAACCGC TGGGCCAATG CCCTCCTGGC
pSerAsnArg TrpAlaAsnA laLeu
31 36

Figure 7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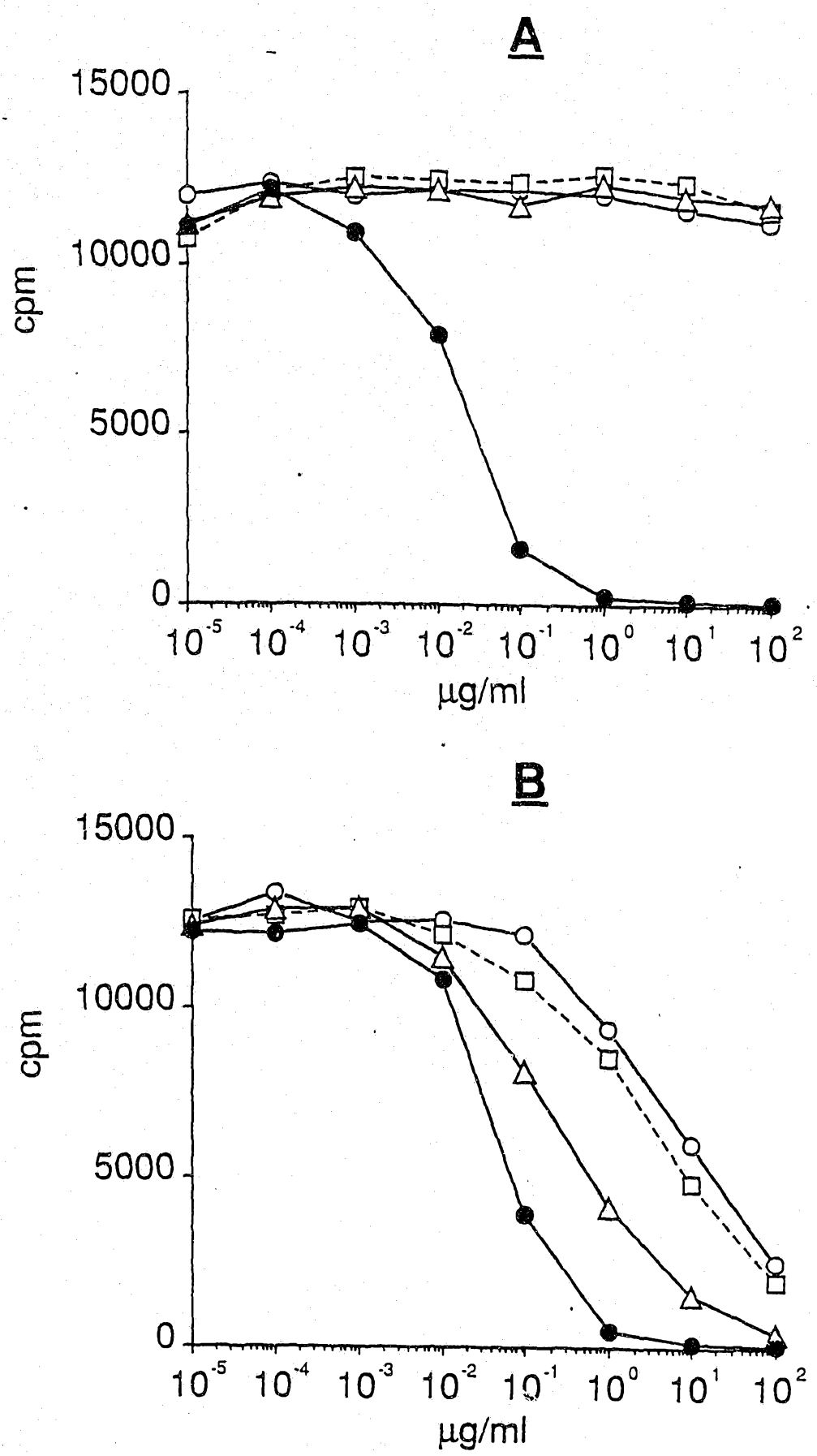
15000
10000
5000
0
cpm
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}
µg/ml
B
25000
20000
15000
10000
5000
0
cpm
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}
µg/ml

Figure 8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5000
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10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}
µg/ml
B
15000
10000
5000
0
cpm
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}
µg/ml