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(57) **Abrégé/Abstract:**

Novel analogs of proteins of KGF are provided comprising a charge-change by the deletion or substitution of one or more of amino acid residues 41-154 of Figure 2 (amino acids 72-185 of SEQ ID NO:2). These analogs are more stable than the corresponding parent molecule KGF.



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(54) Title: **KERATINOCYTE GROWTH FACTOR ANALOGS**

(57) Abstract

Novel analogs of proteins of KGF are provided comprising a charge-change by the deletion or substitution of one or more of amino acid residues 41-154 (amino acids 72-185 of SEQ ID NO:2). These analogs are more stable than the corresponding parent molecule KGF.

KERATINOCYTE GROWTH FACTOR ANALOGS

Field of the Invention

5 The present invention relates to recombinant
DNA technology and protein engineering. Specifically,
recombinant DNA methodologies have been applied to
generate polypeptide analogs of keratinocyte growth
factor (KGF), a potent mitogen of non-fibroblast
10 epithelial cell growth, wherein the analogs have
improved stability as compared to that of the parent
KGF.

Background

15 The complex process of tissue generation and
regeneration is mediated by a number of protein factors
sometimes referred to as soft tissue growth factors.
These molecules are generally released by one cell
20 type and act to influence proliferation of other cell
types. (Rubin *et al.* (1989), *Proc. Nat'l. Acad. Sci.*
USA, 86:802-806). Some soft tissue growth factors are
secreted by particular cell types and influence the
proliferation, differentiation and/or maturation of
25 responsive cells in the development of multicellular
organisms (Finch *et al.* (1989), *Science*, 245:752-755).
In addition to their roles in developing organisms, some
are significant in the continued health and maintenance
of more mature systems. For instance, in mammals there
30 are many systems where rapid cell turnover occurs. Such
systems include the skin and the gastrointestinal tract,
both of which are comprised of epithelial cells.
Included within this group of soft tissue growth factors
is a protein family of fibroblast growth factors (FGFs).

35 There are currently eight known FGF family
members which share a relatedness among primary

structures: basic fibroblast growth factor, bFGF
(Abraham et al. (1986), *EMBO J.*, 5:2523-2528); acidic
fibroblast growth factor, aFGF (Jaye et al. (1986),
Science, 233:541-545); int-2 gene product, int-2
5 (Dickson & Peters (1987), *Nature*, 326:833); hst/kFGF
(Delli-Bovi et al. (1987), *Cell*, 50:729-737 and
Yoshida et al. (1987), *Proc. Natl. Acad. Sci. USA*,
84:7305-7309); FGF-5 (Zhan et al. (1988), *Mol. Cell.*
Biol., 8:3487-3495); FGF-6 (Marics et al. (1989),
10 *Oncogene*, 4:335-340); keratinocyte growth factor (Finch
et al. (1989), *Science*, 24:752-755) and hisactophilin
(Habazzettl et al. (1992), *Nature*, 359:855-858).

Among the FGF family of proteins, keratinocyte
growth factor ("KGF") is a unique effector of non-
15 fibroblast epithelial (particularly keratinocyte) cell
proliferation derived from mesenchymal tissues. The
term "native KGF" refers to a natural human (hKGF) or
recombinant (rKGF) polypeptide (with or without a signal
sequence) as depicted by the amino acid sequence
20 presented in SEQ ID NO:2 or an allelic variant thereof.
[Unless otherwise indicated, amino acid numbering for
molecules described herein shall correspond to that
presented for the mature form of the native molecule
(i.e., minus the signal sequence), as depicted by amino
25 acids 32 to 194 of SEQ ID NO:2.]

Native KGF may be isolated from natural human
sources (hKGF) or produced by recombinant DNA techniques
(rKGF) (Finch et al. (1989), *supra*; Rubin et al. (1989),
supra; Ron et al. (1993), *The Journal of Biological*
30 *Chemistry*, 268(4):2984-2988; and Yan et al. (1991), *In*
Vitro Cell. Dev. Biol., 27A:437-438).

It is known that native KGF is relatively
unstable in the aqueous state and that it undergoes
chemical and physical degradation resulting in a loss of
35 biological activity during processing and storage (Chen
et al. (1994), *Pharmaceutical Research*, 11:1582-1589).

Native KGF is prone also to aggregation at elevated temperatures and it becomes inactivated under acidic conditions (Rubin et al. (1989), *Proc. Natl. Acad. Sci. USA*, 86:802-806). Aggregation of native KGF in aqueous solution also results in inactivated protein. This is disadvantageous because such loss of activity makes it impractical to store aqueous formulations of native KGF proteins for extended periods of time or to administer the protein over extended periods. Moreover, this is particularly problematic when preparing pharmaceutical formulations, because aggregated proteins have been known to be immunogenic (Cleland et al. (1993), *Crit. Rev. Therapeutic Drug Carrier Systems*, 10:307-377; Robbins et al. (1987), *Diabetes*, 36:838-845; and Pinckard et al. (1967), *Clin. Exp. Immunol.*, 2:331-340).

Recombinant DNA technology has been utilized to modify the sequences of various FGF family members. For example, bFGF and aFGF have been modified by deleting or substituting positively-charged residues, which are important for heparin binding with neutral or negatively-charged amino acids. It was reported that the modified molecules resulted in reduced heparin binding activity. Accordingly, it was taught that the amount of modified molecule sequestered by heparin and/or heparin-like molecules in a patient would be reduced, thereby increasing potency as more of the FGF will reach its targeted receptor (EP 0 298 723).

In order to improve or otherwise alter one or more of the characteristics of native KGF, protein engineering may be employed. Ron et al. (1993), *J. Biol. Chem.*, 268(4):2984-2988 reported modified KGF polypeptides having 3, 8, 27, 38 or 49 amino acids deleted from the N-terminus. Those polypeptides missing 3, 8, or 27 N-terminal residues retained heparin binding ability; the others did not. Also, the polypeptides missing 3 and 8 residues were reported as being fully

active, whereas the form missing 27 residues was 10-20 fold less mitogenic, and the forms lacking 38 or 49 amino acids did not have mitogenic activity. The stability of the modified KGF polypeptides was not
5 discussed or otherwise reported.

Published PCT application no. 90/08771, *supra*, also reported the production of a chimeric protein wherein about the first 40 N-terminal amino acids of mature form of native KGF were combined with the C-
10 terminal portion (about 140 amino acids) of aFGF. The chimera was reported to target keratinocytes like KGF, but it lacked susceptibility to heparin, a characteristic of aFGF but not KGF. The stability of the chimera was not discussed or otherwise reported.

15 Thus, the literature has not reported a modified KGF molecule having significantly improved stability relative to native KGF. Moreover, the literature has not reported sufficient teachings or evidence to provide a reasonable expectation of
20 successfully generating KGF molecules with such desirable characteristics.

It is not currently possible to predict the characteristics of a protein based upon the knowledge of only its primary structure. For example, the mitogenic
25 activity of aFGF is substantially increased in the presence of heparin, but the mitogenic activity of bFGF in the presence of heparin is only minimally increased, despite the fact that heparin tightly binds to bFGF [(Burgess and Maciag (1989), *Annu. Rev. Biochem.*,
30 58:575-606; Schreiber, et al. (1985), *Proc Natl. Acad. Sci. USA*, 82:6138-6142; and Gospodarowicz and Cheng (1986), *J. Cell Physiol.*, 128:475-485); and PCT 90/00418)]. In contrast, thymidine incorporation by BALB/MK cells is inhibited when heparin is included with
35 KGF in the culture medium.

Generally, the effects upon biological activity of any amino acid change upon the protein will vary depending upon a number of factors, including the three-dimensional structure of the protein and whether
5 or not the modification is to either the heparin binding region or the receptor binding region on the primary sequence of the protein. As neither the three-dimensional structure nor the heparin binding region and the receptor binding region on the primary sequence of
10 native KGF has been published, the knowledge within the art does not permit generalization about the effects of amino acid modifications to native KGF based upon the effects of amino acid modifications on even commonly categorized proteins.

15 It is the object of this invention to provide polypeptide analogs of KGF and nucleic acid molecules encoding such analogs that exhibit enhanced stability (e.g., when subjected to typical pH, thermal and/or other storage conditions) as compared to native KGF.

20

Summary of the Invention

The present invention provides novel, biologically active polypeptide analogs of KGF. For
25 purposes of this invention, the term "KGF" includes native KGF and proteins characterized by a peptide sequence substantially the same as the peptide sequence of native KGF which retain some or all of the biological activity of native KGF, particularly non-fibroblast
30 epithelial cell proliferation.

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According to the invention, there is provided a polypeptide analog of native keratinocyte growth factor (which corresponds to amino acid residues 32-194 of SEQ ID NO:2),
5 termed "KGF", wherein the analog comprises the amino acid sequence of KGF having a charge-change by the deletion or substitution of one or more of amino acid residues 41-154 (amino acid residues 72-185 of SEQ ID NO:2), optionally further having an N-terminal methionine or a signal sequence.

10 By "characterized by a peptide sequence substantially the same as the peptide sequence of native KGF" is meant a peptide sequence which retains residues corresponding to Arg⁴¹, Gln⁴³, Lys⁵⁵, Lys⁹⁵, Asn¹³⁷, Gln¹³⁸, Lys¹³⁹,
15 Arg¹⁴⁴, Lys¹⁴⁷, Gln¹⁵², Lys¹⁵³ and Thr¹⁵⁴ and which is encoded by a DNA sequence capable of hybridizing to

nucleotides 201 to 684 of SEQ. ID. NO:1, preferably under stringent hybridization conditions.

The determination of a corresponding amino acid position between two amino acid sequences may be determined by aligning the two sequences to maximize matches of residues including shifting the amino and/or carboxyl terminus, introducing gaps as required and/or deleting residues present as inserts in the candidate. Database searches, sequence analysis and manipulations may be performed using one of the well-known and routinely used sequence homology/identity scanning algorithm programs (e.g., Pearson and Lipman (1988), *Proc. Natl. Acad. Sci. U.S.A.*, 85:2444-2448; Altschul et al. (1990), *J. Mol. Biol.*, 215:403-410; Lipman and Pearson (1985), *Science*, 222:1435 or Devereux et al. (1984), *Nuc. Acids Res.*, 12:387-395).

Stringent conditions, in the hybridization context, will be stringent combined conditions of salt, temperature, organic solvents and other parameters typically controlled in hybridization reactions. Exemplary stringent hybridization conditions are hybridization in 4 X SSC at 62-67° C., followed by washing in 0.1 X SSC at 62-67° C. for approximately an hour. Alternatively, exemplary stringent hybridization conditions are hybridization in 45-55% formamide, 4 X SSC at 40-45°C. [See, T. Maniatis et. al., *Molecular Cloning* (A Laboratory Manual); Cold Spring Harbor Laboratory (1982), pages 387 to 389].

Thus, the proteins include allelic variations, or deletion(s), substitution(s) or insertion(s) of amino acids, including fragments, chimeric or hybrid molecules of native KGF. One example of KGF includes proteins having residues corresponding to Cys¹ and Cys¹⁵

replaced or deleted, with the resultant molecule having improved stability as compared with the parent molecule (as taught in commonly owned International

application Publication WO 96/11949). Specifically disclosed molecules include: C(1,15)S, a KGF having substitutions of serine for cysteine at amino acid positions 1 and 15; Δ N15- Δ N24, KGFs having a deletion of
5 any one of from the first 15 to 24 amino acids of the N-terminus of native KGF; Δ N3/C(15)S, a KGF having a deletion of the first 3 amino acids of the N-terminus of native KGF and a substitution of serine for cysteine at amino acid position 15; Δ N3/C(15)-, a KGF having a
10 deletion of the first 3 amino acids of the N-terminus of native KGF and a deletion of cysteine at amino acid position 15; Δ N8/C(15)S, a KGF having a deletion of the first 8 amino acids of the N-terminus of native KGF and a substitution of serine for cysteine at amino acid
15 position 15; Δ N8/C(15)-, a KGF having a deletion of the first 8 amino acids of the N-terminus of native KGF and a deletion of cysteine at amino acid position 15; C(1,15,40)S, a KGF having a substitution of serine for cysteine at amino acid positions 1, 15 and 40;
20 C(1,15,102)S, a KGF having a substitution of serine for cysteine at amino acid positions 1, 15 and 102; and C(1,15,102,106)S, a KGF having a substitution of serine for cysteine at amino acid positions 1, 15, 102 and 106.

Another example of KGF includes proteins
25 generated by substituting at least one amino acid having a higher loop-forming potential for at least one amino acid within a loop-forming region of Asn¹¹⁵-His¹¹⁶-Tyr¹¹⁷-Asn¹¹⁸-Thr¹¹⁹ of native KGF

30 specifically including H(116)G, a KGF having a substitution of glycine for histidine at amino acid position 116 of native KGF.

A still further example includes proteins having one or more amino acid substitutions, deletions
35 or additions within a region of 123-133 (amino acids

154-164 of SEQ ID NO:2) of native KGF; these proteins may have agonistic or antagonistic activity.

Surprisingly, it has been discovered that by replacing the more positively charged residues with
5 neutral or negatively charged residues (i.e., substituting positively charged residues for neutral or negatively charged residues, or neutral residues for positively charged residues) of a KGF molecule (i.e., parent molecule), the resultant KGF analog has improved
10 stability as compared to the parent molecule. Preferably, in addition to having increased stability, the invention is directed to those analogs which also exhibit full biological activity (i.e., at least substantially similar receptor binding or affinity) as
15 compared to native KGF.

In another aspect of the invention, purified and isolated nucleic acid molecules encoding the various biologically active polypeptide analogs of KGF are described. In one embodiment, such nucleic acids
20 comprise DNA molecules cloned into biologically functional plasmid or viral vectors. In another embodiment, nucleic acid constructs may then be utilized to stably transform a procaryotic or eucaryotic host cell. In still another embodiment, the invention
25 involves a process wherein either a procaryotic (preferably *E. coli*) or eucaryotic host cell stably transformed with a nucleic acid molecule is grown under suitable nutrient conditions in a manner allowing the expression of the KGF analog. Following expression, the
30 resultant recombinant polypeptide can be isolated and purified.

A further aspect of the invention concerns pharmaceutical formulations comprising a therapeutically effective amount of a KGF analog and an
35 acceptable pharmaceutical carrier. Such formulations

will be useful in treating patients afflicted with epithelial diseases and injuries.

In this vein, another aspect relates to methods of stimulating epithelial cell growth by administering to a patient a therapeutically effective amount of a KGF analog. In one embodiment, non-fibroblast epithelial cells are the cells whose proliferation is stimulated. Such epithelial cells include various adnexal cells, pancreatic cells, liver cells, and mucosal epithelium in the respiratory and gastrointestinal tracts.

Brief Description of the Figures

Figure 1 shows the nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of native KGF (the nucleotides encoding the mature form of native KGF are depicted by bases 201 to 684 of SEQ ID NO:1 and the mature form of KGF is depicted by amino acid residues 32 to 194 of SEQ ID NO:2).

Figures 2A, 2B and 2C show the plasmid maps of pCFM1156, pCFM1656 and pCFM3102, respectively.

Figure 3 shows the nucleotide (SEQ ID NO:3) and amino acid (SEQ ID NO:4) sequences of the construct RSH-KGF.

Figure 4 shows the nucleotide (SEQ ID NO:5) and amino acid (SEQ ID NO:6) sequences of the construct contained in plasmid KGF.

Figure 5 shows the chemically synthesized OLIGOs (OLIGO#6 through OLIGO#11; SEQ ID NO:12-17, respectively) used to substitute the DNA sequence between a *KpnI* site and an *EcoRI* site (from amino acid positions 46 to 85 of SEQ ID No:6) in the construct contained in plasmid KGF to produce the construct in plasmid KGF(dsd).

Figure 6 shows the chemically synthesized OLIGOs (OLIGO#12 through OLIGO#24; SEQ ID NO:18-30, respectively) used to construct KGF (codon optimized).

Figure 7 shows the nucleotide (SEQ ID NO:31) and amino acid sequences (SEQ ID NO:32) of R(144)Q, a KGF analog having a substitution of glutamine for arginine at amino acid position 144 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

Figure 8 shows the nucleotide (SEQ ID NO:33) and amino acid sequences (SEQ ID NO:34) of C(1,15)S/R(144)E, a KGF analog having substitutions of serine for cysteine at amino acid positions 1 and 15 and a substitution of glutamic acid for arginine at amino acid position 144 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

Figure 9 shows the nucleotide (SEQ ID NO:35) and amino acid (SEQ ID NO:36) sequences of C(1,15)S/R(144)Q, a KGF analog having substitutions of serine for cysteine at amino acid positions 1 and 15 and a substitution of glutamine for arginine at amino acid position 144 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

Figure 10 shows the nucleotide (SEQ ID NO:37) and amino acid (SEQ ID NO:38) sequences of Δ N23/R(144)Q, a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of glutamine for arginine at amino acid position 144 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

Figure 11 shows the amount of soluble protein, determined by size exclusion HPLC, as a function of incubation time at 37°C.

Figure 12 shows the estimated melting temperature (T_m) as a function of pH for native KGF, C(1,15)S, C(1,15)S/R(144)Q and C(1,15)S/R(144)E.

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Figure 13 shows a typical profile of mitogenic activity of R(144)Q, determined by measuring the incorporation of [³H]-Thymidine during DNA synthesis and by comparing it to a native KGF standard curve.

5 Figure 14 shows a typical profile of the mitogenic activity of ΔN23/R(144)Q, determined by measuring the incorporation of [³H]-Thymidine during DNA synthesis and by comparing it to a native KGF standard curve.

10 Figure 15 shows a typical profile of the mitogenic activity of C(1,15)S/R(144)Q, determined by measuring the incorporation of [³H]-Thymidine during DNA synthesis and by comparing it to a native KGF standard curve.

15 Figure 16 shows a typical profile of the mitogenic activity of C(1,15)S/R(144)E, determined by measuring the incorporation of [³H]-Thymidine during DNA synthesis and by comparing it to a native KGF standard curve.

20 Figure 17 shows the nucleotide (SEQ ID NO:41) and amino acid (SEQ ID NO:42) sequences of ΔN23/N(137)E, a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of glutamic acid for asparagine at amino acid position 137 of native
25 KGF wherein the initial methionine in the sequence should be considered residue number "0".

30 Figure 18 shows the nucleotide (SEQ ID NO:43) and amino acid (SEQ ID NO:44) sequences of ΔN23/K(139)E, a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of glutamic acid for lysine at amino acid position 139 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

35

Figure 19 shows the nucleotide (SEQ ID NO:45) and amino acid (SEQ ID NO:46) sequences of Δ N23/K(139)Q, a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of glutamine for lysine at amino acid position 139 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

Figure 20 shows the nucleotide (SEQ ID NO:47) and amino acid (SEQ ID NO:48) sequences of Δ N23/R(144)A, a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of alanine for arginine at amino acid position 144 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

Figure 21 shows the nucleotide (SEQ ID NO:49) and amino acid (SEQ ID NO:50) sequences of Δ N23/R(144)L, a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of leucine for arginine at amino acid position 144 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

Figure 22 shows the nucleotide (SEQ ID NO:51) and amino acid (SEQ ID NO:52) sequences of Δ N23/K(147)E, a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of glutamic acid for lysine at amino acid position 147 of native KGF wherein the initial methionine in the sequence should be considered residue number "0".

Figure 23 shows the nucleotide (SEQ ID NO:53) and amino acid (SEQ ID NO:54) sequences of Δ N23/K(147)Q, a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of glutamine for lysine at amino acid position 147 of native KGF

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wherein the initial methionine in the sequence should be considered residue number "0".

5 Figure 24 shows the nucleotide (SEQ ID NO:55)
and amino acid (SEQ ID NO:56) sequences of Δ N23/K(153)E,
a KGF analog having a deletion of the first 23 amino
acids of the N-terminus and a substitution of glutamic
acid for lysine at amino acid position 153 of native
KGF wherein the initial methionine in the sequence should be
10 considered residue number "0".

 Figure 25 shows the nucleotide (SEQ ID NO:57)
and amino acid (SEQ ID NO:58) sequences of Δ N23/K(153)Q,
a KGF analog having a deletion of the first 23 amino
acids of the N-terminus and a substitution of glutamine
15 for lysine at amino acid position 153 of native KGF.
wherein the initial methionine in the sequence should be
considered residue number "0".

 Figure 26 shows the nucleotide (SEQ ID NO:59)
and amino acid (SEQ ID NO:60) sequences of
20 Δ N23/Q(152)E/K(153)E, a KGF analog having a deletion of
the first 23 amino acids of the N-terminus and a
substitution of glutamic acid for glutamine at amino
acid position 152 of native KGF and glutamic acid for
lysine at amino acid position 153 of native KGF
25 wherein the initial methionine in the sequence should be
considered residue number "0".

30

35

Detailed Description

In accordance with the present invention, novel analogs of KGF are provided. The KGF analogs are produced by deleting or substituting one or more specific, positively-charged residues in KGF.

The KGF analogs have, among other properties, an improved stability under at least one of a variety of purification and/or storage conditions. For example, the KGF analogs will generally be purified in a greater yield of soluble, correctly folded protein. Moreover, once the material is purified, it will be more stable to pH, temperature, etc. as compared to the stability of the parent molecule. As described in the Examples section below (modified by the substitution of Gln and Glu for arginine at position 144 [R(144)Q and R(144)E, respectively] and in some instances modified at the N-terminus as well) exhibit, relative to native KGF, (1) a 35 to 37.2 day increase of half-life upon storage at 37°C, (2) a 7.5-9.5% higher thermal melting temperatures over the course of thermal unfolding, and (3) an increase in T_m over a range of pH values.

Although not intended to be bound by theory, a possible reason for the enhanced stability of the R(144)Q and R(144)E may be due to a reduction in overall charge density of a cluster of basic residues, which is inherently unstable due to charge repulsion, in the absence of heparin. The results set forth below suggest that the arginine residue at position 144 may correspond to a residue in bFGF, as determined by X-ray crystallography, which is reported to be within or near a cluster of basic residues that mediate heparin binding (Ago, et al. (1991), *J. Biochem.*, 110:360-363; and Eriksson et al. (1993), *Protein Science*, 2:1274-1284).

Native KGF contains 46 charged residues, 27 of which carry a positive charge. In view of the results

obtained with the KGF analogs, a comparison of the native KGF primary sequence with the primary sequence of bFGF suggests that some of the 27 positively charged residues form a cluster similar to a cluster found in the tertiary structure of bFGF. Depending on the location of such residues in the protein's three-dimensional structure, substitution of one or more of these clustered residues with amino acids carrying a negative or neutral charge may alter the electrostatic interactions of adjacent residues and may be useful to achieve increased stability.

Thus other analogs, in addition to the preferred R(144)Q specifically set forth herein, are contemplated by the present invention. As used in this invention, a "KGF analog" or a "polypeptide analog of KGF" shall mean charge-change polypeptides wherein one or more of amino acid residues 41-154 (amino acids 72-185 of SEQ ID NO:2), specifically including amino acid residues 123-133 (amino acids 154-164 of SEQ ID NO:2), are deleted or substituted with a neutral residue or negatively charged residue selected to effect a protein with a reduced positive charge. Preferred residues for modification are Arg⁴¹, Gln⁴³, Lys⁵⁵, Lys⁹⁵, Lys¹²⁸, Asn¹³⁷, Gln¹³⁸, Lys¹³⁹, Arg¹⁴⁴, Lys¹⁴⁷, Gln¹⁵², Lys¹⁵³ or Thr¹⁵⁴, with Gln¹³⁸, Lys¹³⁹, Arg¹⁴⁴, Lys¹⁴⁷, Gln¹⁵² or Lys¹⁵³ being more preferred and Arg¹⁴⁴ being most preferred. Preferred amino acids for substitution include glutamic acid, aspartic acid, glutamine, asparagine, glycine, alanine, valine, leucine, isoleucine, serine and threonine, with glutamic acid, glutamine, aspartic acid, asparagine and with alanine being particularly preferred.

Any modification should give consideration to minimizing charge repulsion in the tertiary structure of the molecule; most preferably the analog will have increased stability compared with the parent molecule.

Obviously, the deletions or substitutions should not be so numerous nor be made to residues of such close proximity so as to set up charge repulsion between two negatively-charged residues.

5 When the KGF analogs are biologically generated, i.e., are the products of cellular expression as opposed to the products of solid state synthesis, proteolytic or enzymatic derivatization of naturally-
10 such polypeptides will differ in one or more nucleotides as compared to the native KGF nucleotide sequence. Such polynucleotides may be expressed and the resultant polypeptide purified by any one of a number of
15 recombinant technology methods known to those skilled in the art.

 DNA sequences coding for all or part of the KGF analogs may include, among other things, the incorporation of codons "preferred" for expression in selected host cells (e.g., "*E. coli* expression codons");
20 the provision of sites for cleavage by restriction enzymes; and the provision of additional initial, terminal, or intermediate nucleotide sequences (e.g., as an initial methionine amino acid residue for expression in *E. coli* cells), to facilitate construction of readily
25 expressed vectors.

 The present invention also provides for recombinant molecules or vectors for use in the method of expression of the polypeptides. Such vectors may be comprised of DNA or RNA and can be circular, linear,
30 single-stranded or double-stranded in nature and can be naturally-occurring or assemblages of a variety of components, be they naturally-occurring or synthetic.

 Many examples of such expression vectors are known. The components of the vectors, e.g. replicons,
35 selection genes, enhancers, promoters, and the like, may be obtained from natural sources or synthesized by known

procedures. In each case, expression vectors useful in this invention will contain at least one expression control element functionally associated with the inserted nucleic acid molecule encoding the KGF polypeptide analog. This control element is responsible for regulating polypeptide expression from the nucleic acid molecules of the invention. Useful control elements include, for example, the *lac* system, the *trp* system, the operators and promoters from phage λ , a glycolytic yeast promoter, a promoter from the yeast acid phosphatase gene, a yeast alpha-mating factor, and promoters derived from adenovirus, Epstein-Barr virus, polyoma, and simian virus, as well as those from various retroviruses. However, numerous other vectors and control elements suitable for procaryotic or eucaryotic expression are known in the art and may be employed in the practice of this invention.

Examples of suitable procaryotic cloning vectors may include plasmids from *E. coli* (e.g. pBR322, col E1, pUC, and the F-factor), with preferred plasmids being pCFM1156 (ATCC 69702), pCFM1656 (ATCC 69576) and pCFM3102 (described in the Examples section, below). Other appropriate expression vectors of which numerous types are known in the art for mammalian, insect, yeast, fungal and bacterial expression can also be used for this purpose. The transfection of these vectors into appropriate host cells can result in expression of the KGF analog polypeptides.

Host microorganisms useful in this invention may be either procaryotic or eucaryotic. Suitable procaryotic hosts include various *E. coli* (e.g., FM5, HB101, DH5 α , DH10, and MC1061), *Pseudomonas*, *Bacillus*, and *Streptomyces* strains, with *E. coli* being preferred. Suitable eucaryotic host cells include yeast and other fungi, insect cells, plant cells, and animal cells, such as COS (e.g., COS-1 and COS-7) and CV-1 monkey cell

lines, 3T3 lines derived from Swiss, Balb-c or NIH cells, HeLa and L-929 mouse cells, and CHO, BHK or HaK hamster cells. Depending upon the host employed, recombinant polypeptides produced in accordance herewith
5 will be glycosylated with mammalian or other eucaryotic carbohydrates or may be non-glycosylated.

The preferred production method will vary depending upon many factors and considerations; the optimum production procedure for a given situation will
10 be apparent to those skilled in the art through minimal experimentation. The resulting expression product may then be purified to near homogeneity using procedures known in the art. A typical purification procedure for procaryotic cell production involves rupturing the cell
15 walls by high pressure or other means, centrifugation or filtration to remove cellular debris, followed by ion exchange chromatography of supernatant or filtrate and, finally, hydrophobic interaction chromatography. If the analog is expressed in insoluble form, another
20 purification technique involves first solublizing the inclusion bodies containing the analogs followed by ion exchange chromatography, then refolding of the protein, and, finally, hydrophobic interaction chromatography. Exemplary purification techniques are taught in commonly
25 owned U.S. Patent 6,008,328. Generally, U.S. Patent 6,008,328 teaches a method for purifying a keratinocyte growth factor comprising: (a) obtaining a solution comprising the KGF; (b) binding the KGF from the solution of part (a) to a cation exchange
30 resin; (c) eluting the KGF in an eluate solution from the cation exchange resin; (d) either passing the eluate solution from part (c) through an appropriate molecular weight exclusion matrix or performing hydrophobic interaction chromatography on the eluate solution of
35 part (c); and (e) recovering the KGF from the molecular

weight exclusion matrix or hydrophobic interaction chromatography.

Of course, the analogs may be rapidly screened to assess their physical properties. The Examples sets forth various well-known stability assays, although the specific assay used to test the analog is not critical. Moreover, the level of biological activity (e.g., receptor binding and/or affinity, mitogenic, cell proliferative and/or *in vivo* activity) may also be tested using a variety of assays, some of which are set forth in the Examples section. Numerous assays are well-known and can be used to quickly screen the KGF analogs to determine whether or not they possess acceptable biological activity. One such assay specifically tests the KGF analogs for the ability to bind to the KGF receptor (KGFR) by competing with ¹²⁵I-KGF binding (Bottaro *et al.* (1990), *J. Biol. Chem.*, 265:12767-12770; Ron *et al.* (1993), *J. Biol. Chem.*, 268:2984-2988). An alternative method for assaying KGFR/KGF analog interactions involves the use of techniques such as real time biospecific interaction analysis (BIA) (Felder *et al.* (1993), *Molecular & Cellular Biology*, 13:1449-1455). Additionally a mitogenic assay can be utilized to test the ability of the KGF analogs to stimulate DNA synthesis (Rubin *et al.* (1989), *supra*). Finally, cell proliferative assays can be utilized to test the ability of the KGF analogs to stimulate cell proliferation (Falco, *et al.* (1988), *Oncogene*, 2:573-578). Using any of the aforementioned assay systems, KGF analogs can be rapidly screened for their biological activity.

The KGF analogs may be further modified to contain additional chemical moieties not normally a part of the peptide. Such derivatized moieties may improve the solubility, absorption, biological half life, and the like of the KGF analog. The moieties may

alternatively eliminate or attenuate any undesirable side effects of the protein and the like. Moieties capable of mediating such effects are disclosed, for example, in *REMINGTON'S PHARMACEUTICAL SCIENCES*, 18th ed., Mack Publishing Co., Easton, PA (1990). Covalent modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues (T.E. Creighton (1983), *PROTEINS: STRUCTURE AND MOLECULE PROPERTIES*, W.H. Freeman & Co., San Francisco, pp. 79-86). Polyethylene glycol ("PEG") is one such chemical moiety which has been used in the preparation of therapeutic protein products. For some proteins, the attachment of polyethylene glycol has been shown to protect against proteolysis, Sada, et al. (1991), *J. Fermentation Bioengineering*, 71:137-139, and methods for attachment of certain polyethylene glycol moieties are available. See U.S. Patent No. 4,179,337, Davis et al., "Non-Immunogenic Polypeptides," issued December 18, 1979; and U.S. Patent No. 4,002,531, Royer, "Modifying enzymes with Polyethylene Glycol and Product Produced Thereby," issued January 11, 1977. For a review, see Abuchowski et al., in *Enzymes as Drugs*. (Holcerberg and Roberts, (eds.) pp. 367-383 (1981)). For polyethylene glycol, a variety of means have been used to attach the polyethylene glycol molecules to the protein. Generally, polyethylene glycol molecules are connected to the protein via a reactive group found on the protein. Amino groups, such as those on lysine residues or at the N-terminus, are convenient for such attachment. For example, Royer (U.S. Pat. No. 4,002,531, above) states that reductive alkylation was used for attachment of polyethylene glycol molecules to an enzyme. EP 0 539 167, published April 28, 1993, Wright, "PEG Imidates and Protein Derivates Thereof"

states that peptides and organic compounds with free amino group(s) are modified with an imidate derivative of PEG or related water-soluble organic polymers. U.S. Patent No. 4,904,584, Shaw, issued February 27, 1990, relates to the modification of the number of lysine residues in proteins for the attachment of polyethylene glycol molecules via reactive amine groups.

In yet another embodiment, the present invention is directed to a single-dose administration unit of a medicinal formulation which can be safely administered parenterally or orally to treat a disease in a warm-blooded animal (such as a human). Such medicinal formulation may be in the form of a lyophilized or otherwise dehydrated therapeutic or diagnostic which can be reconstituted by the addition of a physiologically acceptable solvent. The solvent may be any media such as sterile water, physiological saline solution, glucose solution or other aqueous carbohydrates (e.g., polyols such as mannitol, xylitol, glycerol) which is capable of dissolving the dried composition, is compatible with the selected administration route and which does not negatively interfere with the active principle and the reconstitution stabilizers employed. In a specific embodiment, the present invention is directed to a kit for producing the single-dose administration unit. The kit contains both a first container having a dried protein and a second container having an aqueous formulation comprising a reconstitution stabilizer. As for the concentration of the protein in the solution, the solution volume which is charged into each container, and the capacity of the containers (interrelated parameters which can be suitably modified, depending upon the desired concentration of active principle in the end-dosage unit), these may vary within wide ranges well-known to skilled artisans.

KGF analogs according to the invention may be useful as therapeutic and diagnostic agents and as research reagents. Thus the KGF analogs may be used in *in vitro* and/or *in vivo* diagnostic assays to quantify the amount of KGF in a tissue or organ sample or to determine and/or isolate cells which express KGFR (Bottaro et al. (1990), *J. Biol. Chem.*, 265:12767-12770; Ron et al. (1993), *J. Biol. Chem.*, 268:2984-2988). In assays of tissues or organs there will be less radioactivity from ^{125}I -KGF analog binding to KGFR, as compared to a standardized binding curve of ^{125}I -KGF analog, due to unlabeled native KGF binding to KGFR. Similarly, the use of ^{125}I -KGF analog may be used to detect the presence of KGFR in various cell types.

This invention also contemplates the use of a KGF analog in the generation of antibodies made against the peptide, which antibodies also bind to native KGF. In this embodiment, the antibodies are monoclonal or polyclonal in origin and are generated using a KGF analog. The resulting antibodies bind preferentially to native KGF, preferably when that protein is in its native (biologically active) conformation. These antibodies can be used for detection or purification of the KGF.

Moreover, the invention contemplates the use of KGF analogs in the discovery of high affinity or low affinity KGF binding molecules having therapeutical applications, for example, as a way for efficient KGF delivery or as an inhibitor for KGF activity. The thermal stability of the KGF analogs is important to identify such binding molecules in physiological conditions (i.e., at 37°C) since their affinity for KGF could be strongly temperature-dependent and may be unpredictable from the affinity observed at 4°C.

For *in vivo* uses, the KGF analogs may be formulated with additives. Such additives include

5 buffers, carriers, stabilizers, excipients, preservatives, tonicity adjusting agents, anti-oxidants and the like (e.g., viscosity adjusting agents or extenders). The selection of specific additives will depend upon the storage form (i.e., liquid or lyophilized) and the modes of administering the KGF analog. Suitable formulations, known in the art, can be found in *REMBINGTON'S PHARMACEUTICAL SCIENCES* (latest edition), Mack Publishing Company, Easton, PA.

10 The KGF analogs may be applied in therapeutically effective amounts to tissues specifically characterized by having damage to or clinically insufficient numbers of non-fibroblast epithelium cells. Since KGF binds to heparin, it is likely that heparin, heparin sulfate, heparin-like glycosaminoglycans and heparin-like glycosaminoglycans, which are present in the extracellular environment may bind KGF *in vivo*. It follows that KGF analogs with reduced heparin binding ability will have enhanced potency, as more KGF will reach its targeted receptor and will not be sequestered by heparin and heparin-like compounds in the extracellular environment. These analogs will be more useful therapeutically, as lower dosages of a particular KGF analog will be required per treatment.

25 The KGF analogs may be applied in therapeutically effective amounts to tissues specifically characterized by having damage to or clinically insufficient numbers of non-fibroblast epithelium cells. Areas in which KGF analogs may be successfully administered include, but are not limited to: the stimulation, proliferation and differentiation of adnexal structures such as hair follicles, sweat glands, and sebaceous glands in patients with burns and other partial and full-thickness injuries; accelerated reepithelialization of lesions caused by epidermolysis

bullosa, which is a defect in adherence of the epidermis to the underlying dermis, resulting in frequent open, painful blisters which can cause severe morbidity; preventing chemotherapy-induced alopecia and treating male-pattern baldness, or the progressive loss of hair in men and women; treating gastric and duodenal ulcers; treating inflammatory bowel diseases, such as Crohn's disease (affecting primarily the small intestine) and ulcerative colitis (affecting primarily the large bowel); preventing or reducing gut toxicity in radiation and chemotherapy treatment regimes through treatment (e.g., pretreatment and/or posttreatment) to induce a cytoprotective effect or regeneration or both; stimulating the production of mucus throughout the gastrointestinal tract; inducing the proliferation and differentiation of type II pneumocytes, which may help treat or prevent diseases such as hyaline membrane disease (i.e., infant respiratory distress syndrome and bronchopulmonary dysplasia) in premature infants; stimulating the proliferation and differentiation of the bronchiolar and/or alveolar epithelium with acute or chronic lung damage or insufficiency due to inhalation injuries (including high oxygen levels), emphysema, use of lung damaging chemotherapeutics, ventilator trauma or other lung damaging circumstances; increasing liver function to treat or prevent hepatic cirrhosis, fulminant liver failure, damage caused by acute viral hepatitis and/or toxic insults to the liver; inducing corneal cell regeneration, for example in the treatment of corneal abrasion; inducing epithelial cell regeneration to treat progressive gum disease; inducing regeneration of tympanic epithelial cells to treat ear drum damage and treating or preventing the onset of diabetes mellitus or as an adjunct in the setting of islet cell transplantation.

A patient in need of proliferation of non-fibroblast epithelial cells will be administered an effective amount of a KGF analog. An "effective amount" is that amount of KGF analog required to elicit the desired response in the patient being treated and will, thus, generally be determined by the attending physician. Factors influencing the amount of KGF analog administered will include the age and general condition of the patient, the disease being treated, etc. Typical dosages will range from 0.001 mg/kg body weight to 500 mg/kg body weight.

The KGF analog may be safely administered parenterally (e.g., via IV, IT, IM, SC, or IP routes), orally or topically to warm-blooded animals (such as humans). The KGF analog may be used once or administered repeatedly, depending on the disease and condition of the patient. In some cases, the KGF analog may be administered as an adjunct to other therapy and also with other pharmaceutical preparations.

The following examples are included to more fully illustrate the present invention. It is understood that modifications can be made in the procedures set forth, without departing from the spirit of the invention.

EXAMPLES

Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely recognized manuals of molecular biology such as, for example, *Molecular Cloning*, Second Edition, Sambrook et al., Cold Spring Harbor Laboratory Press (1987) and *Current Protocols in Molecular Biology*, Ausabel et al., Greene Publishing Associates/Wiley Interscience, New York (1990).

EXAMPLE 1: Preparation of DNA Coding for KGF and KGF Analogs

5 The cloning of the full-length human KGF gene (encoding a polypeptide with the sequence of native KGF) was carried out both by polymerase chain reaction (PCR) of RNA from an animal cell and by PCR of chemically synthesized (*E. coli* optimized codon) oligonucleotides ("OLIGOs"). Both procedures are described below:

10 PCR amplification using RNA isolated from cells known to produce the polypeptide was performed. Initially, cells from a human fibroblast cell line AG1523A (obtained from Human Genetic Mutant Cell Culture Repository Institute For Medical Research, Camden, New
15 Jersey) were disrupted with guanidium thiocyanate, followed by extraction (according to the method of Chomyzinski et al. (1987), *Anal. Biochem.*, 172:156). Using a standard reverse transcriptase protocol for total RNA, the KGF cDNA was generated. PCR (PCR#1)
20 amplification of the KGF gene was carried out using the KGF cDNA as template and primers OLIGO#1 and OLIGO#2 that encode DNA sequences immediately 5' and 3' of the KGF gene [Model 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, CT); 28 cycles; each cycle consisting of one
25 minute at 94°C for denaturation, two minutes at 60°C for annealing, and three minutes at 72°C for elongation]. A small aliquot of the PCR#1 product was then used as template for a second KGF PCR (PCR#2) amplification identical to the cycle conditions described above except
30 for a 50°C annealing temperature. For expression cloning of the KGF gene, nested PCR primers were used to create convenient restriction sites at both ends of the KGF gene. OLIGO#3 and OLIGO#4 were used to modify the KGF DNA product from PCR#2 to include *MluI* and *BamHI*
35 restriction sites at the 5' and 3' ends of the gene, respectively [PCR#3; 30 cycles; each cycle consisting of

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one minute at 94°C for denaturation, two minutes at 60°C for annealing, and three minutes at 72°C for elongation]. This DNA was subsequently cut with *MluI* and *BamHI*, phenol extracted, and ethanol precipitated. It was then resuspended and ligated (using T4 ligase) into a pCFM1156 plasmid (Figure 2A) that contained a "RSH" signal sequence to make construct RSH-KGF (Figure 3).

The ligation products were transformed (according to the method of Hanahan (1983), *J. Mol. Biol.*, 166:557) into *E. coli* strain FM5 (ATCC: 53911) and plated onto LB+kanamycin at 28°C. Several transformants were selected and grown in small liquid cultures containing 20 µg/mL kanamycin. The RSH-KGF plasmid was isolated from the cells of each culture and DNA sequenced. Because of an internal *NdeI* site in the KGF gene, it was not possible to directly clone the native gene sequence into the desired expression vector with the bracketed restriction sites of *NdeI* and *BamHI*. This was accomplished as a three-way ligation. Plasmid RSH-KGF was cut with the unique restriction sites of *BsmI* and *SstI*, and a ~3 kbp DNA fragment (containing the 3' end of the KGF gene) was isolated following electrophoresis through a 1% agarose gel. A PCR (PCR#4) was carried out as described for PCR#3 except for the substitution of OLIGO#5 for OLIGO#3. The PCR DNA product was then cut with *NdeI* and *BsmI* and a 311 bp DNA fragment was isolated following electrophoresis through a 4% agarose gel. The third piece of the ligation is a 1.8 kbp DNA fragment of pCFM1156 cut with *NdeI* and *SstI* which was isolated following electrophoresis through a 1% agarose gel. Following ligation (T4 ligase), transformation, kanamycin selection and DNA sequencing as described above, a clone was picked containing the construct in Figure 4 and the plasmid designated KGF. Because of an internal ribosomal binding site that

produced truncated products, the KGF DNA sequence between the unique *KpnI* and *EcoRI* sites was replaced with chemically synthesized OLIGOs (OLIGO#6 through OLIGO#11) to minimize the use of the internal start site (Figure 5).

OLIGO#1 (SEQ ID NO:7): 5'-CAATGACCTAGGAGTAACAATCAAC-3'
 OLIGO#2 (SEQ ID NO:8): 5'-AAAACAAACATAAATGCACAAGTCCA-3'
 OLIGO#3 (SEQ ID NO:9): 5'-ACAACGCGTGCAATGACATGACTCCA-3'
 10 OLIGO#4 (SEQ ID NO:10):
 5'-ACAGGATCCTATTAAGTTATTGCCATAGGAA-3'
 OLIGO#5 (SEQ ID NO:11):
 5'-ACACATATGTGCAATGACATGACTCCA-3'
 OLIGO#6 (SEQ ID NO:12):
 15 5'-CTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCC-3'
 OLIGO#7 (SEQ ID NO:13):
 5'-AAGAGATGAAAAACAACACTACAATATTATGGAAATCCGTACTGTT-3'
 OLIGO#8 (SEQ ID NO:14):
 5'-GCTGTTGGTATCGTTGCAATCAAAGGTGTTGAATCTG-3'
 20 OLIGO#9 (SEQ ID NO:15):
 5'-TCTTGGGTGCCCTTGACTTTGCCGCGTTTGTGATACGCAGGTAC-3'
 OLIGO#10 (SEQ ID NO:16):
 5'-ACAGCAACAGTACGGATTTCCATAATATTGTAGTTGTTTTTCATC-3'
 OLIGO#11 (SEQ ID NO:17):
 25 5'-AATTCAGATTCAACACCTTTGATTGCAACGATACCA-3'

The OLIGOs were phosphorylated with T4 polynucleotide kinase and then heat denatured. The single-stranded (ss) OLIGOs were then allowed to form a
 30 ds DNA fragment by allowing the temperature to slowly decrease to room temperature. T4 ligase was then used to covalently link both the internal OLIGO sticky-ends and the whole ds OLIGO fragment to the KGF plasmid cut with *KpnI* and *EcoRI*. The new plasmid was designated
 35 KGF(dsd).

A completely *E. coli* codon-optimized KGF gene was constructed by PCR amplification of chemically synthesized OLIGOs #12 through 24.

5 OLIGO#12 (SEQ ID NO:18): 5'-AGTTTTGATCTAGAAGGAGG-3'
 OLIGO#13 (SEQ ID NO:19): 5'-TCAAACTGGATCCTATTAA-3'
 OLIGO#14 (SEQ ID NO:20):
 5'-AGTTTTGATCTAGAAGGAGGAATAACATATGTGCAACGACATG-
 ACTCCGGAACAGATGGCTACCAACGTTAAGTCTCCAGCCCGGAACGT-3'
 10 OLIGO#15 (SEQ ID NO:21):
 5'-CACACCCGTAGCTACGACTACATGGAAGGTGGTGACATCCGT-
 GTTCGTCGTCTGTTCTGCCGTACCCAGTGGTACCTGCGTATCGACAAA-3'
 OLIGO#16 (SEQ ID NO:22):
 5'-CGTGGTAAAGTTAAAGGTACCCAGGAAATGAAAAACAACATC-
 15 ATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAA-3'
 OLIGO#17 (SEQ ID NO:23):
 5'-GGTGTGAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAACT-
 GTACGCAAAAAAAGAATGCAACGAAGACTGCAACTTCAAAGAA-3'
 OLIGO#18 (SEQ ID NO:24):
 20 5'-CTGATCCTGAAAACCACTACAACACCTACGCATCTGCTAAATGGAC-
 CCACAACGGTGGTGAAATGTTCGTTGCTCTGAACCAGAAAGGT-3'
 OLIGO#19 (SEQ ID NO:25):
 5'-ATCCCGGTTCTGTTGTAATAAACCAGAAAGAAACCGCTC-
 ACTTCCTGCCGATGGCAATCACTTAATAGGATCCAGTTTGA-3'
 25 OLIGO#20 (SEQ ID NO:26): 5'-TACGGGTGTGACGTTCCGGG-3'
 OLIGO#21 (SEQ ID NO:27): 5'-CTTTACCACGTTTGTCGATA-3'
 OLIGO#22 (SEQ ID NO:28): 5'-ATTCAACACCTTTGATTGCA-3'
 OLIGO#23 (SEQ ID NO:29): 5'-CCAGGATCAGTTCTTTGAAG-3'
 30 OLIGO#24 (SEQ ID NO:30): 5'-GAACCGGGATACCTTTCTGG-3'

 OLIGOs #12 through 24 were designed so that the entire DNA sequence encoding native KGF was represented by OLIGOs from either the "Watson" or the "Crick" strand and upon PCR amplification would produce the desired double-stranded DNA sequence (Figure 6)

35 [PCR#5, Model 9600 thermocycler, Perkin-Elmer Cetus]; 21

cycles, each cycle consisting of 31 seconds at 94°C for denaturation, 31 seconds at 50°C for annealing, and 31 seconds at 73°C for elongation; following the 21 cycles the PCR was finished with a final elongation step of 7 minutes]. After PCR amplification, the DNA fragment was cut with *XbaI* and *BamHI* and the 521 bp fragment ligated into the expression plasmid pCFM1156 cut with the same enzymes. PCR#5 utilized the outside primers (100 pmoles/100 µl rxn) OLIGO#12 and OLIGO#13 and 1 µl/100 µl rxn of a KGF template derived by ligation (by T4 ligase) of OLIGO #14 through OLIGO#19 (OLIGO#15 through OLIGO#18 were phosphorylated with T4 polynucleotide kinase) using OLIGO#20 through OLIGO#24 as band-aid oligos (Jayaraman et al. (1992), *Biotechniques*, 12:392) for the ligation. The final construct was designated KGF (codon optimized).

All of the KGF analogs described herein are composed in part from DNA sequences found in KGF(dsd) or KGF(codon optimized), or a combination of the two. The sequences are further modified by the insertion into convenient restriction sites of DNA sequences that encode the particular KGF analog amino acids made utilizing one or more of the above-described techniques for DNA fragment synthesis. Any of the analogs can be generated in their entirety by the above described techniques. However, as a part of the general OLIGO design optimized *E. coli* codons were used where appropriate, although the presence of *E. coli* optimized codons in part or *in toto* of any of the genes where examined did not significantly increase the yield of protein that could be obtained from cultured bacterial cells. Figures 7 to 10 and 17 to 26 set forth by convenient example particular KGF analog nucleotide and amino acid sequence constructions: R(144)Q (Figure 7); C(1,15)S/R(144)E (Figure 8); C(1,15)S/R(144)Q

(Figure 9); Δ N23/R(144)Q (Figure 10); Δ N23/N(137)E (Figure 17); Δ N23/K(139)E (Figure 18); Δ N23/K(139)Q (Figure 19); Δ N23/R(144)A (Figure 20); Δ N23/R(144)L (Figure 21); Δ N23/K(147)E (Figure 22); Δ N23/K(147)Q (Figure 23); Δ N23/K(153)E (Figure 24); Δ N23/K(153)Q; (Figure 25) and Δ N23/Q(152)E/K(153)E (Figure 26). All the KGF analog constructions described herein were DNA sequence confirmed.

10 EXAMPLE 2: Production in *E. coli*

Three different expression plasmids were utilized in the cloning of the KGF analog genes. They were pCFM1156 (ATCC# 69702), pCFM1656 (ATCC# 69576), and pCFM3102 (Figures 2A, 2B and 2C, respectively). The plasmid p3102 can be derived from the plasmid pCFM1656 by making a series of site-directed base changes with PCR overlapping oligo mutagenesis. Starting with the *Bgl*III site (pCFM1656 plasmid bp # 180) immediately 5' to the plasmid replication promoter, *P*_{copB}, and proceeding toward the plasmid replication genes, the base pair changes are as follows:

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	<u>pCFM1656 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pCFM3102</u>
	# 204	T/A	C/G
	# 428	A/T	G/C
	# 509	G/C	A/T
5	# 617	- -	insert two G/C bp
	# 677	G/C	T/A
	# 978	T/A	C/G
	# 992	G/C	A/T
	# 1002	A/T	C/G
10	# 1005	C/G	T/A
	# 1026	A/T	T/A
	# 1045	C/G	T/A
	# 1176	G/C	T/A
	# 1464	G/C	T/A
15	# 2026	G/C	bp deletion
	# 2186	C/G	T/A
	# 2479	A/T	T/A
20	# 2498-2501	<u>AGTG</u> TCAC	<u>GTCA</u> CAGT
	# 2641-2647	<u>TCCGAGC</u> AGGCTCG	bp deletion
25	# 3441	G/C	A/T
	# 3452	G/C	A/T
	# 3649	A/T	T/A
30	# 4556	--	insert bps
	(SEQ ID NO:39)	5'- <u>GAGCTCACTAGTGTCTCGACCTGCAG</u> -3'	
	(SEQ ID NO:40)	3'-CTCGAGTGATCACAGCTGGACGTC-5'	

As seen above, pCFM1156, pCFM1656 and pCFM3102 are very similar to each other and contain many of the same restriction sites. The plasmids were chosen by convenience, and the vector DNA components can be easily exchanged for purposes of new constructs. The host used for all cloning was *E. coli* strain FM5 (ATCC: 53911) and the transformations were carried out (according to the method of Hanahan (1983), *supra*) or by electroelution with a Gene Pulser™ transfection apparatus (BioRad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions.

Initially, a small, freshly cultured inoculum of the desired recombinant *E. coli* clone harboring the

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desired construct on one of the three pCFM vectors was started by transferring 0.1 mL of a frozen glycerol stock of the appropriate strain into a 2 L flask containing 500 mL of Luria broth. The culture was
5 shaken at 30°C for 16 hours, after which the culture was transferred to a 15 L fermentor containing 8 L of sterile batch medium (Tsai, et al. (1987), *J. Industrial Microbiol.*, 2:181-187).

Feed batch fermentation starts with the
10 feeding of Feed # 1 medium (Tsai, et al. (1987), *supra*). When the OD600 reached 35, expression of the desired KGF analog was induced by rapidly raising the culture temperature to 37°C for two hours then up to 42°C to denature the CI repressor. The addition of Feed
15 1 was discontinued in favor of Feed 2, the addition rate of which was initiated at 300 mL/hr. Feed 2 comprised 175 g/L trypticase-peptone, 87.5 g/L yeast extract, and 260 g/L glucose. After one hour at 42°C, the culture temperature was decreased to 36°C, where this
20 temperature was then maintained for another 6 hours.

The fermentation was then halted and the cells were harvested by centrifugation into plastic bags placed within 1 L centrifuge bottles. The cells were pelleted by centrifugation at 400 rpm for 60 minutes,
25 after which the supernatants were removed and the cell paste frozen at -90°C.

Following expression of the various KGF analogs, in *E. coli*, native KGF, R(144)Q, C(1,15)S/R(144)E, C(1,15)S/R(144)Q and ΔN23/R(144)Q
30 proteins were purified using the following procedure. Cell paste from a high cell density fermentation was suspended at 4°C in 0.2 M NaCl, 20 mM NaPO₄, pH 7.5 as a 10-20% solution (weight per volume) using a suitable high shear mixer. The suspended cells were then lysed
35 by passing the solution through a homogenizer (APV Gaulin, Inc., Everett, MA) three times. The outflowing

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homogenate was cooled to 4-8°C by using a suitable heat exchanger. Debris was then removed by centrifuging the lysate in a J-6B™ centrifuge (Beckman Instruments, Inc., Brea, CA) equipped with a JS 4.2 rotor at 4,200 rpm for
5 30-60 min. at 4°C. Supernatants were then carefully decanted and loaded onto a previously prepared 450 mL (5 cm x 23 cm) column of S-Sepharose Fast Flow™ resin (Pharmacia, Piscataway, NJ) equilibrated with 0.2 M NaCl, 20 mM NaPO₄, pH 7.5 at 4°C. Next, the column was
10 washed with five column volumes (2250 mL) of 0.4 M NaCl, 20 mM NaPO₄, pH 7.5 at 4°C. The desired protein was eluted by washing the column with 5 L of 0.5 M NaCl, 20 mM NaPO₄, pH 7.5. Then, 50 mL fractions were collected and the A₂₈₀ of the effluent was continuously monitored.
15 Fractions identified by A₂₈₀ as containing eluted material were then analyzed by SDS-PAGE through 14% gels to confirm the presence of the desired polypeptide.

Those fractions containing proteins of interest were then pooled, followed by the addition of
20 an equal volume of distilled water. The diluted sample was then loaded onto a previously prepared 450 mL (5 cm x 23 cm) column of S-Sepharose Fast Flow equilibrated with 0.4 M NaCl, 20 mM NaPO₄, pH 6.8 at 4°C. The column was washed with 2250 mL of 0.4 M NaCl, 20 mM NaPO₄, pH
25 6.8 and the protein eluted using a 20 column volume linear gradient ranging from 0.4 M NaCl, 20 mM NaPO₄, pH 6.8 to 0.6 M NaCl, 20 mM NaPO₄, pH 6.8. Again, 50 mL fractions were collected under constant A₂₈₀ monitoring of the effluent. Those fractions containing the protein
30 (determined by 14% SDS-PAGE) were then pooled, followed by concentration through a YM-10 membrane (10,000 molecular weight cutoff) in a 350cc stirring cell (Amicon, Inc. Mayberry, MA) to a volume of 30-40 mL.

The concentrate was then loaded onto a
35 previously generated 1,300 mL (4.4 cm x 85 cm) column of Superdex-75™ resin (Pharmacia) equilibrated in column

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buffer comprising 1X PBS (Dulbecco's Phosphate Buffered Saline, "D-PBS", calcium and magnesium-free) or 0.15 M NaCl, 20 mM NaPO₄, pH 7.0. After allowing the sample to run into the column, the protein was eluted from the gel filtration matrix using column buffer. Thereafter, 10 mL fractions were recovered and those containing the analog (determined by 14% SDS-PAGE) were pooled. Typically, the protein concentration was about 5-10 mg/mL in the resultant pool. All of the above procedures were performed at 4-8°C, unless otherwise specified.

Analysis

Analysis was conducted on *E. coli*-derived, native KGF; R(144)Q; C(1,15)S/R(144)E; C(1,15)S/R(144)Q and ΔN23/R(144)Q.

Conformational Stability

The polypeptides were compared by their storage stability, thermal unfolding transition temperatures (T_m), and stability in a broad range of pH conditions.

The ability of native KGF, C(1,15)S, R(144) Q, C(1,15)S/R(144)Q, C(1,15)S/R(144)E and ΔN23/R(144)Q to prevent aggregation at elevated temperatures was also examined. Samples containing 0.5 mg/mL of protein were prepared in D-PBS. 0.5 mL of each sample was aliquoted into 3 cc type-1 glass vials. The vials were sealed with rubber stoppers and 13 mm flip-off aluminum seals were crimped on. These vials were then placed in a 37°C incubator. At predetermined time intervals, vials were withdrawn and analyzed for the loss of soluble protein. Visible precipitates were removed by centrifuging 250 μL of each sample through a 0.22 μm Spin-X™ filter unit

(Costar, Cambridge, MA). Soluble protein in the filtered solutions was subsequently analyzed by size exclusion HPLC. The amount of soluble protein was determined by integrating the HPLC peak area and plotting the result as a function of incubation time at 37°C. The results of native KGF, C(1,15)S, C(1,15)S/R(144)Q, and C(1,15)S/R(144)E are shown in Figure 11. The data for R(144)Q and Δ N23/R(144)Q are not shown.

The half-lives for the loss of soluble, monomeric protein were then estimated from these kinetic curves. Table 1 shows the half-life for remaining soluble KGF upon storage at 37°C for these proteins.

Table 1

Half-life for the Loss of Soluble, Monomeric Proteins

Protein	t _{1/2} (day)
native KGF	0.6
R(144)Q	4.1
C(1,15)S/R(144)Q	13.3
Δ N23/R(144)Q	22.3
C(1,15)S/R(144)E	38.0

As seen in Table 1, above, and Figure 11, the native KGF aggregated the most rapidly, with a half-life of 0.6 days. R(144)Q increased the half-life to 4.1 days. C(1,15)S/R(144)Q, Δ N23/R(144)Q and C(1,15)S/R(144)E showed substantial increases in the solubility half-life to 13.3, 22.3 and 38 days, respectively.

Thermal Unfolding

Thermal unfolding was monitored by circular dichroism (CD) at 230 nm using a J-720[™] spectropolarimeter (Jasco, Inc., Easton, MD) equipped with a PTC-343 Peltier-type temperature control system. For CD analysis, separate samples containing 0.1 mg/mL

of the polypeptide to be analyzed were prepared in D-PBS (Life Technologies, Inc., Grand Island, NY). For each sample, about 2.5 mL was loaded into a 10 mm path length rectangular Suprasil™ quartz (Heraeus Quarzschmelze, GmbH, Hanau, Germany) fluorescent cell (Hellma Cells, Inc., Jamaica, NY). The cell was then placed into the Peltier-type temperature control system in the spectropolarimeter. Thermal unfolding was carried out at a rate of 50°C/hr. Changes in ellipticity were monitored at 230 nm to indicate unfolding. The T_m of each sample was estimated by identifying a temperature at which 50% of protein molecules in the solution were unfolded (*Biophysical Chemistry*, Cantor and Schimmel (eds), W.H. Freeman and Co. San Francisco (1980)). The estimated T_m for each of the five proteins is listed in Table 2.

Table 2
Estimated Melting Temperatures

Protein	T_m (°C)
native KGF	54.0
R(144)Q	61.5
C(1,15)S/R(144)Q	62.5
Δ N23/R(144)Q	63.0
C(1,15)S/R(144)E	63.5

As the results show, R(144)Q has a greater than 7°C increase in the T_m as compared with native KGF. The substitution of R(144)Q to C(1,15)S/R(144)Q or Δ N23 adds at least another 1°C increase in T_m and more than 8°C as compared with native KGF. Moreover, the C(1,15)S/R(144)E is greater than 9°C more stable than native KGF. Therefore, switching a positively charged residue (Arg) at amino acid position 144 to a neutrally

or negatively charged residue substantially stabilized the polypeptide.

pH

5

The acid stabilities of C(1,15)S/R(144)Q, C(1,15)S, and C(1,15)S/R(144)E were also compared to that of native KGF, by adjusting D-PBS to different pH values by adding concentrated HCl or NaOH. Approximately 2.35 mL of D-PBS at different pH values was mixed with 100 μ L of 2.45 mg/mL KGF protein in a quartz cell. These samples were thermally unfolded at a rate of 50°C/hr and monitored by CD at 230 nm. Figure 12 shows the T_m as a function of pH for native KGF, C(1,15)S/R(144)Q and C(1,15)S/R(144)E. In the pH range tested, the C(1,15)S/R(144)Q and C(1,15)S/R(144)E always have a higher T_m than the native KGF.

10

15

In vitro Biological Activity

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In vitro mitogenic activities of R(144)Q, Δ N23/R(144)Q, C(1,15)S/R(144)Q, C(1,15)S, and C(1,15)S/R(144)E were also determined as a function of protein concentration and the half-maximal concentrations by measurement of [^{3} H]-thymidine uptake by Balb/MK cells (according to the methods of Rubin et al. (1989), *supra*).

25

Generally, the concentrations of each of the KGF analogs relative to a known standard native KGF was determined using an *in vitro* biological assay. Each KGF analog was then diluted and assayed for biological activity using a Balb/MK mitogenic assay. The samples were first diluted in a bioassay medium consisting of 50% customer-made Eagle's MEM, 50% customer-made F12, 5 μ G/mL transferrin, 5 ng/ml sodium selenite, 0.0005% HSA and 0.005% Tween 20. KGF samples were then added into Falcon Primeria 96-well plates seeded with Balb/MK

30

35

cells. Incorporation of [³H]-Thymidine during DNA synthesis was measured and converted to input native KGF concentration by comparison to a native KGF standard curve. The results are presented in Figures 13 to 16.

5 As seen in Figures 13 to 16, each of the KGF analogs has mitogenic activity.

While the present invention has been described above both generally and in terms of preferred embodiments, it is understood that other variations and
10 modifications will occur to those skilled in the art in light of the description above.

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**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

1. A polypeptide analog of native keratinocyte growth factor (which corresponds to amino acid residues 32-194 of SEQ ID NO: 2), termed "KGF", wherein the analog comprises the amino acid sequence of KGF having a charge-change by the substitution of Arg¹⁴⁴ with a neutral or negatively charged residue, optionally further having an N-terminal methionine or a signal sequence, wherein the polypeptide analog has increased stability over native KGF.

2. The polypeptide analog according to claim 1, further comprising an N-terminal methionine.

3. The polypeptide analog according to claim 1, further comprising a signal sequence.

4. The polypeptide analog according to any of claims 1, 2 or 3 further comprising one or more deleted or substituted amino acid residues selected from the arginine residue at amino acid position 72 of SEQ ID NO:2, the glutamine residue at amino acid position 74 of SEQ ID NO:2, the lysine residue at amino acid position 86 of SEQ ID NO:2, the lysine residue at amino acid position 126 of SEQ ID NO:2, the asparagine residue at amino acid position 168 of SEQ ID NO:2, the glutamine residue at amino acid position 169 of SEQ ID NO:2, the lysine residue at amino acid position 170 of SEQ ID NO:2, the arginine residue at amino acid position 175 of SEQ ID NO:2, the lysine residue at amino acid position 178 of SEQ ID NO:2, the glutamine residue at amino acid position 183 of SEQ ID NO:2, the lysine residue at amino acid position 184 of SEQ ID NO:2, and the threonine residue at amino acid position 185 of SEQ ID NO:2.

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5. A polypeptide analog of native keratinocyte growth factor selected from: R(144)Q (which corresponds to SEQ ID NO:32); C(1,15)S/R(144)E (which corresponds to SEQ ID NO:34); C(1,15)S/R(144)Q (which corresponds to SEQ ID NO:36); Δ N23/R(144)Q (which corresponds to SEQ ID NO:38); Δ N23/N(137)E (which corresponds to SEQ ID NO:42); Δ N23/K(139)E (which corresponds to SEQ ID NO:44); Δ N23/K(139)Q (which corresponds to SEQ ID NO:46); Δ N23/R(144)A (which corresponds to SEQ ID NO:48); Δ N23/R(144)E (a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of glutamic acid for arginine at amino acid position 144 of native KGF); Δ N23/R(144)L (which corresponds to SEQ ID NO:50); Δ N23/K(147)E (which corresponds to SEQ ID NO:52); Δ N23/K(147)Q (which corresponds to SEQ ID NO:54); Δ N23/K(153)E (which corresponds to SEQ ID NO:56); Δ N23/K(153)Q (which corresponds to SEQ ID NO:58); and Δ N23/Q(152)E/K(153)E (which corresponds to SEQ ID NO:60).

6. A polypeptide analog of native keratinocyte growth factor selected from: R(144)Q (which corresponds to SEQ ID NO:32, wherein the initial methionine residue is not included); C(1,15)S/R(144)E (which corresponds to SEQ ID NO:34, wherein the initial methionine residue is not included); C(1,15)S/R(144)Q (which corresponds to SEQ ID NO:36, wherein the initial methionine residue is not included); Δ N23/R(144)Q (which corresponds to SEQ ID NO:38, wherein the initial methionine residue is not included); Δ N23/N(137)E (which corresponds to SEQ ID NO:42, wherein the initial methionine residue is not included); Δ N23/K(139)E (which corresponds to SEQ ID NO:44, wherein the initial methionine residue is not included); Δ N23/K(139)Q (which corresponds to SEQ ID NO:46, wherein the initial methionine residue is not included); Δ N23/R(144)A (which corresponds to SEQ ID

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NO:48, wherein the initial methionine residue is not included); Δ N23/R(144)E (a KGF analog having a deletion of the first 23 amino acids of the N-terminus and a substitution of glutamic acid for arginine at amino acid position 144 of native KGF, wherein the initial methionine residue is not included); Δ N23/R(144)L (which corresponds to SEQ ID NO:50, wherein the initial methionine residue is not included); Δ N23/K(147)E (which corresponds to SEQ ID NO:52, wherein the initial methionine residue is not included); Δ N23/K(147)Q (which corresponds to SEQ ID NO:54, wherein the initial methionine residue is not included); Δ N23/K(153)E (which corresponds to SEQ ID NO:56, wherein the initial methionine residue is not included); Δ N23/K(153)Q (which corresponds to SEQ ID NO:58, wherein the initial methionine residue is not included); and Δ N23/Q(152)E/K(153)E (which corresponds to SEQ ID NO:60, wherein the initial methionine residue is not included).

7. The polypeptide analog according to any of claims 1 to 6, wherein said polypeptide analog is covalently attached to a chemical moiety.

8. The polypeptide analog according to claim 7, wherein said chemical moiety is polyethylene glycol.

9. The polypeptide analog according to any of claims 1 to 8, wherein said polypeptide analog is lyophilized.

10. A pharmaceutical formulation comprising a therapeutically effective amount of a polypeptide analog according to any of claims 1 to 9, and a pharmaceutically acceptable carrier.

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11. A recombinant nucleic acid molecule encoding a polypeptide analog according to any of claims 1 to 6.

12. A biologically functional plasmid comprising a recombinant nucleic acid molecule according to claim 11.

13. A biologically functional viral vector comprising a recombinant nucleic acid molecule according to claim 11.

14. An isolated host cell containing material selected from the group comprising: a recombinant nucleic acid according to claim 11, a biologically functional plasmid according to claim 12, and a biologically functional viral vector according to claim 13.

15. The isolated host cell according to claim 14, wherein the host cell is a procaryotic cell.

16. The isolated host cell according to claim 15, wherein the procaryotic cell is *E. coli*.

17. The isolated host cell according to claim 14, wherein the host cell is a eukaryotic cell.

18. The isolated host cell according to claim 17, wherein the eukaryotic cell is a mammalian cell.

19. The isolated host cell according to claim 18, wherein the mammalian cell is a Chinese hamster ovary cell.

20. A process for the production of a polypeptide analog according to any of claims 1 to 9, comprising the steps of:

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growing a host cell according to any of claims 14 to 19 under suitable nutrient conditions in a manner allowing expression of the encoded polypeptide analog, and isolating the polypeptide analog so produced.

5

21. An *in vitro* method of stimulating the production of non-fibroblast epithelial cells comprising contacting non-fibroblast epithelial cells with an effective amount of a polypeptide analog according to any of claims 1 to 9.

10

22. An *in vitro* method of stimulating the production of non-fibroblast epithelial cells comprising contacting non-fibroblast epithelial cells with an effective amount of a pharmaceutical formulation according to claim 10.

15

23. A use of an effective amount of the polypeptide analog according to any of claims 1 to 9 for stimulating production of non-fibroblast epithelial cells.

20

24. A use of an effective amount of the pharmaceutical formulation according to claim 10 for stimulating production of non-fibroblast epithelial cells.

25

25. A use of an effective amount of the polypeptide analog according to claims 1 to 9 for stimulating production of non-fibroblast epithelial cells in a patient to prevent or treat a condition wherein said condition is selected from burns and other partial and full-thickness injuries; epidermolysis bullosa; chemotherapy-induced alopecia; male-pattern baldness, progressive loss of hair in men and women; gastric and duodenal ulcers; inflammatory bowel disease; gut toxicity

30
35

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in radiation and chemotherapy treatment regimes; hyaline
membrane disease; acute or chronic lung damage; hepatic
cirrhosis; fulminant liver failure; acute viral
hepatitis; toxic insults to the liver; corneal abrasion;
5 progressive gum disease and ear drum damage.

26. A use of an effective amount of the
pharmaceutical formulation according to claim 10 for
stimulating production of non-fibroblast epithelial cells
10 in a patient to prevent or treat a condition wherein said
condition is selected from burns and other partial and
full-thickness injuries; epidermolysis bullosa;
chemotherapy-induced alopecia; male-pattern baldness,
progressive loss of hair in men and women; gastric and
15 duodenal ulcers; inflammatory bowel disease;

gut toxicity in
radiation and chemotherapy treatment regimes; hyaline
membrane disease; acute or chronic lung damage; hepatic
cirrhosis; fulminant liver failure; acute viral
20 hepatitis; toxic insults to the liver; corneal abrasion;
progressive gum disease and ear drum damage.

27. A use of an effective amount of the polypeptide
analog according to any of claims 1 to 9 for the
25 production of a medicament for stimulating production of
non-fibroblast epithelial cells.

28. A use of an effective amount of the
pharmaceutical formulation according to claim 10 for the
30 production of a medicament for stimulating production of
non-fibroblast epithelial cells.

29. A use of an effective amount of a polypeptide
35 analog according to any of claims 1 to 9 for the

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production of a medicament for stimulating production of non-fibroblast epithelial cells in a patient for the prevention or treatment of a condition, wherein said condition is selected from burns and other partial and full-thickness injuries; epidermolysis bullosa; chemotherapy-induced alopecia; male-pattern baldness, progressive loss of hair in men and women; gastric and duodenal ulcers; inflammatory bowel disease;

gut toxicity in radiation and chemotherapy treatment regimes; hyaline membrane disease; acute or chronic lung damage; hepatic cirrhosis; fulminant liver failure; acute viral hepatitis; toxic insults to the liver; corneal abrasion; progressive gum disease and ear drum damage.

30. A use of an effective amount of the pharmaceutical formulation according to claim 10 for the production of a medicament for stimulating production of non-fibroblast epithelial cells in a patient for the prevention or treatment of a condition wherein said condition is selected from burns and other partial and full-thickness injuries; epidermolysis bullosa; chemotherapy-induced alopecia; male-pattern baldness, progressive loss of hair in men and women; gastric and duodenal ulcers; inflammatory bowel disease;

gut toxicity in radiation and chemotherapy treatment regimes; hyaline membrane disease; acute or chronic lung damage; hepatic cirrhosis; fulminant liver failure; acute viral hepatitis; toxic insults to the liver; corneal abrasion; progressive gum disease and ear drum damage.

31. The use according to any of claims 23 to 30, wherein said non-fibroblast epithelial cells are selected from adnexal structures, liver cells, mucosal epithelium

in the respiratory and gastrointestinal tracts, corneal cells and tympanic epithelial cells.

5 32. The use according to claims 23 or 24 wherein the non-fibroblast epithelial cells are stimulated in a patient in need thereof.

10 33. The use of claim 32 wherein said non-fibroblast epithelial cells are selected from adnexal structures, liver cells, mucosal epithelium in the respiratory and gastrointestinal tracts, corneal cells and tympanic epithelial cells.

15 34. The use according to any of claims 23 to 33 wherein said polypeptide analog is used by IV, IT, IM, SC or IP injection.

20 35. A kit comprising a polypeptide analog according to any of claims 1 to 9 and an aqueous formulation comprising a stabilizer.

 36. A kit comprising a pharmaceutical formulation of claim 10 and an aqueous formulation comprising a stabilizer.

25 37. The use of Claim 25 wherein the inflammatory bowel disease is selected from the group consisting of Crohn's disease and ulcerative colitis.

 38. The use of claims 26, 29 or 30 wherein the inflammatory bowel disease is selected from the group consisting of Crohn's disease and ulcerative colitis.

FIG. 1A

human KGF (+ signal sequence)

|-----OLIGO#1-----|
 5' -CAATGACCTAGGAGTAACAATCAAC-3'

5' -CAATCTACAATTACAGATAGGAAGAGGTCAATGACCTAGGAGTAACAATCAACTCAAGA-
 -----+-----+-----+-----+-----+-----+-----+ 60

-TTCATTTTCATTATGTTATTCATGAACACCCGGAGCACTACACTATAATGCACAAATGGA-
 -----+-----+-----+-----+-----+-----+-----+ 120
M H K W I

-TACTGACATGGATCCTGCCAACTTTGCTCTACAGATCATGCTTTCACATTATCTGTCTAG-
 -----+-----+-----+-----+-----+-----+-----+ 180
L T W I L P T L L Y R S C F H I I C L V

-TGGGTACTATATCTTTAGCTTGCAATGACATGACTCCAGAGCAAATGGCTACAAATGTGA-
 -----+-----+-----+-----+-----+-----+-----+ 240
G T I S L A C N D M T P E Q M A T N V N

-ACTGTTCCAGCCCTGAGCGACACACAAGAAGTTATGATTACATGGAAGGAGGGGATATAA-
 -----+-----+-----+-----+-----+-----+-----+ 300
C S S P E R H T R S Y D Y M E G G D I R

-GAGTGAGAAGACTCTTCTGTCTGAACACAGTGGTACCTGAGGATCGATAAAAGAGGCAAAG-
 -----+-----+-----+-----+-----+-----+-----+ 360
V R R L F C R T Q W Y L R I D K R G K V

-TAAAAGGGACCCAAGAGATGAAGAATAATTACAATATCATGGAAATCAGGACAGTGGCAG-
 -----+-----+-----+-----+-----+-----+-----+ 420
K G T Q E M K N N Y N I M E I R T V A V

-TTGGAATTGTGGCAATCAAAGGGGTGGAAAGTGAATTCTATCTTGCAATGAACAAGGAAG-
 -----+-----+-----+-----+-----+-----+-----+ 480
G I V A I K G V E S E F Y L A M N K E G

-GAAACTCTATGCAAAGAAAGAATGCAATGAAGATTGTAAGTTCAAAGAACTAATTCTGG-
 -----+-----+-----+-----+-----+-----+-----+ 540
K L Y A K K E C N E D C N F K E L I L E

-AAAACCATTAACAACACATATGCATCAGCTAAATGGACACACAACGGAGGGGAAATGTTTG-
 -----+-----+-----+-----+-----+-----+-----+ 600
N H Y N T Y A S A K W T H N G G E M F V

-TTGCCTTAAATCAAAGGGGATTCCTGTAAGAGGAAAAAAAAACGAAGAAAGAACAAAAAA-
 -----+-----+-----+-----+-----+-----+-----+ 660
A L N Q K G I P V R G K K T K K E Q K T

-CAGCCCACTTTCTTCCTATGGCAATAACTTAATTGCATATGGTATATAAGAACCCAGTT
 -----+-----+-----+-----+-----+-----+-----+ 720
A H F L P M A I T *

FIG.1 B

-CCAGCAGGGAGATTCTTTAAGTGGACTGTTTTCTTTCTTCTCAAATTTCTTTCCTTT
-----+-----+-----+-----+-----+-----+ 780

-TATTTTTTAGTAATCAAGAAAGGCTGGAAAACTACTGAAAACTGATCAAGCTGGACTT
-----+-----+-----+-----+-----+-----+ 840

3'-ACCTGAA-
|-----

-GTGCATTTATGTTTGTTTAAAG-3'
-----+-----+-- 862

-CACGTAAATACAAACAAAA-5'
---OLIGO#2-----|

FIG.2A

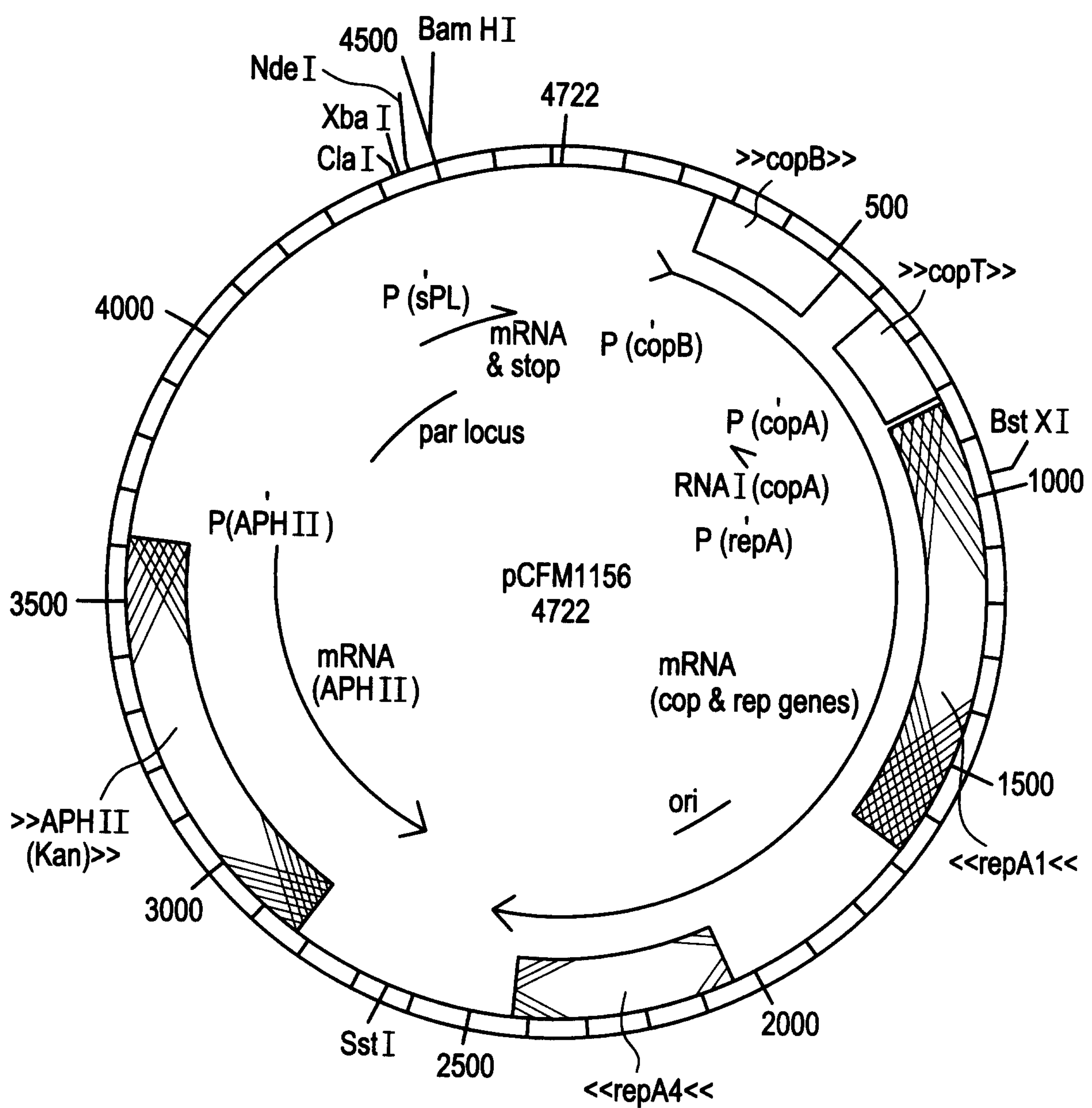


FIG.2B

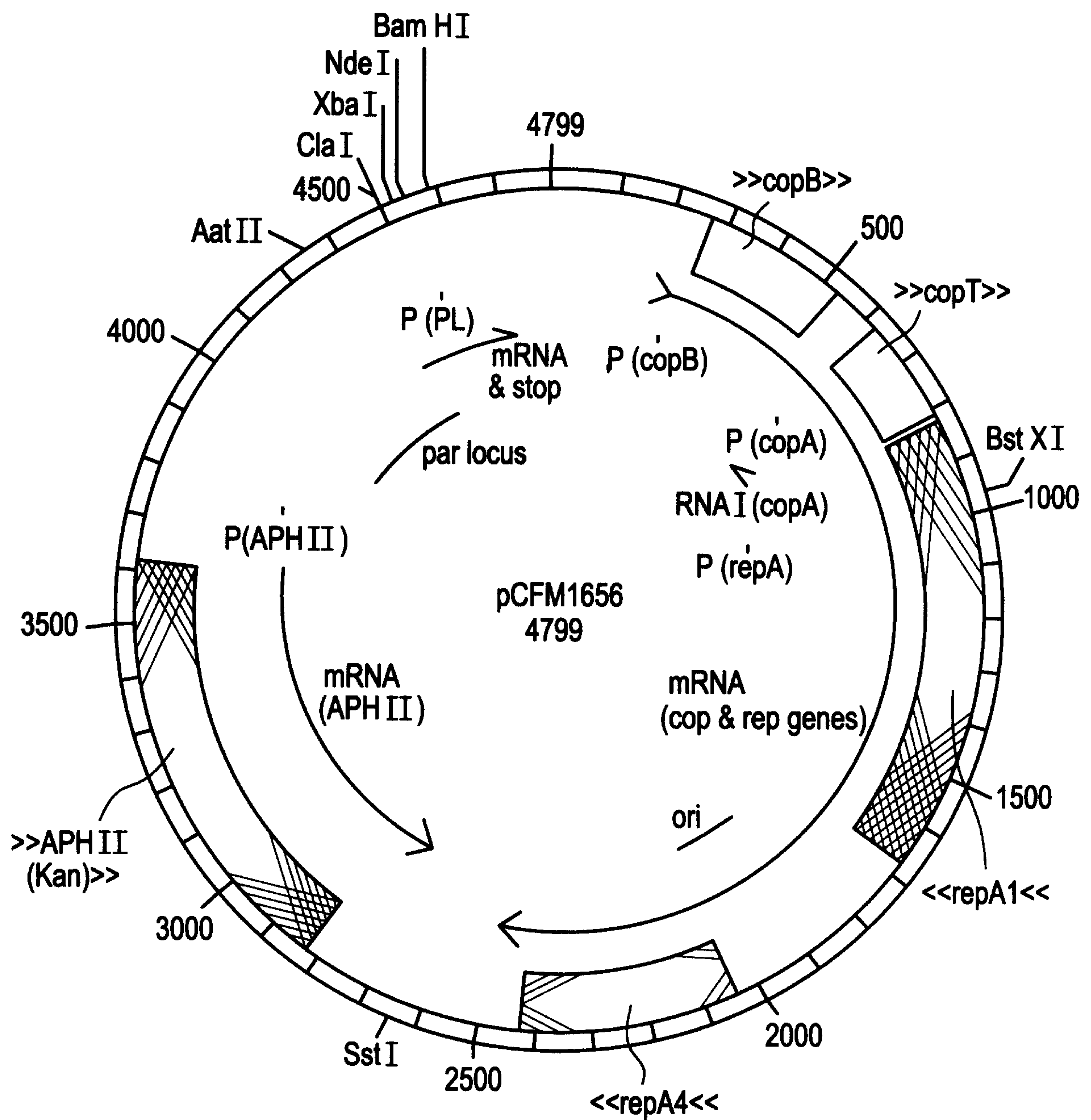


FIG.2C

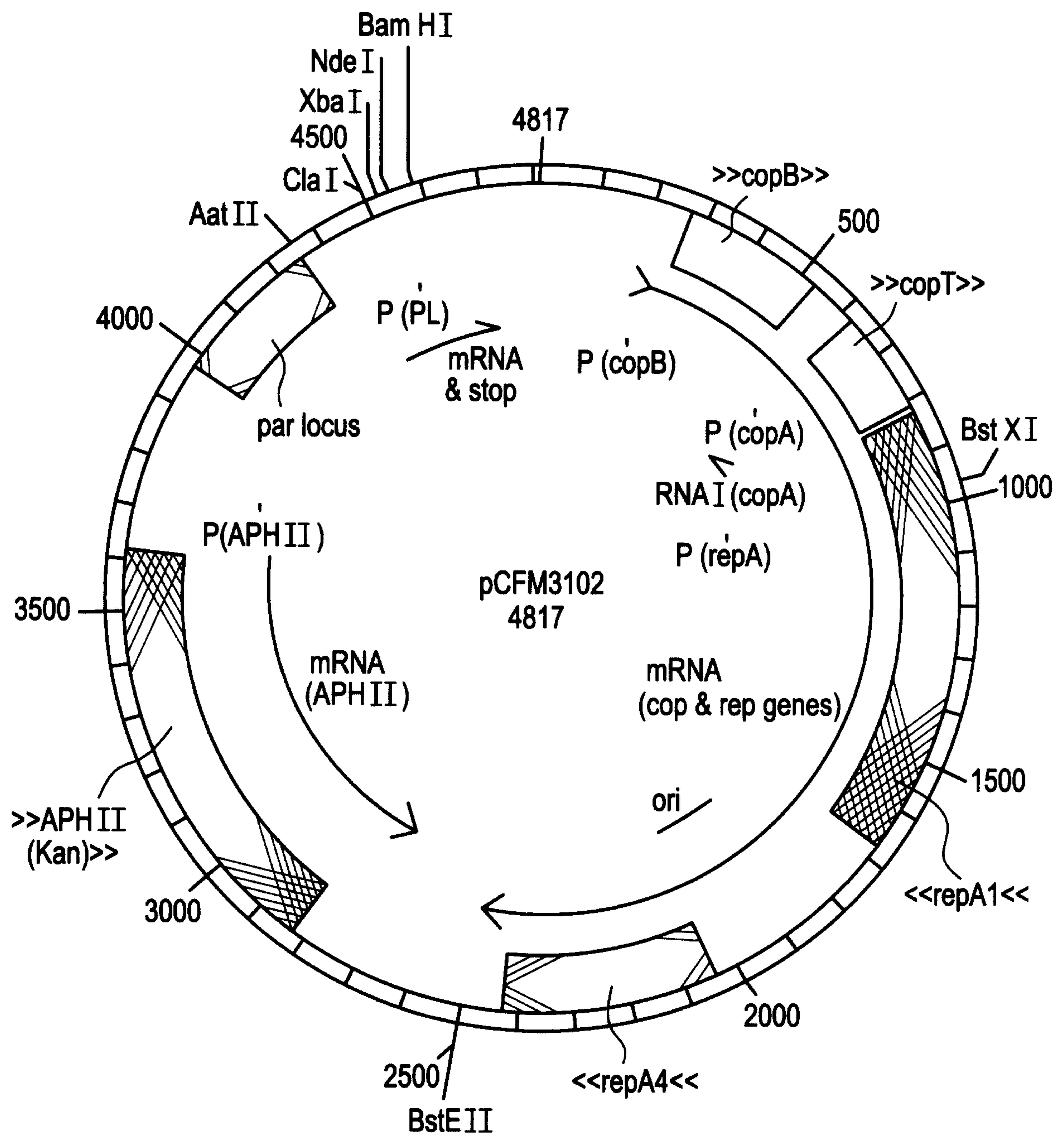


FIG.3
RSH-KGF

plasmid DNA
sequence

ClaI *XbaI* *NdeI*

5'-ATCGATTTGATTCTAGAAGGAGGAATAACATATGAAAAAG-
M K K

RSH signal sequence

MluI

-CGCGCACGTGCTATCGCCATTGCTGTGGCTCTGGCAGGTTTCGCAACTAGTGCACA-3'
R A R A I A I A V A L A G F A T S A H A -

MluI

5'-CGCGTGCAATGACATGACTCCAGAGCAAATGGCTACAAATGTGAACTGTTCCAGCCCTGA-
-----+-----+-----+-----+-----+ 60
- C N D M T P E Q M A T N V N C S S P E

-GCGACACACAAGAAGTTATGATTACATGGAAGGAGGGGATATAAGAGTGAGAAGACTCTT-
-----+-----+-----+-----+-----+ 120
R H T R S Y D Y M E G G D I R V R R L F

KpnI *ClaI*

-CTGTCGAACACAGTGGTACCTGAGGATCGATAAAAGAGGCAAAGTAAAAGGGACCCAAGA-
-----+-----+-----+-----+-----+ 180
C R T Q W Y L R I D K R G K V K G T Q E

-GATGAAGAATAATTACAATATCATGGAAATCAGGACAGTGGCAGTTGGAATTGTGGCAAT-
-----+-----+-----+-----+-----+ 240
M K N N Y N I M E I R T V A V G I V A I

EcoRI

-CAAAGGGGTGGAAAGTGAATTCTATCTTGCAATGAACAAGGAAGGAAAACCTCTATGCAAA-
-----+-----+-----+-----+-----+ 300
K G V E S E F Y L A M N K E G K L Y A K

BsmI

-GAAAGAATGCAATGAAGATTGTAAGTTCAAAGAACTAATTCTGGAAAACCATTACAACAC-
-----+-----+-----+-----+-----+ 360
K E C N E D C N F K E L I L E N H Y N T

NdeI

-ATATGCATCAGCTAAATGGACACACAACGGAGGGGAAATGTTTGTTCCTTAAATCAAAA-
-----+-----+-----+-----+-----+ 420
Y A S A K W T H N G G E M F V A L N Q K

-GGGGATTCCTGTAAGAGGAAAAAAACGAAGAAAGAACAAAAACAGCCCACTTTCTTCC-
-----+-----+-----+-----+-----+ 480
G I P V R G K K T K K E Q K T A H F L P

BamHI

-TATGGCAATAACTTAATAG-3'
-----+-----+----- 503
M A I T * *

-plasmid DNA
-sequence

FIG.4

KGF

NdeI

5' -TATGTGCAATGACATGACTCCAGAGCAAATGGCTACAAATGTGAACTGTTCCAGCCCTGA-
 -----+-----+-----+-----+-----+-----+ 60
 M C N D M T P E Q M A T N V N C S S P E

-GCGACACACAAGAAGTTATGATTACATGGAAGGAGGGGATATAAGAGTGAGAAGACTCTT-
 -----+-----+-----+-----+-----+-----+ 120
 R H T R S Y D Y M E G G D I R V R R L F

*KpnI**ClaI*

-CTGTCGAACACAGTGGTACCTGAGGATCGATAAAAGAGGCAAAGTAAAAGGGACCCAAGA-
 -----+-----+-----+-----+-----+-----+ 180
 C R T Q W Y L R I D K R G K V K G T Q E

-GATGAAGAATAATTACAATATCATGGAAATCAGGACAGTGGCAGTTGGAATTGTGGCAAT-
 -----+-----+-----+-----+-----+-----+ 240
 M K N N Y N I M E I R T V A V G I V A I

EcoRI

-CAAAGGGGTGGAAAGTGAATTCTATCTTGCAATGAACAAGGAAGGAAAACCTATGCAAA-
 -----+-----+-----+-----+-----+-----+ 300
 K G V E S E F Y L A M N K E G K L Y A K

BsmI

-GAAAGAATGCAATGAAGATTGTAACCTCAAAGAACTAATTCTGGAAAACCATTACAACAC-
 -----+-----+-----+-----+-----+-----+ 360
 K E C N E D C N F K E L I L E N H Y N T

NdeI

-ATATGCATCAGCTAAATGGACACACAACGGAGGGGAAATGTTTGTTCCTTAAATCAAAA-
 -----+-----+-----+-----+-----+-----+ 420
 Y A S A K W T H N G G E M F V A L N Q K

-GGGGATTCCTGTAAGAGGAAAAAAAACGAAGAAAGAACAAAAACAGCCCACTTTCTTCC-
 -----+-----+-----+-----+-----+-----+ 480
 G I P V R G K K T K K E Q K T A H F L P

BamHI

-TATGGCAATAACTTAATAG-3'
 -----+-----+----- 503
 M A I T *

FIG.5

substitution of KpnI to EcoRI sequence to make KGF(dsd)

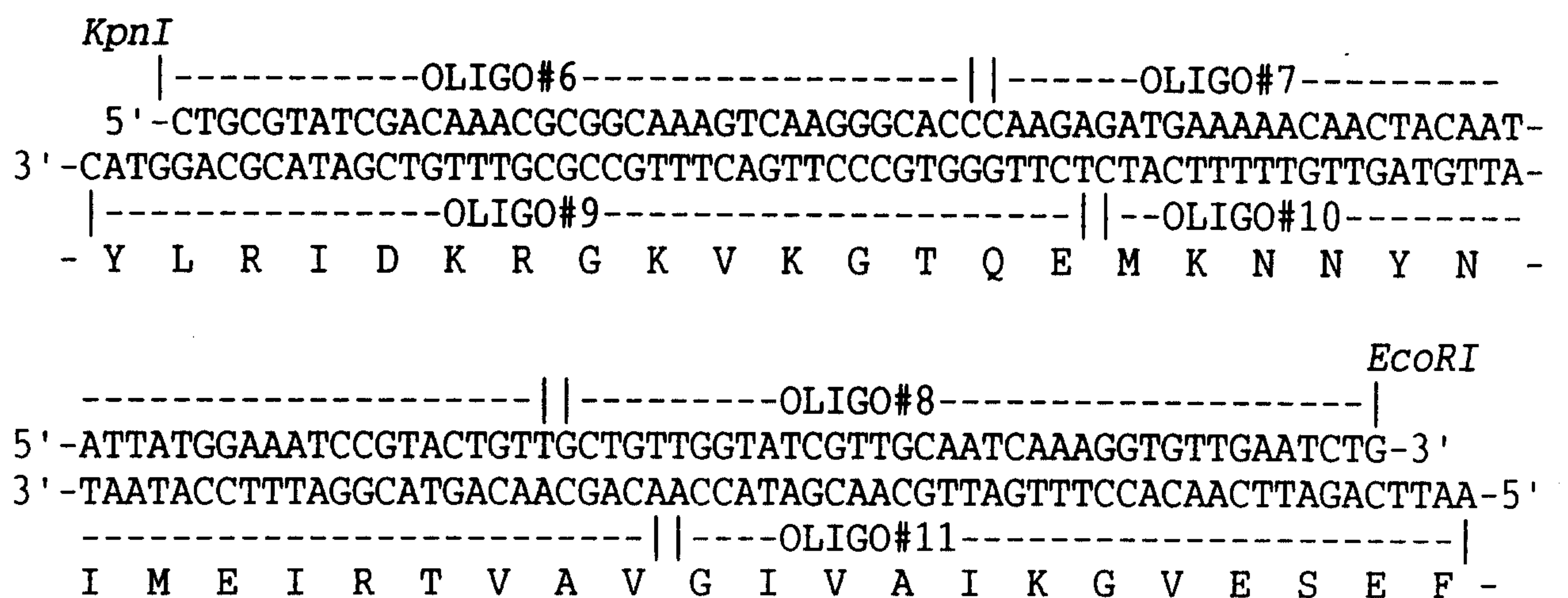


FIG.6

KGF (codon optimized)

XbaI

5' -AGTTTGTGATCTAGAAGGAGG 3' |-----OLIGO#12-----|

5' -AGTTTGTGATCTAGAAGGAGGAATAACATATGTGCAACGACATGACTCCGGAACAGATGGCT-
 -----OLIGO#14-----
 -----OLIGO#15-----
 -ACCAACGTTAACTGCTCCAGCCCGGAACGTCACACCCGTAGCTACGACTACATGGAAGGTG-
 3' -GGGCCTTGCAGTGTGGGCAT-5'
 |-----OLIGO#20-----|

-----OLIGO#15-----||-
 -GTGACATCCGTGTTTCGTCTGTTCTGCCGTACCCAGTGGTACCTGCGTATCGACAAACG-
 3' -ATAGCTGTTTGC-
 |-----OLIGO#21-----

-----OLIGO#16-----
 -TGGTAAAGTTAAAGGTACCCAGGAAATGAAAAACAACATCATGGAAATCCGTACT-
 -ACCATTTTC-5'
 -----|

-----OLIGO#17-----
 -GTTGCTGTTGGTATCGTTGCAATCAAAGGTGTTGAATCTGAATTCTACCTGGCAATGAACA-
 3' -ACGTTAGTTTCCACAACCTTA-5'
 |-----OLIGO#22-----|

-----OLIGO#17-----||-----
 -AAGAAGGTAAACTGTACGCAAAAAAGAATGCAACGAAGACTGCAACTTCAAAGAACTGAT-
 3' -GAAGTTTCTTGACTA-
 |-----OLIGO#23-----

-----OLIGO#18-----
 -CCTGGAAAACCACTACAACACCTACGCATCTGCTAAATGGACCCACAACGGTGGTGAAATG-
 -GGACC-5'
 -----|

-----OLIGO#19-----
 -TTCGTTGCTCTGAACCAGAAAGGTATCCCGGTTTCGTGGTAAAAAAACCAAAAAGAACAGA-
 3' -GGTCTTTCCATAGGGCCAAG -5'
 |-----OLIGO#24-----|

-----OLIGO#19-----|
 -AAACCGCTCACTTCCTGCCGATGGCAATCACTTAATAGGATCCAGTTTGA-3'
 3' -AATTATCCTAGGTCAAACT-5'
 |-----OLIGO#13-----|

BamHI

FIG.7

KGF R(144)Q

5' -ATGTGCAATGATATGACTCCTGAACAAATGGCTACCAATGTCAACTGTTCTCTCCGGAG-
 -----+-----+-----+-----+-----+-----+-----+ 60
 M C N D M T P E Q M A T N V N C S S P E

 -CGCCACACCCGGAGTTACGATTACATGGAAGGTGGGGATATTCGCGTACGTCGTCTGTTC-
 -----+-----+-----+-----+-----+-----+-----+ 120
 R H T R S Y D Y M E G G D I R V R R L F

 -TGCCGTACCCAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAG-
 -----+-----+-----+-----+-----+-----+-----+ 180
 C R T Q W Y L R I D K R G K V K G T Q E

 -ATGAAAACAACACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATC-
 -----+-----+-----+-----+-----+-----+-----+ 240
 M K N N Y N I M E I R T V A V G I V A I

 -AAAGGTGTTGAATCTGAATTCTATCTTGCAATGAACAAGGAAGGAAAACCTCTATGCAAAG-
 -----+-----+-----+-----+-----+-----+-----+ 300
 K G V E S E F Y L A M N K E G K L Y A K

 -AAAGAATGCAATGAAGATTGTAACCTCAAAGAACTAATTCTGGAAAACCATTAACAACA-
 -----+-----+-----+-----+-----+-----+-----+ 360
 K E C N E D C N F K E L I L E N H Y N T

 -TATGCATCTGCTAAATGGACCCACAACGGTGGTGAATGTTCGTTGCTCTGAACCAGAAA-
 -----+-----+-----+-----+-----+-----+-----+ 420
 Y A S A K W T H N G G E M F V A L N Q K

 -GGTATCCCTGTTCAAGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCG-
 -----+-----+-----+-----+-----+-----+-----+ 480
 G I P V Q G K K T K K E Q K T A H F L P

 -ATGGCAATCACTTAA-3'
 -----+----- 495
 M A I T *

FIG.8

KGF C(1,15)S/R(144)E

```

5' -ATGTCTAATGATATGACTCCGGAACAGATGGCTACCAACGTTAACTCCTCCTCCCCGGAA-
-----+-----+-----+-----+-----+-----+ 60
M S N D M T P E Q M A T N V N S S S P E

-CGTCACACGCGTTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTC-
-----+-----+-----+-----+-----+-----+ 120
R H T R S Y D Y M E G G D I R V R R L F

-TGCCGTACCCAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAG-
-----+-----+-----+-----+-----+-----+ 180
C R T Q W Y L R I D K R G K V K G T Q E

-ATGAAAAACAAC TACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATC-
-----+-----+-----+-----+-----+-----+ 240
M K N N Y N I M E I R T V A V G I V A I

-AAAGGTGTTGAATCTGAATTCTATCTTGCAATGAACAAGGAAGGAAACTCTATGCAAAG-
-----+-----+-----+-----+-----+-----+ 300
K G V E S E F Y L A M N K E G K L Y A K

-AAAGAATGCAATGAAGATTGTAAC TTCAAAGAACTAATTCTGGAAAACCATTACAACACA-
-----+-----+-----+-----+-----+-----+ 360
K E C N E D C N F K E L I L E N H Y N T

-TATGCATCTGCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGAAA-
-----+-----+-----+-----+-----+-----+ 420
Y A S A K W T H N G G E M F V A L N Q K

-GGTATCCCTGTTGAAGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCG-
-----+-----+-----+-----+-----+-----+ 480
G I P V E G K K T K K E Q K T A H F L P

-ATGGCAATCACTTAA-3'
-----+----- 495
M A I T *
```

FIG.9

KGF C(1,15)S/R(144)Q

```

5'-ATGTCTAATGATATGACTCCGGAACAGATGGCTACCAACGTTAACTCCTCCTCCCCGGAA-
-----+-----+-----+-----+-----+-----+-----+ 60
M S N D M T P E Q M A T N V N S S S P E

-CGTCACACGCGTTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTC-
-----+-----+-----+-----+-----+-----+-----+ 120
R H T R S Y D Y M E G G D I R V R R L F

-TGCCGTACCCAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAG-
-----+-----+-----+-----+-----+-----+-----+ 180
C R T Q W Y L R I D K R G K V K G T Q E

-ATGAAAACAACACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATC-
-----+-----+-----+-----+-----+-----+-----+ 240
M K N N Y N I M E I R T V A V G I V A I

-AAAGGTGTTGAATCTGAATTCTATCTTGCAATGAACAAGGAAGGAAACTCTATGCAAAG-
-----+-----+-----+-----+-----+-----+-----+ 300
K G V E S E F Y L A M N K E G K L Y A K

-AAAGAATGCAATGAAGATTGTAACCTCAAAGAACTAATTCTGGAAAACCATTACAACACA-
-----+-----+-----+-----+-----+-----+-----+ 360
K E C N E D C N F K E L I L E N H Y N T

-TATGCATCTGCTAAATGGACCCACAACGGTGGTGAAATGTTCGTTGCTCTGAACCAGAAA-
-----+-----+-----+-----+-----+-----+-----+ 420
Y A S A K W T H N G G E M F V A L N Q K

-GGTATCCCTGTTCAAGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCG-
-----+-----+-----+-----+-----+-----+-----+ 480
G I P V Q G K K T K K E Q K T A H F L P

-ATGGCAATCACTTAA-3'
-----+----- 495
M A I T *
```

FIG.10

KGF ΔN23/R(144)Q

```

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
-----+-----+-----+-----+-----+-----+-----+ 60
M S Y D Y M E G G D I R V R R L F C R T

-CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
-----+-----+-----+-----+-----+-----+-----+ 120
Q W Y L R I D K R G K V K G T Q E M K N

-AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
-----+-----+-----+-----+-----+-----+-----+ 180
N Y N I M E I R T V A V G I V A I K G V

-GAATCTGAATTCTATCTTGCAATGAACAAGGAAGGAAAACCTCTATGCAAAGAAAGAATGC-
-----+-----+-----+-----+-----+-----+-----+ 240
E S E F Y L A M N K E G K L Y A K K E C-

-AATGAAGATTGTAACCTCAAAGAACTAATTCTGGAAAACCATTACAACACATATGCATCT-
-----+-----+-----+-----+-----+-----+-----+ 300
N E D C N F K E L I L E N H Y N T Y A S

-GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGAAAGGTATCCCT-
-----+-----+-----+-----+-----+-----+-----+ 360
A K W T H N G G E M F V A L N Q K G I P

-GTTCAAGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCGATGGCAATC-
-----+-----+-----+-----+-----+-----+-----+ 420
V Q G K K T K K E Q K T A H F L P M A I

-ACTTAA-3'
----- 426
T *
```

FIG. 11

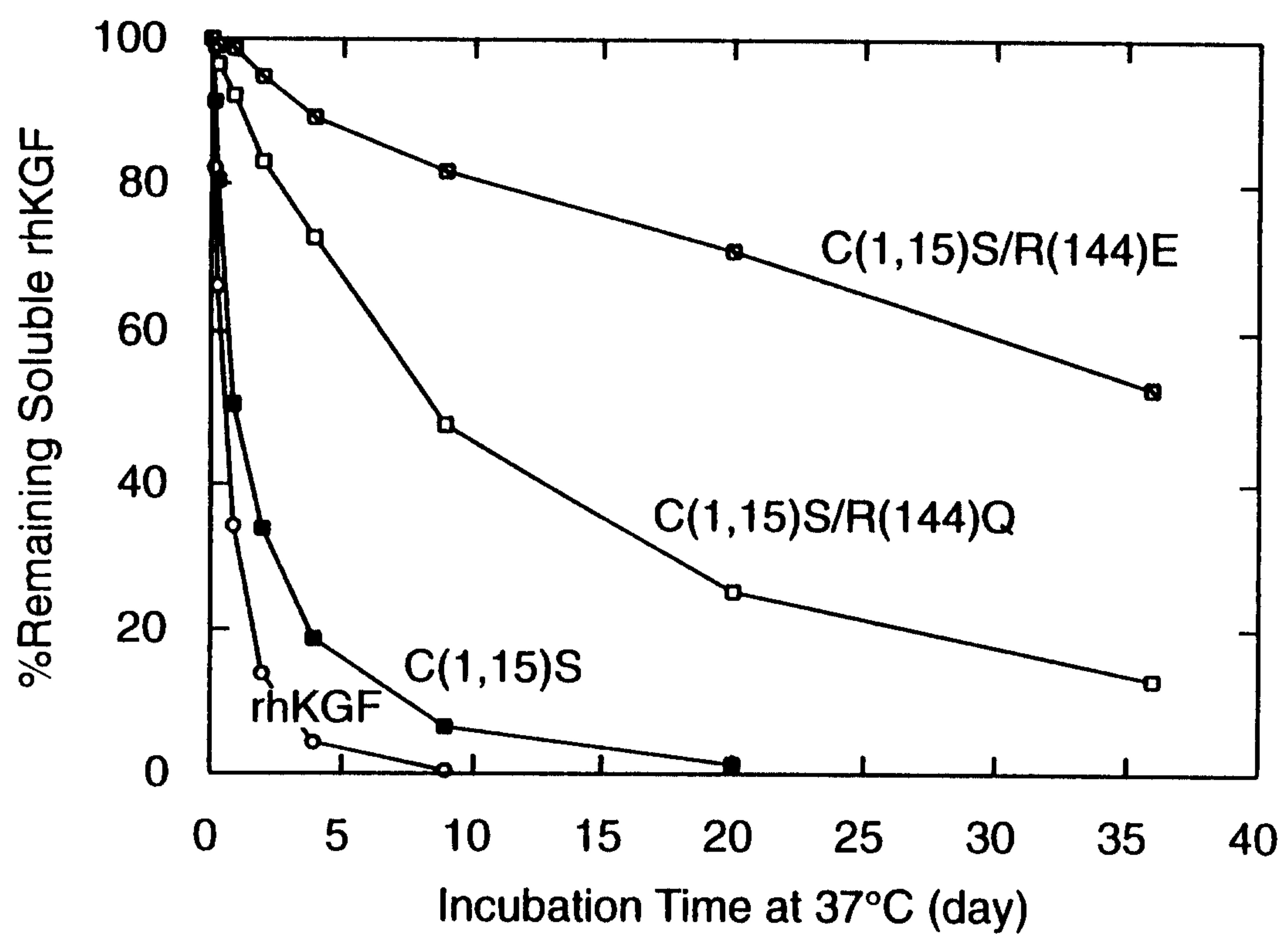


FIG.12

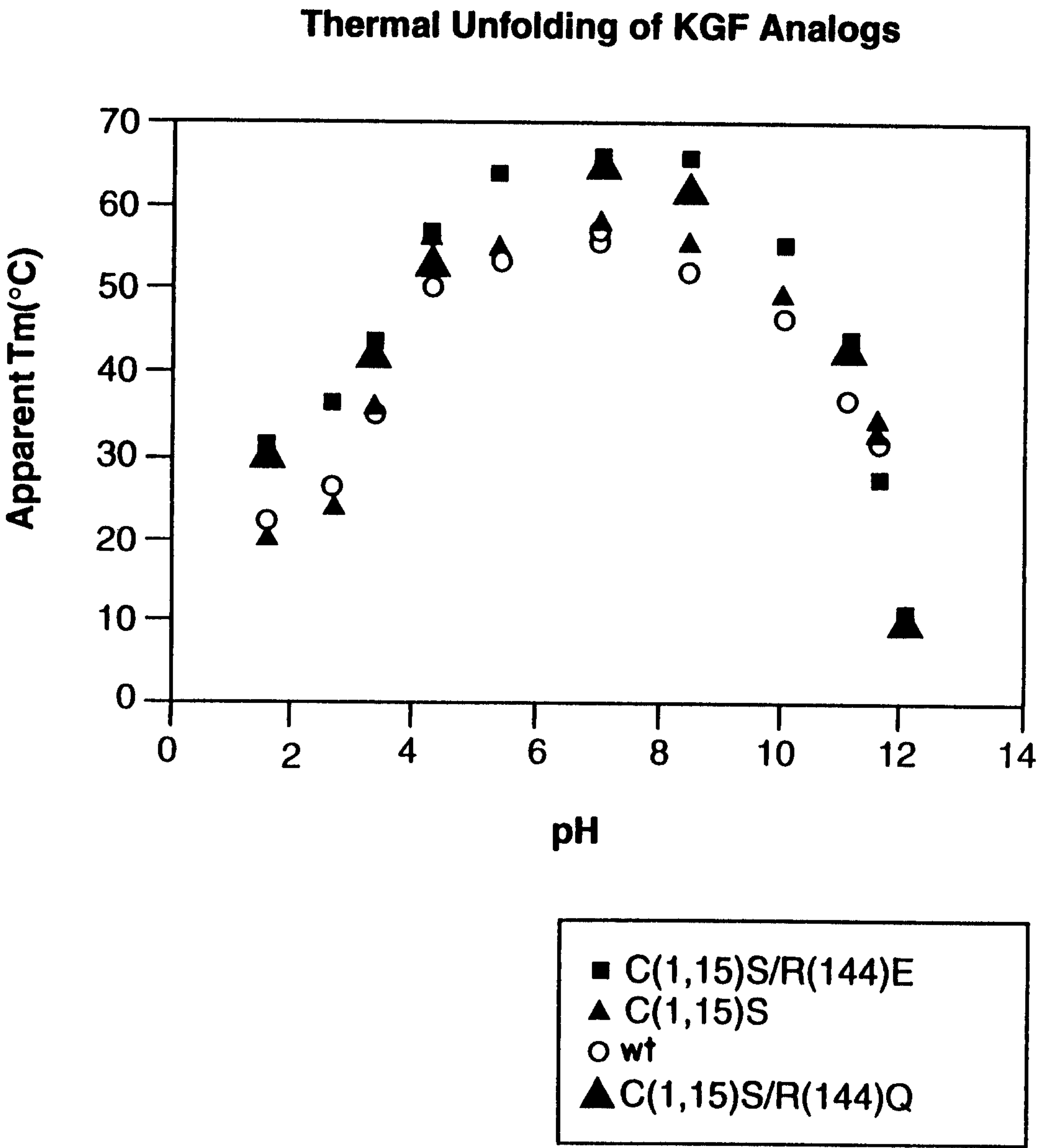


FIG.13

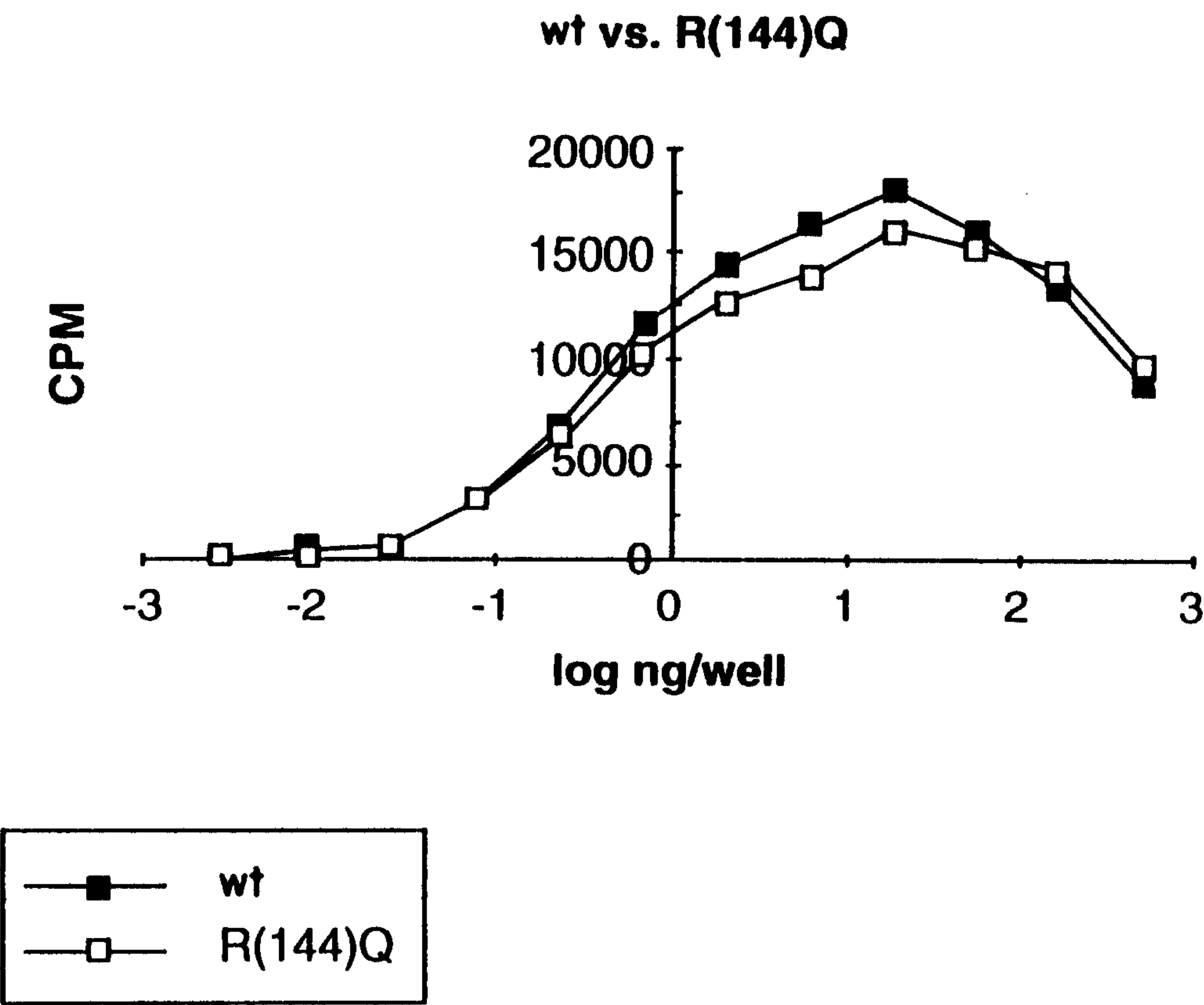


FIG.14

wt vs. Δ N23/R(144)Q

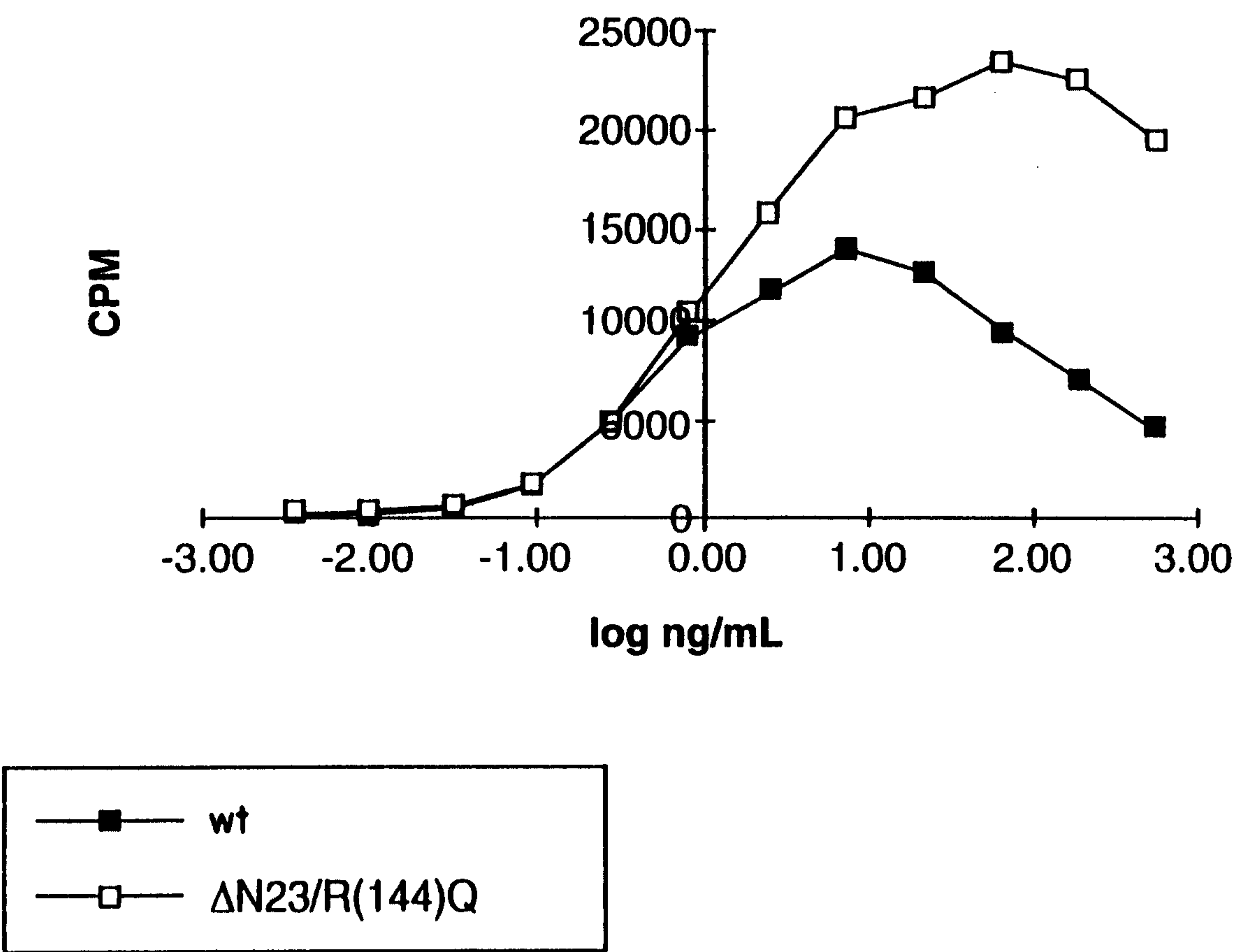


FIG.15

wt vs. C(1,15)S/R(144)Q

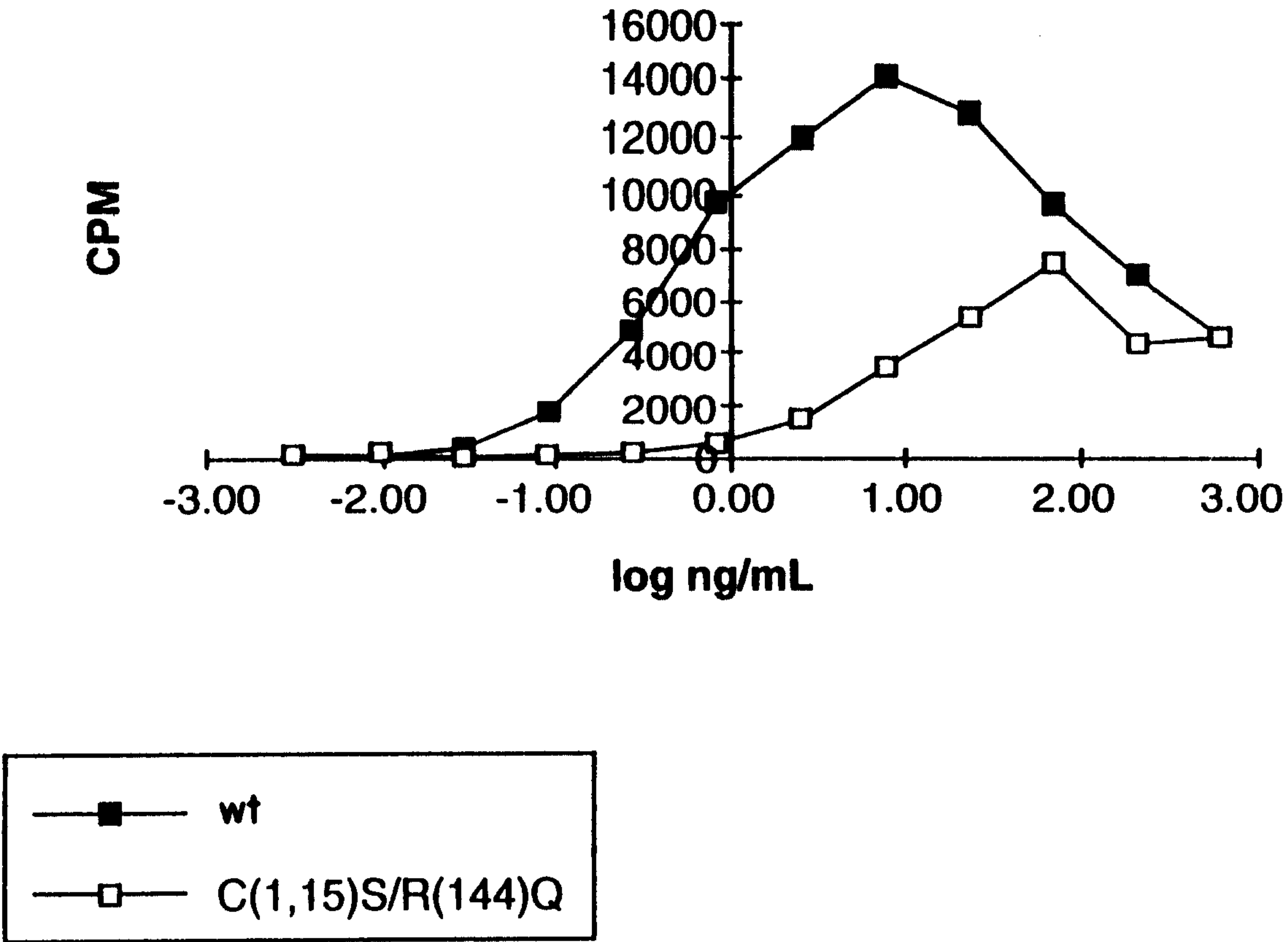


FIG.16

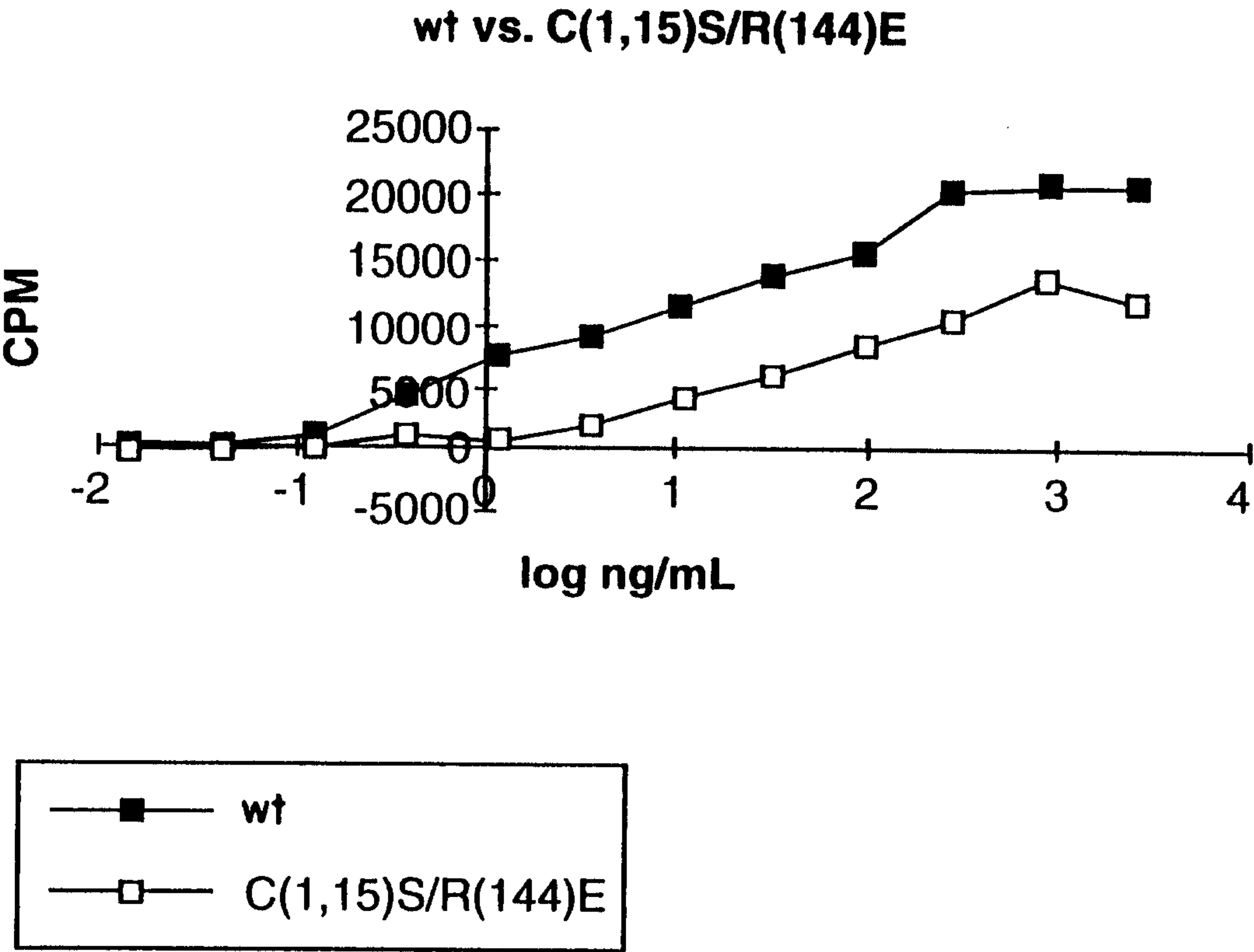


FIG.17

 Δ N23/N(137)E

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
 +-----+-----+-----+-----+-----+-----+
 M S Y D Y M E G G D I R V R R L F C R T -
 -CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
 +-----+-----+-----+-----+-----+-----+
 Q W Y L R I D K R G K V K G T Q E M K N -
 -AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
 +-----+-----+-----+-----+-----+-----+
 N Y N I M E I R T V A V G I V A I K G V -
 -GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAAGAATGC-
 +-----+-----+-----+-----+-----+-----+
 E S E F Y L A M N K E G K L Y A K K E C -
 -AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT-
 +-----+-----+-----+-----+-----+-----+
 N E D C N F K E L I L E N H Y N T Y A S -
 -GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGGAACAGAAAGGTATCCCT-
 +-----+-----+-----+-----+-----+-----+
 A K W T H N G G E M F V A L E Q K G I P -
 -GTTTCGTGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCGATGGCAATC-
 +-----+-----+-----+-----+-----+-----+
 V R G K K T K K E Q K T A H F L P M A I -
 -ACTTAA-3'
 +-----
 T *

FIG.18

 Δ N23/K(139)E

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
+-----+-----+-----+-----+-----+-----+
M S Y D Y M E G G D I R V R R L F C R T -
-CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC
+-----+-----+-----+-----+-----+-----+
Q W Y L R I D K R G K V K G T Q E M K N -
-AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
+-----+-----+-----+-----+-----+-----+
N Y N I M E I R T V A V G I V A I K G V -
-GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAAGAATGC-
+-----+-----+-----+-----+-----+-----+
E S E F Y L A M N K E G K L Y A K K E C -
-AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT
+-----+-----+-----+-----+-----+-----+
N E D C N F K E L I L E N H Y N T Y A S -
-GCTAAATGGACCCACAACGGTGGTGAAATGTTCGTTGCTCTGAACCAGGAAGGTATCCCT-
+-----+-----+-----+-----+-----+-----+
A K W T H N G G E M F V A L N Q E G I P -
-GTTCGTGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCGATGGCAATC-
+-----+-----+-----+-----+-----+-----+
V R G K K T K K E Q K T A H F L P M A I -
-ACTTAA-3'
+-----
T *

FIG.19

ΔN23/K(139)Q

```

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
+-----+-----+-----+-----+-----+-----+-----+
M  S  Y  D  Y  M  E  G  G  D  I  R  V  R  R  L  F  C  R  T  -
-CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
+-----+-----+-----+-----+-----+-----+-----+
Q  W  Y  L  R  I  D  K  R  G  K  V  K  G  T  Q  E  M  K  N  -
-AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
+-----+-----+-----+-----+-----+-----+-----+
N  Y  N  I  M  E  I  R  T  V  A  V  G  I  V  A  I  K  G  V  -
-GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAAGAATGC-
+-----+-----+-----+-----+-----+-----+-----+
E  S  E  F  Y  L  A  M  N  K  E  G  K  L  Y  A  K  K  E  C  -
-AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT-
+-----+-----+-----+-----+-----+-----+-----+
N  E  D  C  N  F  K  E  L  I  L  E  N  H  Y  N  T  Y  A  S  -
-GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGCAAGGTATCCCT-
+-----+-----+-----+-----+-----+-----+-----+
A  K  W  T  H  N  G  G  E  M  F  V  A  L  N  Q  Q  G  I  P  -
-GTTCGTGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCGATGGCAATC-
+-----+-----+-----+-----+-----+-----+-----+
V  R  G  K  K  T  K  K  E  Q  K  T  A  H  F  L  P  M  A  I  -
-ACTTAA-3'
+-----
T  *
```

FIG.20

 Δ N23/R(144)A

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
+-----+-----+-----+-----+-----+-----+-----+-----+
M S Y D Y M E G G D I R V R R L F C R T -
-CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
+-----+-----+-----+-----+-----+-----+-----+-----+
Q W Y L R I D K R G K V K G T Q E M K N -
-AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
+-----+-----+-----+-----+-----+-----+-----+-----+
N Y N I M E I R T V A V G I V A I K G V -
-GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAAGAATGC-
+-----+-----+-----+-----+-----+-----+-----+-----+
E S E F Y L A M N K E G K L Y A K K E C -
-AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT-
+-----+-----+-----+-----+-----+-----+-----+-----+
N E D C N F K E L I L E N H Y N T Y A S -
-GCTAAATGGACCCACAACGGTGGTGAAATGTTCGTTGCTCTGAACCAGAAAGGTATCCCT-
+-----+-----+-----+-----+-----+-----+-----+-----+
A K W T H N G G E M F V A L N Q K G I P -
-GTTGCTGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCGATGGCAATC-
+-----+-----+-----+-----+-----+-----+-----+-----+
V A G K K T K K E Q K T A H F L P M A I -
-ACTTAA-3'
+-----
T *

FIG.21

Δ N23/R(144)L

```

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
  +-----+-----+-----+-----+-----+-----+
  M S Y D Y M E G G D I R V R R L F C R T -
- CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
  +-----+-----+-----+-----+-----+-----+
  Q W Y L R I D K R G K V K G T Q E M K N -
- AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
  +-----+-----+-----+-----+-----+-----+
  N Y N I M E I R T V A V G I V A I K G V -
- GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAAGAATGC-
  +-----+-----+-----+-----+-----+-----+
  E S E F Y L A M N K E G K L Y A K K E C -
- AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT-
  +-----+-----+-----+-----+-----+-----+
  N E D C N F K E L I L E N H Y N T Y A S -
- GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGAAAGGTATCCCT-
  +-----+-----+-----+-----+-----+-----+
  A K W T H N G G E M F V A L N Q K G I P -
- GTTCTGGGTAAGAAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCGATGGCAATC-
  +-----+-----+-----+-----+-----+-----+
  V L G K K T K K E Q K T A H F L P M A I -
- ACTTAA-3'
  +-----
  T *
```

FIG.22

 Δ N23/K(147)E

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
 +-----+-----+-----+-----+-----+-----+
 M S Y D Y M E G G D I R V R R L F C R T -

 -CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
 +-----+-----+-----+-----+-----+-----+
 Q W Y L R I D K R G K V K G T Q E M K N -

 -AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
 +-----+-----+-----+-----+-----+-----+
 N Y N I M E I R T V A V G I V A I K G V -

 -GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAGAATGC-
 +-----+-----+-----+-----+-----+-----+
 E S E F Y L A M N K E G K L Y A K K E C -

 -AACGAAGACTGCAACTTCAAAGAAGTATCCTGGAAAACCACTACAACACCTACGCATCT-
 +-----+-----+-----+-----+-----+-----+
 N E D C N F K E L I L E N H Y N T Y A S -

 -GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGAAAGGTATTCCT-
 +-----+-----+-----+-----+-----+-----+
 A K W T H N G G E M F V A L N Q K G I P -

 -GTTTCGTGGTAAGGAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCGATGGCAATC-
 +-----+-----+-----+-----+-----+-----+
 V R G K E T K K E Q K T A H F L P M A I -

 -ACTTAA-3'
 +-----
 T *

FIG.23

 Δ N23/K(147)Q

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
+-----+-----+-----+-----+-----+-----+-----+-----
M S Y D Y M E G G D I R V R R L F C R T -
-CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
+-----+-----+-----+-----+-----+-----+-----+-----
Q W Y L R I D K R G K V K G T Q E M K N -
-AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
+-----+-----+-----+-----+-----+-----+-----+-----
N Y N I M E I R T V A V G I V A I K G V -
-GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAAGAATGC-
+-----+-----+-----+-----+-----+-----+-----+-----
E S E F Y L A M N K E G K L Y A K K E C -
-AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT-
+-----+-----+-----+-----+-----+-----+-----+-----
N E D C N F K E L I L E N H Y N T Y A S -
-GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGAAAGGTATCCCT-
+-----+-----+-----+-----+-----+-----+-----+-----
A K W T H N G G E M F V A L N Q K G I P -
-GTTTCGTGGTAAGCAAACCAAGAAAGAACAGAAAACCGCTCACTTCCTGCCGATGGCAATC-
+-----+-----+-----+-----+-----+-----+-----+-----
V R G K Q T K K E Q K T A H F L P M A I -
-ACTTAA-3'
+-----
T *

FIG.24

 Δ N23/K(153)E

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
 +-----+-----+-----+-----+-----+-----+-----+
 M S Y D Y M E G G D I R V R R L F C R T -
 -CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
 +-----+-----+-----+-----+-----+-----+-----+
 Q W Y L R I D K R G K V K G T Q E M K N -
 -AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
 +-----+-----+-----+-----+-----+-----+-----+
 N Y N I M E I R T V A V G I V A I K G V -
 -GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAGAATGC-
 +-----+-----+-----+-----+-----+-----+-----+
 E S E F Y L A M N K E G K L Y A K K E C -
 -AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT-
 +-----+-----+-----+-----+-----+-----+-----+
 N E D C N F K E L I L E N H Y N T Y A S -
 -GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGAAAGGTATCCCT-
 +-----+-----+-----+-----+-----+-----+-----+
 A K W T H N G G E M F V A L N Q K G I P -
 -GTTCGTGGTAAGAAAACCAAGAAAGAACAGGAAACCGCTCACTTCCTGCCGATGGCAATC-
 +-----+-----+-----+-----+-----+-----+-----+
 V R G K K T K K E Q E T A H F L P M A I -
 -ACTTAA-3'
 +-----
 T *

FIG.25

ΔN23/K(153)Q

```

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
+-----+-----+-----+-----+-----+-----+
M S Y D Y M E G G D I R V R R L F C R T -
- CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
+-----+-----+-----+-----+-----+-----+
Q W Y L R I D K R G K V K G T Q E M K N -
-AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
+-----+-----+-----+-----+-----+-----+
N Y N I M E I R T V A V G I V A I K G V -
-GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAAGAATGC-
+-----+-----+-----+-----+-----+-----+
E S E F Y L A M N K E G K L Y A K K E C -
-AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT-
+-----+-----+-----+-----+-----+-----+
N E D C N F K E L I L E N H Y N T Y A S -
-GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGAAAGGTATCCCT-
+-----+-----+-----+-----+-----+-----+
A K W T H N G G E M F V A L N Q K G I P -
-GTTCGTGGTAAGAAAACCAAGAAAGAACAGCAAACCGCTCACTTCCTGCCGATGGCAATC-
+-----+-----+-----+-----+-----+-----+
V R G K K T K K E Q Q T A H F L P M A I -
-ACTTAA-3'
+-----
T * -

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FIG.26

 Δ N23/Q(152)E/K(153)E

5' -ATGTCCTACGACTACATGGAAGGTGGTGACATCCGCGTACGTCGTCTGTTCTGCCGTACC-
 +-----+-----+-----+-----+-----+-----+
 M S Y D Y M E G G D I R V R R L F C R T -
 -CAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAAC-
 +-----+-----+-----+-----+-----+-----+
 Q W Y L R I D K R G K V K G T Q E M K N -
 -AACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTT-
 +-----+-----+-----+-----+-----+-----+
 N Y N I M E I R T V A V G I V A I K G V -
 -GAATCTGAATTCTACCTGGCAATGAACAAAGAAGGTAAACTGTACGCAAAAAAAGAATGC-
 +-----+-----+-----+-----+-----+-----+
 E S E F Y L A M N K E G K L Y A K K E C -
 -AACGAAGACTGCAACTTCAAAGAACTGATCCTGGAAAACCACTACAACACCTACGCATCT-
 +-----+-----+-----+-----+-----+-----+
 N E D C N F K E L I L E N H Y N T Y A S -
 -GCTAAATGGACCCACAACGGTGGTGAAATGTTTCGTTGCTCTGAACCAGAAAGGTATCCCT-
 +-----+-----+-----+-----+-----+-----+
 A K W T H N G G E M F V A L N Q K G I P -
 -GTTTCGTGGTAAGAAAACCAAGAAAGAAGAGGAAACCGCTCACTTCCTGCCGATGGCAATC-
 +-----+-----+-----+-----+-----+-----+
 V R G K K T K K E E E T A H F L P M A I -
 -ACTTAA-3'
 +-----
 T *