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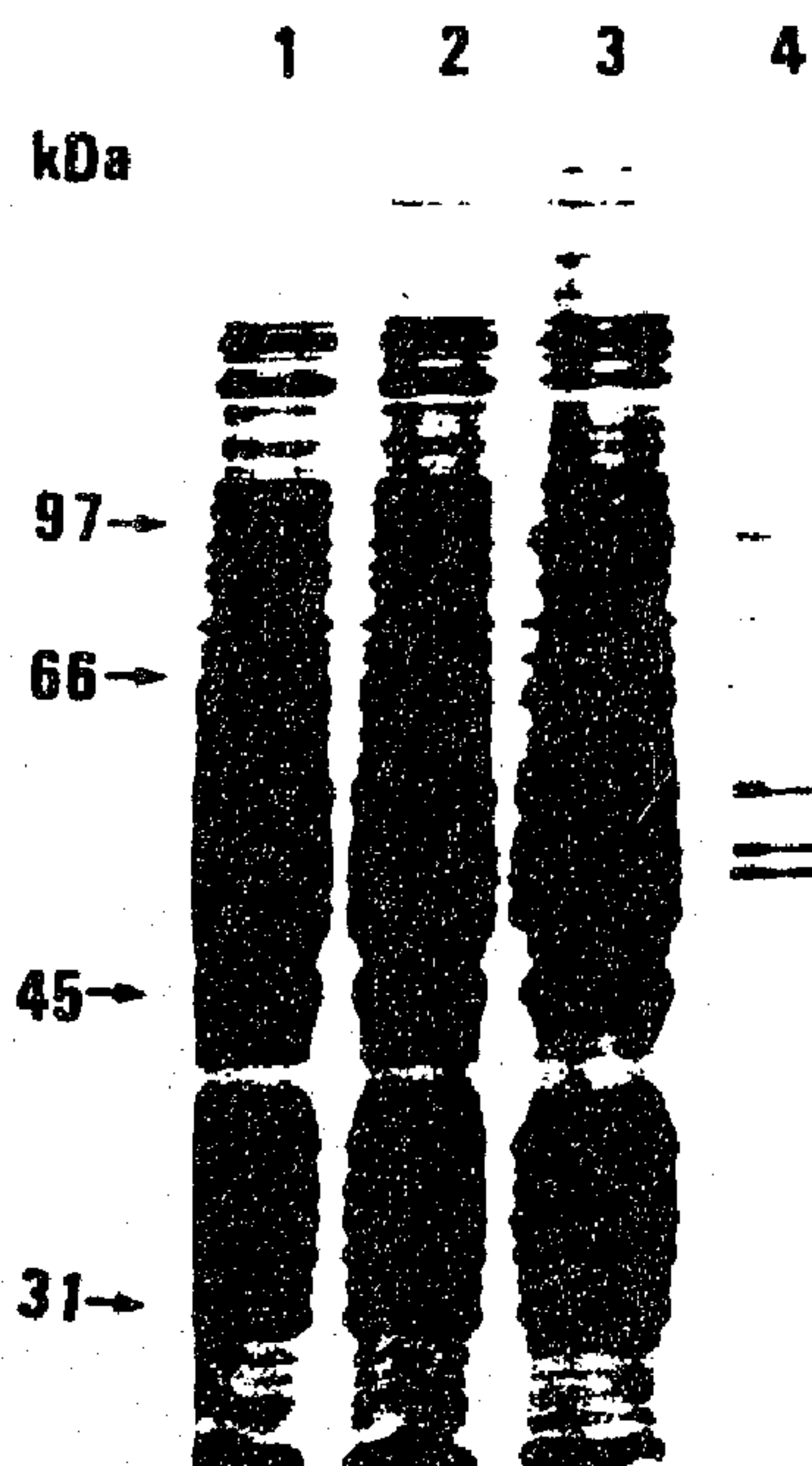
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(54) **CARBOXYLASE DEPENDANTE DE LA VITAMINE K**

(54) **VITAMIN K-DEPENDENT CARBOXYLASE**



(57) L'invention se rapporte a un ADN isolé qui code pour une carboxylase dépendante de la vitamine K. La carboxylase est choisie dans le groupe constitué: (a) par un ADN isolé qui code pour une carboxylase bovine ou humaine dépendante de la vitamine K; (b) par un ADN isolé qui s'hybride avec l'ADN isolé mentionné en (a) et qui code pour une carboxylase dépendante de la vitamine K; et (c) par un ADN isolé différent des ADN isolés mentionnés en (a) et en (b) dans la séquence des nucléotides en raison de la dégénérescence du code génétique, et qui code pour une carboxylase dépendante de la vitamine K. L'invention décrit également des vecteurs et des cellules hôtes contenant les ADN mentionnés, à des procédés d'utilisation de ceux-ci, et à des protéines purifiées pour lesquelles codes les ADN mentionnés.

(57) Isolated DNA encoding a vitamin K dependent carboxylase is disclosed. The carboxylase is selected from the group consisting of: (a) isolated DNA which encodes bovine or human vitamin K dependent carboxylase; (b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a vitamin K dependent carboxylase; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encodes a vitamin K dependent carboxylase: Also disclosed are vectors and host cells containing the aforesaid DNA, methods of using the same, and purified protein coded for by the aforesaid DNA.



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(21) International Application Number: PCT/US92/03853 (22) International Filing Date: 8 May 1992 (08.05.92) (30) Priority data: 697,427 8 May 1991 (08.05.91) US 756,250 9 September 1991 (09.09.91) US (71) Applicant: THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; 300 Bynum Hall, Campus Box 4100, Chapel Hill, NC 27599-4100 (US). (72) Inventors: STAFFORD, Darrell, E. ; WU, Sheue-Mei ; 300 Rainbow Drive, Carrboro, NC 27510 (US). (74) Agents: SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).		(81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (Euro- pean patent), MC (European patent), NL (European pa- tent), SE (European patent). 2102702 Published <i>With international search report.</i>
(54) Title: VITAMIN K-DEPENDENT CARBOXYLASE (57) Abstract Isolated DNA encoding a vitamin K dependent carboxylase is disclosed. The carboxylase is selected from the group consisting of: (a) isolated DNA which encodes bovine or human vitamin K dependent carboxylase; (b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a vitamin K dependent carboxylase; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encodes a vitamin K dependent carboxylase. Also disclosed are vectors and host cells containing the aforesaid DNA, methods of using the same, and purified protein coded for by the aforesaid DNA.		

VITAMIN K-DEPENDENT CARBOXYLASE

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Field of the Invention

5 This invention relates to DNA sequences encoding enzymes, known as the vitamin K-dependent carboxylases, which carry out the γ -carboxylation of glutamic acid residues in vitamin K-dependent proteins such as Factor VII, Factor IX, Factor X, Protein C, Protein S, and Prothrombin.

Background of the Invention

10 A number of blood coagulation proteins require a post-translational, vitamin K-dependent modification for biological activity. In 1974, Stenflo et al., Nelsestuen et al. and Magnusson et al. reported that the prototype of these vitamin K-dependent proteins, prothrombin, contained the modified amino acid, γ -carboxyglutamic acid (Gla). See J. Stenflo et al., *Proc. Natl. Acad. Sci. USA*
15 71:2730-2733 (1974); G. Nelsestuen et al., *J. Biol. Chem.* **249**:6347-6350 (1974); S. Magnusson et al., *FEBS Lett.* **44**:189-193 (1974). Prothrombin from animals treated with the vitamin K antagonist warfarin lacked this Gla modification. It was inferred from these observations that the blood-clotting activity of the vitamin K-dependent proteins required γ -carboxylation of specific glutamic acid residues.
20 Shortly thereafter, Esmon et al. demonstrated an enzyme activity, vitamin K-dependent carboxylase (hereafter called

carboxylase), capable of making this Gla modification. See C. Esmon et al., *J. Biol. Chem.* **250**:4744-4748 (1975).

After cDNA sequences were obtained for several of the vitamin K-dependent proteins, Pan and Price compared the deduced amino-acid sequences and suggested that the propeptide consensus sequence preceding the amino terminus of the vitamin K-dependent protein was a recognition site for the carboxylase. See L. Pan and P. Price, *Proc. Natl. Acad. Sci. USA* **82**:6109-6113 (1985). This suggestion was confirmed by Knobloch and Suttie, who demonstrated the importance of the propeptide in carboxylation by showing that the synthetic propeptide sequence of human factor X stimulated the activity of the carboxylase for a small substrate (Boc-Glu-Glu-Leu-OMe) *in vitro*. J. Knobloch and J. Suttie, *J. Biol. Chem.* **262**:16157-16163 (1987). Jorgensen extended this observation by showing that factor IX with its propeptide deleted was not carboxylated. M. Jorgensen et al., *Cell* **48**:185-191 (1987).

In spite of its importance, the carboxylase has not been previously purified. Purification of 400-fold has been reported. See J.-M. Girardot, *J. Biol. Chem.* **257**:15008-15011 (1982). Comparison of Girardot's results to later purifications is complicated because, in our hands, ammonium sulfate and the propeptide stimulate the incorporation of CO₂ into the synthetic peptide substrate FLEEL by 13-fold. If one corrects for the lack of ammonium sulfate and propeptide in Girardot's assay mix, then he achieved a specific activity of 1.1×10^7 cpm/mg/hr. Soute et al. demonstrated that an immobilized factor X antibody would bind the carboxylase, presumably through a factor X precursor-carboxylase complex, and that the bound carboxylase retained its activity for the synthetic peptide substrate FLEEL. See B. Soute et al., *Biochem. Biophys. Acta.* **676**:101-107 (1981). Harbeck et al. extended this method by eluting the carboxylase from a prothrombin antibody column with a synthetic propeptide achieving a 500-fold purification and a final specific activity of 6.6

x 10^6 cpm/mg/hr. See M. Harbeck et al., *Thromb. Res.* **56**:317-323 (1989). Hubbard et al. reported the purification of the carboxylase to homogeneity using a synthetic propeptide sequence as an affinity ligand. See B. Hubbard
5 et al., *Proc. Natl. Acad. Sci. USA* **86**:6893-6897 (1989). However, the reported final specific activity, 1.3×10^7 cpm/mg/hr, was still not significantly different than that reported by Girardot. Numerous studies with the crude
10 carboxylase have yielded important information about its properties and mode of action. See J. Suttie, *Ann. Rev. Biochem.* **54**:459-77 (1985). It is clear, however, that for detailed mechanistic studies and physical characterization of the enzyme, purification is necessary.

We recently reported the production in *E. coli* of
15 four different 59-residue peptides containing the propeptide and Gla domain of human factor IX. See S.-M. Wu et al., *J. Biol. Chem.* **265**:13124-13129 (1990). We report here that one of these peptides, FIXQ/S (SEQ ID NO:1), is an excellent affinity ligand for purification of the
20 carboxylase. A 7,000-fold purification of the carboxylase to approximately 80%-90% apparent purity and final specific activity of about 2.4×10^9 cpm/mg/hr was obtained. The apparent molecular weight was 94,000 by reducing SDS-PAGE analysis.

25 Summary of the Invention

A first object of an aspect of the present invention is isolated DNA encoding a vitamin K dependent carboxylase selected from the group consisting of: (a) isolated DNA selected from the group consisting of DNA
30 which encodes bovine vitamin K dependent carboxylase and DNA which encodes human vitamin K dependent carboxylase (b) isolated DNA which hybridizes to isolated DNA of (a) above

and which encodes a vitamin K dependent carboxylase; and
(c) isolated DNA differing from the isolated DNAs of (a)
and (b) above in nucleotide sequence due to the degeneracy
of the genetic code, and which encodes a vitamin K
5 dependent carboxylase.

A second object of an aspect of the present
invention is a recombinant DNA sequence comprising vector
DNA and a DNA according encoding vitamin K dependent
carboxylase as given above.

10 A third object of an aspect of the present
invention is a host cell containing a recombinant DNA
sequence as given above and capable of expressing the
encoded vitamin K dependent carboxylase.

A fourth object of an aspect of the present
15 invention is an improved method of making a vitamin K
dependent protein. The method comprises culturing a host
cell which expresses a vitamin K dependent protein in the
presence of vitamin K; and then harvesting said vitamin K
dependent protein from the culture. The improvement
20 comprises employing as the host cell a host cell which
contains a recombinant DNA sequence comprising vector DNA
operable in the host cell and a DNA encoding a vitamin K
dependent carboxylase selected from the group consisting
of: (a) isolated DNA selected from the group consisting of
25 DNA which encodes bovine vitamin K dependent carboxylase
and DNA which encodes human vitamin K dependent
carboxylase; (b) isolated DNA which hybridizes to isolated
DNA of (a) above and which encodes a vitamin K dependent
carboxylase; and (c) isolated DNA differing from the
30 isolated DNAs of (a) and (b) above in nucleotide sequence

due to the degeneracy of the genetic code, and which encodes a vitamin K dependent carboxylase.

The foregoing and other objects of aspects of the present invention are explained in detail in the drawings, Examples, and Detailed Description set forth below.

In accordance with one aspect of the present invention, there is provided isolated DNA encoding a vitamin K dependent carboxylase selected from the group consisting of:

10 (a) isolated DNA selected from the group consisting of DNA which encodes a bovine 94,000 dalton vitamin K dependent carboxylase having the sequence of SEQ ID NO: 9, SEQ ID NO: 11 or SEQ ID NO: 13, and DNA having the sequence given herein as SEQ ID NO: 15 and which
15 encodes human vitamin K dependent carboxylase;

(b) isolated DNA which hybridizes to the complementary strand of isolated DNA of (a) above under stringent conditions represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, and 0.1% SDS at 70°C and
20 which encodes a vitamin K dependent carboxylase at least 75% homologous to isolated DNA of (a) above; and

(c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encodes a vitamin
25 K dependent carboxylase.

In accordance with another embodiment of the present invention, there is provided a method of making a vitamin K dependent protein which comprises culturing a host cell which expresses a vitamin K dependent protein in
30 the presence of vitamin K, and then harvesting the vitamin K dependent protein from the culture, the improvement

comprises:

employing as the host cell a eukaryotic host cell containing a recombinant DNA molecule comprising cloning vector DNA operable in the host cell and a DNA encoding a vitamin K dependent carboxylase selected from the group consisting of:

(a) isolated DNA selected from the group consisting of DNA which encodes a bovine 94,000 dalton vitamin K dependent carboxylase having the sequence of SEQ ID NO: 9, SEQ ID NO: 11 or SEQ ID NO: 13, and DNA having the sequence given herein as SEQ ID NO: 15 and which encodes human vitamin K dependent carboxylase;

(b) isolated DNA which hybridizes to the complementary strand of isolated DNA of (a) above under stringent conditions represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, and 0.1% SDS at 70°C and which encodes a vitamin K dependent carboxylase at least 75% homologous to isolated DNA of (a) above; and

(c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encodes a vitamin K dependent carboxylase;

the host cell expressing increased amounts of the vitamin K dependent protein due to the expression of functional vitamin K dependent carboxylase by the host cell.

Brief Description of the Drawings

Figure 1. Silver-stained, 10% reducing SDS-PAGE analysis of Affi-FIXQ/STM purified carboxylase preparations to demonstrate the effect of sonication. The counts reflect the carboxylase activity in each lane. Lane 1 is the

sonicated loading material (380 cpm/30 min); lane 2 is the
sonicated flow-through (265 cpm/30 min); lane 3 is
unsonicated elution I carboxylase preparation (23,500

cpm/30 min); lane 4 is sonicated elution I carboxylase preparation (5000 cpm/30 min).

Figure 2. Autoradiogram of cross-linking between ^{125}I -FIXQ/S and protein from the partially-purified carboxylase preparation shown in **figure 1**, lane 3. Proteins were separated by 10% non-reducing SDS-PAGE. Lane 1 is proFIXI9 competition of cross-linking; lane 2 is cross-linking in absence of proFIXI9.

Figure 3. Activity profile and reducing SDS-PAGE analysis (10%, silver-stained) of fractions from CM-SepharoseTM chromatography. The fraction numbers are on the X-axis. 7.5 μl from each fraction was analyzed by SDS-PAGE. The counts, shown on the Y-axis, represent the carboxylase activity in each lane. The carboxylase activity was determined by the $^{14}\text{CO}_2$ incorporation into FLEEL in the standard assay. L is loading material; fractions 1-7 are flow-through; fractions 8-27 are wash; fractions 28-37 are elution.

Figure 4. Silver-stained, 10% reducing SDS-PAGE analysis of a CM-purified carboxylase re-chromatographed on Affi-FIXQ/S to show the correlation between the 94,000 M_r protein and activity. Lane 1 is the first Affi-FIXQ/S carboxylase preparation (9750 cpm/30 min); lane 2 is purified CM eluate (6729 cpm/30 min, used for the second Affi-FIXQ/S); lane 3 is flow-through of the second Affi-FIXQ/S (394 cpm/30 min); lane 4 is sample of second Affi-FIXQ/S elute (18853 cpm/30 min, eluate I method). The difference in activity between the carboxylase shown in lanes 2 and 4 is the result of some inactivation of the carboxylase of lane 2.

Figure 5. Activity profile and reducing SDS-PAGE analysis (10%, silver-stained) of fractions from Affi-FIXQ/S chromatography elution II. 5.0 μl of each fraction was used for SDS-PAGE analysis except that the loading material and fraction 8 were diluted 100-fold before analysis. The fraction number is shown on the X-axis. The Y-axis represents the carboxylase activity in each

lane. The carboxylase activity was determined by the $^{14}\text{CO}_2$ incorporation into FLEEL in the standard assay. L is loading material; fractions 1-20 are flow-through (because each fraction is equivalent, only one is shown); fractions 21-30 are wash; fractions 31-49 are Triton X-100 gradient; fractions 56-63 are CHAPS gradient; fractions 64-75 are CHAPS and proFIX19 double gradient; fractions 76-90 are 1% CHAPS/2 μM proFIX19 elution.

Detailed Description of the Invention

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right. Nucleotides and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by three letter code, in accordance with 37 CFR §1.822 and established usage. See, e.g., PatentIn User Manual, 99-102 (Nov. 1990) (U.S. Patent and Trademark Office, Office of the Assistant Commissioner for Patents, Washington, D.C. 20231); U.S. Patent No. 4,871,670 to Hudson et al. at Col. 3 lines 20-43 (applicants specifically intend that the disclosure of this and all other patent references cited herein be incorporated herein by reference).

A. Genetic Engineering Techniques

The production of cloned genes, recombinant DNA, vectors, transformed host cells, proteins and protein fragments by genetic engineering is well known. See, e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6 line 3 to Col. 9 line 65; U.S. Patent No. 4,877,729 to Clark et al. at Col. 4 line 38 to Col. 7 line 6; U.S. Patent No. 4,912,038 to Schilling at Col. 3 line 26 to Col. 14 line 12; and U.S. Patent No. 4,879,224 to Wallner at Col. 6 line 8 to Col. 8 line 59.

A vector is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding Vitamin K

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Dependent Carboxylase and/or to express DNA which encodes Vitamin K Dependent Carboxylase. An expression vector is a replicable DNA construct in which a DNA sequence encoding Vitamin K Dependent Carboxylase is operably linked to
5 suitable control sequences capable of effecting the expression of Vitamin K Dependent Carboxylase in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a
10 transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation.

Amplification vectors do not require expression
15 control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

Vectors comprise plasmids, viruses (e.g.,
20 adenovirus, cytomegalovirus), phage, and integratable DNA fragments (i.e., fragments integratable into the host genome by recombination). The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself.

25 Expression vectors should contain a promoter and RNA binding sites which are operably linked to the gene to be expressed and are operable in the host organism.

DNA regions are operably linked or operably associated when they are functionally related to each
30 other. For example, a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

35 Transformed host cells are cells which have been transformed or transfected with Vitamin K Dependent Carboxylase vectors constructed using recombinant DNA

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techniques. Transformed host cells ordinarily express Vitamin K Dependent Carboxylase, but host cells transformed for purposes of cloning or amplifying Vitamin K Dependent Carboxylase DNA do not need to express Vitamin K Dependent Carboxylase.

Suitable host cells include prokaryote, yeast or higher eukaryotic cells such as mammalian cells and insect cells. Cells derived from multicellular organisms are a particularly suitable host for recombinant Vitamin K Dependent Carboxylase synthesis, and mammalian cells are particularly preferred. Propagation of such cells in cell culture has become a routine procedure (Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)). Examples of useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream from the DNA encoding vitamin K dependent Carboxylase to be expressed and operatively associated therewith, along with a ribosome binding site, an RNA splice site (if intron-containing genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells are often provided by viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and Simian Virus 40 (SV40). See, e.g., U.S. Patent No. 4,599,308.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV 40 or other viral (e.g. Polyoma, Adenovirus, VSV, or BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

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Rather than using vectors which contain viral origins of replication, one can transform mammalian cells by the method of cotransformation with a selectable marker and the Vitamin K Dependent Carboxylase DNA. Examples of
5 suitable selectable markers are dihydrofolate reductase (DHFR) or thymidine kinase. This method is further described in U.S. Pat. No. 4,399,216.

Other methods suitable for adaptation to the synthesis of Vitamin K Dependent Carboxylase in recombinant
10 vertebrate cell culture include those described in M-J. Gething et al., Nature 293, 620 (1981); N. Mantei et al., Nature 281, 40; A. Levinson et al., EPO Application Nos. 117,060A and 117,058A.

Host cells such as insect cells (e.g., cultured
15 *Spodoptera frugiperda* cells) and expression vectors such as the baculovirus expression vector (e.g., vectors derived from *Autographa californica* MNPV, *Trichoplusia ni* MNPV, *Rachiplusia ou* MNPV, or *Galleria ou* MNPV) may be employed in carrying out the present invention, as described in U.S.
20 Patents Nos. 4,745,051 and 4,879,236 to Smith et al. In general, a baculovirus expression vector comprises a baculovirus genome containing the gene to be expressed inserted into the polyhedrin gene at a position ranging from the polyhedrin transcriptional start signal to the ATG
25 start site and under the transcriptional control of a baculovirus polyhedrin promoter.

Prokaryote host cells include gram negative or gram positive organisms, for example *Escherichia coli* (*E. coli*) or *Bacilli*. Higher eukaryotic cells include
30 established cell lines of mammalian origin as described below. Exemplary host cells are *E. coli* W3110 (ATCC 27,325), *E. coli* B, *E. coli* X1776 (ATCC 31,537), *E. coli* 294 (ATCC 31,446). A broad variety of suitable prokaryotic and microbial vectors are available. *E. coli* is typically
35 transformed using pBR322. Promoters most commonly used in recombinant microbial expression vectors include the beta-lactamase (penicillinase) and lactose promoter systems

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(Chang et al., Nature 275, 615 (1978); and Goeddel et al., Nature 281, 544 (1979)), a tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8, 4057 (1980) and EPO App. Publ. No. 36,776) and the tac promoter (H. De Boer et al., Proc. Natl. Acad. Sci. USA 80, 21 (1983)). The promoter and Shine-Dalgarno sequence (for prokaryotic host expression) are operably linked to the DNA encoding the Vitamin K Dependent Carboxylase, i.e., they are positioned so as to promote transcription of Vitamin K Dependent Carboxylase messenger RNA from the DNA.

Eukaryotic microbes such as yeast cultures may also be transformed with Vitamin K Dependent Carboxylase-encoding vectors. see, e.g., U.S. Patent No. 4,745,057. *Saccharomyces cerevisiae* is the most commonly used among lower eukaryotic host microorganisms, although a number of other strains are commonly available. Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding Vitamin K Dependent Carboxylase, sequences for polyadenylation and transcription termination, and a selection gene. An exemplary plasmid is YRp7, (Stinchcomb et al., Nature 282, 39 (1979); Kingsman et al., Gene 7, 141 (1979); Tschemper et al., Gene 10, 157 (1980)). Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255, 2073 (1980) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7, 149 (1968); and Holland et al., Biochemistry 17, 4900 (1978)). Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publ. No. 73,657.

B. Vitamin K-Dependent Carboxylase

Carboxylase enzymes of the present invention include proteins homologous to, and having essentially the same biological properties as, the bovine vitamin K dependent carboxylase and the human vitamin K dependent carboxylase disclosed herein. This definition is intended

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to encompass natural allelic variations in the carboxylase enzymes. Cloned genes of the present invention may code for carboxylase enzyme of any species of origin, including mouse, rat, rabbit, cat, porcine, and human, but preferably
5 code for carboxylase enzyme of mammalian origin. Thus, DNA sequences which hybridize to DNA which encodes bovine or human vitamin K dependent carboxylase and which code on expression for a vitamin K dependent carboxylase are also an aspect of this invention. Conditions which will permit
10 other DNA sequences which code on expression for a vitamin K dependent carboxylase to hybridize to the DNA sequence of bovine or human vitamin K dependent carboxylase can be determined in a routine manner. For example, hybridization of such sequences may be carried out under conditions of
15 reduced stringency or even stringent conditions (e.g., conditions represented by a wash stringency of 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°C or even 70°C to DNA encoding the bovine or human vitamin K dependent carboxylase disclosed herein in a standard in situ
20 hybridization assay. See J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory)). In general, sequences which code for vitamin K dependent carboxylase and hybridize to the DNA encoding bovine or human vitamin K dependent carboxylase
25 disclosed herein will be at least 75% homologous, 85% homologous, or even 95% homologous or more with the sequence of the bovine or human vitamin K dependent carboxylase disclosed herein. Further, DNA sequences which code for bovine or human vitamin K dependent carboxylase,
30 or sequences which code for a carboxylase coded for by a sequence which hybridizes to the DNA sequence which codes for bovine or human vitamin K dependent carboxylase, but which differ in codon sequence from these due to the degeneracy of the genetic code, are also an aspect of this
35 invention. The degeneracy of the genetic code, which allows different nucleic acid sequences to code for the same protein or peptide, is well known in the literature.

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See e.g., U.S. Patent No. 4,757,006 to Toole et al. at Col. 2, Table 1.

By providing the purification procedure set forth below, and making available cloned genes which encode vitamin K dependent carboxylase, the present invention makes available vitamin K dependent carboxylase with purities of at least 80% (percent by weight of total protein), at least 90%, and more. Purified carboxylase of the present invention is useful for carboxylating vitamin K dependent enzymes to increase the activity thereof. The carboxylase enzyme may be purified from lysed cell fractions or microsomes containing the enzyme in accordance with the procedures described herein, optionally followed by other techniques such as ion exchange chromatography. See generally Enzyme Purification and Related Techniques, *Methods in Enzymology* 22, 233-577 (1977).

C. Vitamin K-Dependent Proteins

Numerous vitamin K-dependent proteins can be carboxylated with the carboxylase disclosed herein. A preferred group of vitamin K-dependent proteins for practicing the present invention is the blood coagulation proteins, including, but not limited to, Factor VII, Factor IX, Factor X, Protein C, Protein S, and Prothrombin.

As noted above, the present invention provides a method of carboxylating vitamin K dependent proteins by coexpressing the vitamin K dependent protein and the vitamin K dependent carboxylase disclosed herein in a single host cell. In general, the method comprises culturing a host cell which expresses a vitamin K dependent protein in the presence of vitamin K; and then harvesting the vitamin K dependent protein from the culture. The host cell further contains a recombinant DNA sequence comprising vector DNA operable in the host cell and a DNA encoding a vitamin K dependent carboxylase as described herein. While some host cells may carboxylate the vitamin K dependent protein at basal levels, the vector DNA encoding vitamin K dependent carboxylase is included to enhance

carboxylation. The culture can be carried out in any suitable fermentation vessel, with a growth media and under conditions appropriate for the expression of the vitamin K dependent carboxylase and vitamin K dependent protein by the particular host cell chosen. The vitamin K dependent protein harvested from the culture is found to be carboxylated due to the expression of the vitamin k dependent carboxylase in the host cell. The vitamin K dependent protein can be expressed in the host cell by any of the means described above for expressing the vitamin K dependent carboxylase in the host cell (e.g., by transforming the host cell with an expression vector such as a plasmid comprising DNA which encodes the vitamin K dependent protein). The vitamin K dependent protein can be collected directly from the culture media, or the host cells lysed and the vitamin K dependent protein collected therefrom. The vitamin K dependent protein can then be further purified in accordance with known techniques.

Factor VII DNA sequences, along with vectors and host cells for expression thereof, are disclosed in U.S. Patent No. 4,784,950 to F. Hagen et al.

Factor IX DNA coding sequences, along with vectors and host cells for the expression thereof, are disclosed in European Patent App. 373012, European Patent App. 251874, PCT Patent Appl. 8505376, PCT Patent Appln. 8505125, European Patent Appln. 162782, and PCT Patent Appln. 8400560.

Factor X purification is disclosed in U.S. Patent No. 4,411,794 to H. Schwinn et al.

Protein C DNA coding sequences, along with vectors and host cells for the expression thereof, are disclosed in U.S. Patent No. 4,992,373 to N. Bang et al., U.S. Patent No. 4,968,626 to D. Foster and E. Davie, and U.S. Patent No. 4,959,318 to D. Foster et al.

Protein S DNA coding sequences, along with vectors and host cells for the expression thereof, are

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disclosed in European Patent Appln. 255771 and European Patent Appln. 247843.

Prothrombin purification is disclosed in Japanese Kokai 2019400.

5 **D. Expression of Vitamin-K Dependent
 Carboxylase in Mammalian Milk**

Another aspect of the present invention is transgenic mammals (e.g., cows, goats, pigs) containing an expression system comprising a suitable promoter, such as
10 a casein promoter, operatively linked to a DNA sequence coding for vitamin K dependent carboxylase as disclosed herein through a DNA sequence coding for a signal peptide effective in secreting the carboxylase in mammary tissue. Such animals provide for a process of producing the
15 carboxylase enzyme in which milk can be collected from the animals and the carboxylase enzyme isolated from the milk. The carboxylase isolated from the milk can then be used, among other things, to carboxylase vitamin K dependent proteins in vitro. In the alternative, vitamin K-dependent
20 proteins can be co-expressed with the vitamin K dependent carboxylase in such transgenic animals in like manner, through a like expression system, and the carboxylated proteins collected from the milk. Such animals can be produced and such processes can be carried out in
25 accordance with known procedures. See, e.g., U.S. Patent No. 4,873,316 to H. Meade and N. Lonberg, titled "Isolation of Exogenous Recombinant Proteins from the Milk of Transgenic Mammals"; U.S. Patent No. 4,873,191 to T. Wagner and P. Hoppe, titled "Genetic Transformation of
30 Zygotes."

E. Additional Applications of the Invention

Because the best marker for human liver carcinoma is increased levels of undercarboxylated prothrombin, hybridization probes for mRNA levels of
35 carboxylase are useful for diagnosis of liver cancer, as discussed in detail below. As also discussed below, antibodies to the carboxylase may be prepared, labelled with a suitable detectable group, and used to diagnose

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altered patterns of expression of the carboxylase enzyme in a subject (e.g., a patient potentially afflicted with liver carcinoma). Antibodies can also be used to immobilize the carboxylase for in-vitro carboxylation of undercarboxylated proteins. Furthermore, one can express a soluble form of the carboxylase to enable the in vitro modification of undercarboxylated proteins.

Hybridization probes of the present invention may be cDNA fragments or oligonucleotides, and may be labelled with a detectable group as discussed hereinbelow.

The probes selectively bind to mRNA encoding vitamin K-dependent carboxylase. Pairs of probes which will serve as PCR primers for carboxylase mRNA or a portion thereof may be used in accordance with the process described in U.S. Patents Nos. 4,683,202 and 4,683,195, or modifications thereof which will be apparent to those skilled in the art.

As noted above, the present invention provides for an aqueous solution having the carboxylase enzyme solubilized therein. Soluble carboxylase enzyme can be made by deleting the transmembrane region of the carboxylase cDNA, then expressing the altered enzyme, and then collecting the altered enzyme for solubilization in an aqueous solution. The choice of aqueous solution is not critical, with the solution chosen simply being one appropriate for carrying out the carboxylation of vitamin K dependent proteins in vitro. By "deletion" of the transmembrane region of the cDNA, we mean removal or alteration of a sufficient portion of the hydrophobic transmembrane domain to render the coded-for enzyme soluble in an aqueous solution.

A variety of detectable groups can be employed to label antibodies and probes as disclosed herein, and the term "labelled" is used herein to refer to the conjugating or covalent bonding of any suitable detectable group, including enzymes (e.g., horseradish peroxidase, β -glucuronidase, alkaline phosphatase, and β -D-galactosidase), fluorescent labels (e.g., fluorescein,

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luciferase), and radiolabels (e.g., ^{14}C , ^{131}I , ^3H , ^{32}P , and ^{35}S) to the compound being labelled. Techniques for labelling various compounds, including proteins, peptides, and antibodies, are well known. See, e.g., Morrison, *Methods in Enzymology* 32b, 103 (1974); Syvanen et al., *J. Biol. Chem.* 284, 3762 (1973); Bolton and Hunter, *Biochem. J.* 133, 529 (1973).

Antibodies which specifically bind to the carboxylase enzyme (i.e., antibodies which bind to a single antigenic site or epitope on the carboxylase enzyme) may be polyclonal or monoclonal in origin, but are preferably of monoclonal origin. The antibodies are preferably IgG antibodies of any suitable species, such as rat, rabbit, or horse, but are generally of mammalian origin. Fragments of IgG antibodies which retain the ability to specifically bind the axl receptor, such as F(ab')_2 , F(ab') , and Fab fragments, are intended to be encompassed by the term "antibody" herein. The antibodies may be chimeric, as described by M. Walker et al., *Molecular Immunol.* 26, 403 (1989). Antibodies may be immobilized on a solid support of the type used as a packing in an affinity chromatography column, such as sepharose, silica, or glass beads, in accordance with known techniques.

Monoclonal antibodies which bind to carboxylase enzyme are made by culturing a cell or cell line capable of producing the antibody under conditions suitable for the production of the antibody (e.g., by maintaining the cell line in HAT media), and then collecting the antibody from the culture (e.g., by precipitation, ion exchange chromatography, affinity chromatography, or the like). The antibodies may be generated in a hybridoma cell line in the widely used procedure described by G. Kohler and C. Milstein, *Nature* 256, 495 (1975), or may be generated with a recombinant vector in a suitable host cell such as *Escherichia coli* in the manner described by W. Huse et al., *Generation of a Large Combinatorial Library of the*

Immunoglobulin Repertoire in Phage Lambda, *Science* 246, 1275 (1989).

The present invention is explained in greater detail in the following non-limiting examples. In the examples, mmol means millimoles; ml means milliliters; mCi means milliCuries; μ g means micrograms; μ M means microMolar; gm means grams; hr means hours; cpm means counts per minute; MOPS means 4-Morpholinepropanesulfonic acid; and CHAPS means 3 - [(3 - cholamidopropyl) dimethylammonio] - 1 - propanesulfonate. Temperatures are given in degrees Centigrade.

EXAMPLE 1

Purification to Near Homogeneity of the Vitamin K-dependent Carboxylase

All chemicals used herein are reagent grade. 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) was purchased from Pierce Chemical Co. Aprotinin and Pepstatin A were purchased from Boehringer Mannheim Biochemicals. Leupeptin, phenylmethylsulfonyl fluoride (PMSF), and (3 - ((3-Cholamidopropyl) - dimethylammonio) - 1 - propanesulfonate (CHAPS) were obtained from Sigma. The peptide FLEEL and the protease inhibitors FFRCK and FPRCK were from Bachem. Peptide proFIX19, AVFLDHENANKILNRPKRY, was synthesized by Frank Church of UNC-CH. $\text{NaH}^{14}\text{CO}_3$, specific activity 50.0 mCi/mmol, was from NEN and Aqua Mephyton from Merck Sharp and Dohme. Protease inhibitor cocktail (PIC) was freshly prepared as a 10 X PIC stock with 20 mM dithiothreitol, 20 mM EDTA, 1.25 μ g/ml FFRCK, 1.25 μ g/ml FPRCK, 5 μ g/ml Leupeptin, 7 μ g/ml Pepstatin A, 340 μ g/ml PMSF, and 20 μ g/ml Aprotinin.

Preparation of Affinity Column. Peptide FIXQ/S (SEQ ID NO:1) (residues -18 to 41 of factor IX with mutations Arg to Glu at residue -4 and Arg to Ser at residue -1) was chosen for the affinity ligand because its affinity for the carboxylase is not changed and because it has fewer trypsin cleavage sites than our other peptides and is therefore less likely to be degraded by proteases in the crude extracts used for purification. Peptide FIXQ/S

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was prepared according to S.-M. Wu et al. *supra*. One hundred mg of FIXQ/S was coupled to 25 ml of Affi-Gel 10 (Bio-Rad Inc.) according to the manufacturer. The reaction was done at pH 4.8, which is one unit below the theoretical pI of FIXQ/S. The final concentration of the covalently bound FIXQ/S on Affi-Gel 10 was measured as 442 μ M and the coupled ligand is referred to as Affi-FIXQ/S.

Affinity Purification of Carboxylase.
Preparation of microsomes from bovine liver, solubilization of microsomes, and ammonium-sulfate fractionation were as described by Girardot (*supra*). Protein concentration was measured with the Bio-Rad protein assay kit. See M. Bradford, *Anal. Biochem.* 72:248-254 (1976). Alternatively, protein concentration and relative purity were determined by scanning of SDS-PAGE gels stained with silver or Coomassie blue. See U. Laemmli, *Nature* 227:680-685 (1970); H. Blum, et al., *Electrophoresis* 8:93-99 (1987). In all cases, reference curves were prepared with IgG as a standard. Ammonium-sulfate fractionated microsomal protein (7-8 gm) was resuspended in buffer A (25 mM MOPS, pH 7.0, 500 mM NaCl, 20% glycerol, 0.1% phosphatidylcholine, and 1 X PIC) with 0.1% CHAPS to a total volume of 150 ml. In all purification experiments, except that depicted in lane 3 of figure 1, the suspension was sonicated with a standard ultrasonic probe (Sonicator Model W-220F, Heat Systems Inc.) at scale 9 for 100 2-second pulses in an ice bath. The sonicated material was loaded on a 25-ml Affi-FIXQ/S column equilibrated with 150 ml of 0.1% CHAPS in buffer A at 4° C at a flow rate of 10 ml/hr. The loaded column was washed with 100-200 ml of 0.1% CHAPS in buffer A. The carboxylase was eluted from the Affi-FIXQ/S column by one of the following methods.

Elution I. 150 μ M propeptide in buffer A with 0.1% CHAPS was used to elute the carboxylase. The column was filled with eluant and incubated overnight before collecting the eluate. The propeptide eluate was concentrated with Centricon-30 (Amicon Inc.); the filtrate

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could be reused for elution. Significant amounts of carboxylase activity were continuously eluted for a week.

Elution II. A flow rate of 10 ml/hr was used for all chromatography steps. An extensive wash was carried out by two sequential steps: 100 ml of 0.05% to 0.85% Triton X-100 gradient in buffer B (25 mM MOPS, pH 7.0, 50 mM NaCl, 20% glycerol, 0.1%, and 1 X PIC) containing 0.2% phosphatidylcholine followed by 100 ml of 0.1% to 1% CHAPS gradient in buffer B containing 0.1% phosphatidylcholine. Elution was accomplished by using 100 ml of a double gradient of 0.1% to 1% CHAPS and 0 to 2 μ M proFIX19 in buffer A and continued with another 100 ml of 1% CHAPS and 2 μ M proFIX19 in buffer A.

CM-Sepharose Chromatography. Desalted, concentrated carboxylase (total activity of 2.2×10^7 cpm/30 min) eluted by method I was prepared in buffer C (25 mM MOPS, pH 7.0, 100 mM NaCl, 20% glycerol, 1 X PIC), with 1% Triton X-100, 0.7% phosphatidylcholine, and to a total volume of 3.85 ml. This carboxylase preparation was then sonicated in a bath sonicator for 24 5-second pulses and batch adsorbed to 5.8 ml of CM Sepharose. The CM Sepharose was packed into a column, washed, and eluted with a 10 ml of NaCl gradient from 100 mM to 450 mM in buffer C with 0.05% Triton X-100, 0.2% phosphatidylcholine.

Cross-Linking Reaction. The cross-linking was accomplished with (DTSSP) as described by S. Jung and M. Moroi, *Biochim. Biophys. Acta* 761:152-162 (1983). For competition experiments, 100-fold excess of proFIX19 was included.

Carboxylase assay. The assay was done for 30 min as described in S.-M. Wu et al., *supra*, in 25 mM MOPS, pH 7.0, 500 mM NaCl, with 0.16% CHAPS/phosphatidylcholine. FLEEL at 3.6 mM and 5 μ Ci $\text{NaH}^{14}\text{CO}_3$ were the substrates; 0.8 M ammonium sulfate and 16 μ M proFIX19 were included as activators.

Identification of the Carboxylase. Figure 1 displays a reducing SDS-PAGE analysis of our initial Affi-

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FIXQ/S purification of carboxylase. There is little difference between the protein pattern of the starting material and the flow-through (lane 1 and lane 2). The sample in lane 3 represents protein eluted with 150 μ M proFIX19. There is some enhancement of a protein band at 94,000 M_r , but the purification achieved is only 60-fold. We reasoned that the poor purification might be the result of large micelles which contained at least one carboxylase molecule as well as other integral membrane proteins. The carboxylase molecule would allow binding to the affinity matrix but the other proteins would be co-eluted with the carboxylase. We tried to apply the standard solution to this problem, which is to increase the detergent concentration until one protein per micelle is achieved. However, the carboxylase would not bind to Affi-FIXQ/S in the presence of a high concentration of a number of detergents. Therefore, we sought to reduce the micelle size by sonicating the starting material. As shown in figure 1, lane 4, the relative intensity of the 94,000- M_r band in the proFIX19 eluate is much more prominent after sonication. Furthermore, the amount of carboxylase bound to Affi-FIXQ/S increased from 15% to 30% and the purification increased to 500-fold.

In order to evaluate the binding specificity of FIXQ/S and attain an independent estimate of the molecular weight of the carboxylase, we cross-linked ^{125} I-FIXQ/S to 60-fold affinity-purified material (fig. 1, lane 3). The autoradiogram in figure 2, lane 2 shows one major band, slightly larger than 97,000 M_r , which represents the complex of the cross-linked protein and the synthetic peptide ^{125} I-FIXQ/S. This major band was eliminated when the same experiment was run in the presence of excess non-radioactive proFIX19 (fig. 2, lane 1). The minor bands in lane 2 were unaffected by competition (lane 1). By subtracting the molecular weight of FIXQ/S (7,000 M_r) from the estimated size of the cross-linked complex we estimate that the size of the protein to which the FIXQ/S peptide

binds is approximately 94,000 Mr. This agrees with the size of the band enriched in each of the early purifications (fig. 1), indicating that the 94,000-Mr protein (lanes 3 and 4) is the enzyme carboxylase and that the interaction between the peptide FIXQ/S and carboxylase is very specific.

5 Results shown in **figure 3** indicate that further purification can be achieved by chromatography of the 500-fold affinity-purified material on CM Sepharose. Resolubilization, sonication, and batch adsorption on CM sepharose followed by elution with a salt gradient produced a dramatic improvement in purification. The batch adsorption step is very important as enzymatic activity is
10 lost and significantly less purification is achieved when the material is adsorbed to the top of the column. However, the most important information in this figure is that the carboxylase activity in each fraction is proportional to the amount of 94,000-Mr protein in the corresponding SDS-PAGE analysis. Quantitation of the silver-stained gel by scanning reveals that the protein is 80-90% pure. The
15 specific activity is 2×10^9 cpm/mg/hr.

 Further evidence that the 94,000-Mr band represents the carboxylase is presented in **figure 4**. A carboxylase preparation purified by Affi-FIXQ/S and carboxymethyl sepharose (lane 2) was re-applied to the Affi-FIXQ/S column. Lane 3 shows that the activity of carboxylase was removed when the
20 94,000-Mr protein bound to Affi-FIXQ/S. Lane 4 shows that the activity again co-eluted with the 94,000-Mr protein. This carboxylase is from one of our earlier preparations and contained a significant amount of inactive carboxylase. This explains the observation that the amounts of protein in the 94,000 Mr bands in lanes 2 and 4 are approximately equal while the activity is greater in the sample
25 repurified by Affi-FIXQ/S; only active carboxylase binds to the affinity column.

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Purification of the Carboxylase. One of the problems with using the affinity column is that the elution using the propeptide is very slow and requires several days to achieve an adequate yield. We therefore explored alternative methods to elute the enzyme from the affinity column. As shown in figure 5, we were able to remove essentially all contaminating proteins without loss of carboxylase activity by washing the loaded affinity column extensively with Triton X-100 followed by a wash with CHAPS. The carboxylase could then be eluted with a gradient of CHAPS in a high concentration of NaCl (data not shown) or a gradient of CHAPS and propeptide containing a high concentration of NaCl (fig. 5). The carboxylase activity coincides with the 94,000-M_r protein profile. And, depending upon the fraction chosen, the carboxylase is 80 to 95% pure. If the propeptide is omitted from the elution gradient, the carboxylase in higher concentrations of CHAPS rapidly loses activity. Table 1 is the purification table for our final purification scheme. Note that 30% of the activity in the starting material is bound (obtained by subtracting the activity in the flow-through from the load) and that 34% of total activity is recovered by elution. Thus it appears that very little of the observed increase in specific activity is due to the removal of an inhibitor.

Table 1. Purification of Carboxylase

<u>Sample</u>	Total Carboxylase of Recovery		<u>Specific Activity</u> (cpm/mg/hr)	<u>Purification -fold</u>
	<u>Total Protein</u>	<u>Activity</u> (cpm/30min)		
Solubilized microsomes (load)	8100 mg	1.14 x 10 ⁹	100%	2.81 x 10 ⁵ 1
Flow-through of Affi-FIXQ/S	8090 mg	8.08 x 10 ⁸	70%	2 x 10 ⁵ 0.7
Bound to Affi-FIXQ/S	4.7 mg	3.3 x 10 ⁸	30%	1.4 x 10 ⁸ 502
Affinity-purified carboxylase	.402 mg	3.88 x 10 ⁸	34%	1.93 x 10 ⁹ 7,000

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We have affinity purified the carboxylase to 80-90% purity with a yield of 34%. The procedure is rapid and efficient. The entire purification can be accomplished in four days. It is difficult to compare different purification schemes, but by comparing final specific activities, it appears that our purification results in a carboxylase preparation that is 185-fold higher in specific activity than the best previously published method. From $^{14}\text{CO}_2$ incorporation into the pentapeptide FLEEL, we estimate the specific activity of our carboxylase prepared by Affi-FIXQ/S chromatography eluted by method II (fig. 5) as 2.4×10^9 cpm/mg protein/hr. This represents a 7,000-fold purification. Hubbard et al. reported a specific activity of 1.3×10^7 cpm/mg protein/hr with a 107-fold purification from their affinity purification step. The results should be comparable, because the CO_2 used in the two experiments had the same specific activities.

The major protein that coincides with the carboxylase activity in our preparation has a 94,000 M_r in reducing SDS-PAGE analysis. This is very different from the 77,000 M_r protein reported by Hubbard et al. (*supra*). Flynn et al. demonstrated that many different small peptides could be used for the single-step affinity purification of BiP (or glucose-regulated protein, or hsp 78) from solubilized microsomes. See G. Flynn, *Science* 245:385-390 (1989). Hubbard et al. (*supra*), using a similar starting material and a similar purification scheme, obtained a protein with the same molecular weight as BiP with a relatively low specific activity for carboxylase. We concluded that the 77,000- M_r protein reported by Hubbard et al. was BiP, one of the most abundant proteins in the endoplasmic reticulum.

The method used in this Example for purification of the carboxylase may have general importance for the purification of membrane proteins. A basic strategy for purifying integral membrane proteins is to make micelles containing only one protein. However, high concentrations

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of detergent often result in the inactivation of the protein of interest. By appropriate sonication, one can create smaller mixed micelles without affecting the catalytic or binding ability of the protein. Because the immobilized protein is often more stable, the problems associated with mixed micelles can be solved by carefully choosing the conditions for washing the bound solid phase. This method of washing the bound protein with a high concentration of detergent should also be applicable to integral membrane proteins bound to standard ion exchange matrices.

Carboxylase is an integral membrane protein present in low concentrations that has previously resisted purification. To overcome the inherent difficulties, we chose affinity binding as the first purification step. By manipulating the biophysical properties of micelles we were able to achieve a single-step purification of carboxylase with 80-90% purity. Starting from 8 grams of microsomal protein, we can easily generate 300-400 μ g of carboxylase from a 25-ml Affi-FIXQ/S column, with a final yield of 34% of the starting activity. Glycerol, which is often used to stabilize the membrane protein during purification, proved to be an important stabilizer for carboxylase. It increases the thermal stability of carboxylase and also increases the half life of carboxylase at 4° C. Although 20% glycerol does not inhibit carboxylase binding to Affi-FIXQ/S, it does prevent carboxylase from being eluted by proFIX19 and also inhibits the enzyme activity. The purified carboxylase is very stable and can be stored at -70° C for months without loss of activity.

EXAMPLE 2

Partial Sequencing of Carboxylase Enzyme

Carboxylase enzyme prepared by the two-step purification procedure set forth in Example 1 above was further purified by removing lipid by hexane/isopropanol extraction. The enzyme was then reconcentrated and separated on a preparative reducing SDS-PAGE. After

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electrophoresis, total proteins were transferred onto a nitrocellulose membrane by standard Western blot methodology. The transferred proteins were stained with Ponceau S to locate the carboxylase band, which was then cut out for amino acid sequencing. The amino acid sequencing was performed by Dr. William S. Lane, Harvard Microchem, Harvard University. Amino acid sequence was obtained from several tryptic peptides of the carboxylase. The sequence of the longest peptide, NT77, is given by SEQ ID NO:2. NT77 was 37 amino acids long. The sequence of a second peptide, NT56, is given by SEQ ID NO:3. The sequence of a third peptide, NT49 is given in SEQ ID NO:4. NT56 and NT49 were 12 and 16 amino acids long, respectively.

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EXAMPLE 3**Preparation of Partial cDNA Sequence**

The codons for the amino acid sequence represented by SEQ ID NO:2 above were used to design several redundant oligonucleotides for use in the polymerase chain reaction (PCR). A mixture of 1024 oligonucleotides (SEQ ID NO:5) was synthesized to take into account the redundancy in the codons at the carboxy terminus of the 38 amino acid tryptic peptide NT77 given as SEQ ID NO:2; this mixture was used to synthesize the first strand cDNA with reverse transcriptase.

25

Two degenerate oligonucleotides were synthesized as PCR primers and used for the polymerase chain reaction. Oligonucleotides contained regular bases or contain 5-fluorouridine in order to reduce redundancy and were synthesized by Oligos Etc., Inc., Suite 266, 800 Village Walk, Guilford, CT 06457 USA. One of the oligonucleotide primers had the sequence: AAN NTN GCN TTN GGN MG (SEQ ID NO:6), where the N at residue 3 represents 5-fluorodeoxyuridine (F); the N at residue 4 represents F; the N at residue 6 represents F or G; the N at residue 9 represents F or G; the N at residue 12 represents F; and the N at residue 15 represents A, T, G, or C. The other

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oligonucleotide primer had the sequence: TC NCC NGC NGG YTC RAA (SEQ ID NO:7), where the N at residue 3 represents F or G; the N at residue 6 represents F or G; and the N at residue 9 represents F or G.

5 The polymerase chain reaction was performed by standard procedures (94°C, 1 min; 58°C, 3 min; 72°C, 2 min; 25 cycles) using TaqTM polymerase purchased from Perkin Elmer Cetus (Part No. N801-0046) and a Cetus thermal cycler obtained from Cetus.

10 A product of 87 nucleotides was obtained from the polymerase chain reaction described above and sequenced. A 55 nucleotide probe complementary to the coding sequence of the PCR product was then synthesized; this probe is given as SEQ ID NO:8.

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EXAMPLE 4

Preparation of Bovine cDNA Sequence

 The 55-nucleotide probe represented by SEQ ID NO:8 was synthesized by Oligos Etc., Inc. This probe is labelled with ³²P at the 5' terminus in accordance with known techniques and is used to screen a bovine cDNA library purchased from Stratagene, Inc. A Positive clone is obtained and identified as λZAP-CARB1.6. The positive clone is partially sequenced, and portions of the clone sequence are given as SEQ ID NO:9, SEQ ID NO:11, and SEQ ID NO:13. The peptides coded for by these sequences are given separately as SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, respectively. Note that, in SEQ ID NO:10, amino acid residues 58 to 68 correspond to amino acid residues 1-11 of the tryptic peptide NT77 given as SEQ ID NO:2; in SEQ ID NO:10, amino acid residues 1-12 correspond to amino acid residues 1-12 of the tryptic peptide NT56 given as SEQ ID NO:3; in SEQ ID NO:12, amino acid residues 1-10 correspond to amino acid residues 28-37 of tryptic peptide NT77 given in SEQ ID NO:2; and in SEQ ID NO:14, amino acid residues 1-14 correspond to amino acid residues 1-14 of tryptic peptide NT49 given as SEQ ID NO:4.

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The bacteriophage λ ZAP-CARB1.6 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA in accordance with the provisions of the Budapest Treaty on April 23, 1991, and has been assigned ATCC Accession Number 75002.

EXAMPLE 5

Preparation and Expression of Human cDNA Sequence

The 1.6Kb insert from λ ZAP-CARB1.6 is used to screen a human cDNA library to detect a human cDNA sequence encoding a vitamin K dependent carboxylase. Screening is carried out by a standard in situ hybridization assay, see J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989), under conditions represented by a wash stringency of 0.3 M NaCl, 0.03 M sodium citrate, and 0.1% SDS at a temperature of 60°C or 70°C. A number of positive clones are detected.

None of the foregoing clones coded for the entire sequence of the gamma glutamyl carboxylase. They were important, however, because they allowed us to compare liver carboxylase to the carboxylase of another tissue. We screened a second cDNA library made with mRNA from a human cell line (HEL human erythroleukemia). From the HEL library we obtained a clone, SEQ ID NO:15, which included the entire coding sequence of the human carboxylase and some upstream and downstream sequences. The cDNA codes for a protein of 758 amino acids (SEQ ID NO:16) whose molecular weight is 87,542 (excluding carbohydrates). This clone is the authentic carboxylase and possesses the entire coding sequence.

Conclusive evidence that the clone of SEQ ID NO:15 contains the authentic human carboxylase is from transient expression in human 293 kidney cells. Microsomes from cells transfected with pCMV.hGC+, which is the expression vector pCMV5 (cytomegalovirus promoter) containing the cDNA of SEQ ID NO:15 exhibited a 16-20 fold increase in carboxylase activity compared to microsomes from mock infected cells. The expression vector pCMV5, see

S. Andersson, J. Biol. Chem. **264**, 8222-8229 (1989), was a gift of Dr. David Russell. *Escherichia coli* cells containing pCMV.hGC+, designated *E. coli*, pCMV.hGC+, was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 USA, in accordance with the
5 provisions of the Budapest Treaty on August 15, 1991, and has been assigned ATCC Accession Number 68666.

It is likely that the level of carboxylase can be increased greatly in stable cell lines. This can be done by standard amplification techniques and also by making the translation initiation region conform to the ideal (D. Cavener and S.
10 Ray, Nucleic Acids Res. **19**, 3185 (1991)).

It should be noted that the human carboxylase and bovine carboxylase, over the region where both sequences are known (664 amino acids) are 91% identical. The amino acid sequence of the liver and HEL carboxylase are also identical over the region where both sequences are available (354 amino
15 acids). There are two differences in nucleotide sequence in the two human clones but they result in the same amino acid sequence. It is likely that some polymorphism will exist.

The foregoing examples are illustrative of the present invention, and are not to be taken as limiting thereof. The invention is defined by the following
20 claims, with equivalents of the claims to be included therein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Wu, Sheue-Mei
Stafford, Darrel W.
- (ii) TITLE OF INVENTION: Vitamin K-Dependent Carboxylase
- (iii) NUMBER OF SEQUENCES: 16
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 - (F) ZIP: 28234
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/697,427
 - (B) FILING DATE: 08-MAY-1991
- (viii) ATTORNEY/AGENT INFORMATION:
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 - (B) TELEFAX: 919-881-3175
 - (C) TELEX: 575102

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 59 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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

Ala	Val	Phe	Leu	Asp	His	Glu	Asn	Ala	Asn	Lys	Ile	Leu	Asn	Gln	Pro
1				5					10					15	
Lys	Ser	Tyr	Asn	Ser	Gly	Lys	Leu	Glu	Glu	Phe	Val	Gln	Gly	Asn	Leu
			20					25					30		
Glu	Arg	Glu	Cys	Ile	Glu	Glu	Lys	Cys	Ser	Phe	Glu	Glu	Ala	Arg	Glu
		35					40					45			
Val	Phe	Glu	Asn	Thr	Glu	Arg	Thr	Asn	Glu	Phe					
	50					55									

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Asn	Leu	Ala	Phe	Gly	Arg	Pro	Ser	Leu	Glu	Gln	Leu	Ala	Gln	Glu	Val
1				5					10					15	
Thr	Tyr	Ala	Asn	Leu	Arg	Pro	Phe	Glu	Pro	Ala	Gly	Glu	Pro	Ser	Pro
			20					25				30			
Val	Asn	Thr	Asp	Ser											
			35												

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Gly	Gly	Pro	Glu	Pro	Thr	Pro	Leu	Val	Gln	Thr	Phe
1				5					10		

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

Thr	Gly	Glu	Leu	Gly	Tyr	Leu	Asn	Pro	Gly	Val	Phe	Thr	Gln	Ser	Arg
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

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

SWRTCNGTRT TNACNGG

17

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

AANNTNGCNT TNGGNMG

17

(2) INFORMATION FOR SEQ ID NO:7:

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- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

TCNCCNNCNG GYTCRAA

17

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 55 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

AAGGGTCGCA AGTTGGCATA AGTCACTTCT TGGGCCAGCT GCTCCAGGGA AGGGC

55

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 204 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..204

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

GGC GGC CCT GAG CCA ACA CCA CTG GTC CAG ACC TTC CTT AGA CGC CAG
 Gly Gly Pro Glu Pro Thr Pro Leu Val Gln Thr Phe Leu Arg Arg Gln
 1 5 10 15

48

CAA AGG CTC CAG GAG ATT GAA CGC CGA CGA AAT GCC CCT TTC CAC GAG
 Gln Arg Leu Gln Glu Ile Glu Arg Arg Arg Asn Ala Pro Phe His Glu
 20 25 30

96

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CGA CTT GTC CGC TTC TTG CTG CGA AAG CTC TTT ATC TTT CGC CGT AGC	144
Arg Leu Val Arg Phe Leu Leu Arg Lys Leu Phe Ile Phe Arg Arg Ser	
35 40 45	
TTT CTC ATG ACT TGT ATC TCA CTT CGA AAT CTG GCA TTT GGC CGC CCT	192
Phe Leu Met Thr Cys Ile Ser Leu Arg Asn Leu Ala Phe Gly Arg Pro	
50 55 60	
TCC CTG GAG CAG	204
Ser Leu Glu Gln	
65	

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Gly	Gly	Pro	Glu	Pro	Thr	Pro	Leu	Val	Gln	Thr	Phe	Leu	Arg	Arg	Gln
1				5					10					15	
Gln	Arg	Leu	Gln	Glu	Ile	Glu	Arg	Arg	Arg	Asn	Ala	Pro	Phe	His	Glu
			20					25					30		
Arg	Leu	Val	Arg	Phe	Leu	Leu	Arg	Lys	Leu	Phe	Ile	Phe	Arg	Arg	Ser
		35					40					45			
Phe	Leu	Met	Thr	Cys	Ile	Ser	Leu	Arg	Asn	Leu	Ala	Phe	Gly	Arg	Pro
	50					55					60				
Ser	Leu	Glu	Gln												
65															

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..36

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

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GGA GAG CCG AGT CCT GTA AAC ACA GAT TCT TCT AAT
 Gly Glu Pro Ser Pro Val Asn Thr Asp Ser Ser Asn
 1 5 10

36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Gly Glu Pro Ser Pro Val Asn Thr Asp Ser Ser Asn
 1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..42

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

ACC GGT GAA CTG GGC TAC CTC AAC CCT GGG GTA TTC ACA CAG
 Thr Gly Glu Leu Gly Tyr Leu Asn Pro Gly Val Phe Thr Gln
 1 5 10

42

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Thr Gly Glu Leu Gly Tyr Leu Asn Pro Gly Val Phe Thr Gln
 1 5 10

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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2452 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 87..2360

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

CGGGGCGGAG CCTAGGGAAG CAAATTCTCC TGGCGGCCTC CGTTCAGACG CGGCAGCTGT	60
GACCCACCTG CCTCCTCCGC AGAGCA ATG GCG GTG TCT GCC GGG TCC GCG CGG	113
Met Ala Val Ser Ala Gly Ser Ala Arg	
1 5	
ACC TCG CCC AGC TCA GAT AAA GTA CAG AAA GAC AAG GCT GAA CTG ATC	161
Thr Ser Pro Ser Ser Asp Lys Val Gln Lys Asp Lys Ala Glu Leu Ile	
10 15 20 25	
TCA GGG CCC AGG CAG GAC AGC CGA ATA GGG AAA CTC TTG GGT TTT GAG	209
Ser Gly Pro Arg Gln Asp Ser Arg Ile Gly Lys Leu Leu Gly Phe Glu	
30 35 40	
TGG ACA GAT TTG TCC AGT TGG CGG AGG CTG GTG ACC CTG CTG AAT CGA	257
Trp Thr Asp Leu Ser Ser Trp Arg Arg Leu Val Thr Leu Leu Asn Arg	
45 50 55	
CCA ACG GAC CCT GCA AGC TTA GCT GTC TTT CGT TTT CTT TTT GGG TTC	305
Pro Thr Asp Pro Ala Ser Leu Ala Val Phe Arg Phe Leu Phe Gly Phe	
60 65 70	
TTG ATG GTG CTA GAC ATT CCC CAG GAG CGG GGG CTC AGC TCT CTG GAC	353
Leu Met Val Leu Asp Ile Pro Gln Glu Arg Gly Leu Ser Ser Leu Asp	
75 80 85	
CGG AAA TAC CTT GAT GGG CTG GAT GTG TGC CGC TTC CCC TTG CTG GAT	401
Arg Lys Tyr Leu Asp Gly Leu Asp Val Cys Arg Phe Pro Leu Leu Asp	
90 95 100 105	
GCC CTA CGC CCA CTG CCA CTT GAC TGG ATG TAT CTT GTC TAC ACC ATC	449
Ala Leu Arg Pro Leu Pro Leu Asp Trp Met Tyr Leu Val Tyr Thr Ile	
110 115 120	
ATG TTT CTG GGG GCA CTG GGC ATG ATG CTG GGC CTG TGC TAC CGG ATA	497
Met Phe Leu Gly Ala Leu Gly Met Met Leu Gly Leu Cys Tyr Arg Ile	
125 130 135	

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AGC	TGT	GTG	TTA	TTC	CTG	CTG	CCA	TAC	TGG	TAT	GTG	TTT	CTC	CTG	GAC	545
Ser	Cys	Val	Leu	Phe	Leu	Leu	Pro	Tyr	Trp	Tyr	Val	Phe	Leu	Leu	Asp	
		140					145					150				
AAG	ACA	TCA	TGG	AAC	AAC	CAC	TCC	TAT	CTG	TAT	GGG	TTG	TTG	GCC	TTT	593
Lys	Thr	Ser	Trp	Asn	Asn	His	Ser	Tyr	Leu	Tyr	Gly	Leu	Leu	Ala	Phe	
		155				160					165					
CAG	CTA	ACA	TTC	ATG	GAT	GCA	AAC	CAC	TAC	TGG	TCT	GTG	GAC	GGT	CTG	641
Gln	Leu	Thr	Phe	Met	Asp	Ala	Asn	His	Tyr	Trp	Ser	Val	Asp	Gly	Leu	
170					175					180					185	
CTG	AAT	GCC	CAT	AGG	AGG	AAT	GCC	CAC	GTG	CCC	CTT	TGG	AAC	TAT	GCA	689
Leu	Asn	Ala	His	Arg	Arg	Asn	Ala	His	Val	Pro	Leu	Trp	Asn	Tyr	Ala	
				190					195					200		
GTG	CTC	CGT	GGC	CAG	ATC	TTC	ATT	GTG	TAC	TTC	ATT	GCG	GGT	GTG	AAA	737
Val	Leu	Arg	Gly	Gln	Ile	Phe	Ile	Val	Tyr	Phe	Ile	Ala	Gly	Val	Lys	
			205					210					215			
AAG	CTG	GAT	GCA	GAC	TGG	GTT	GAA	GGC	TAT	TCC	ATG	GAA	TAT	TTG	TCC	785
Lys	Leu	Asp	Ala	Asp	Trp	Val	Glu	Gly	Tyr	Ser	Met	Glu	Tyr	Leu	Ser	
		220					225					230				
CGG	CAC	TGG	CTC	TTC	AGT	CCC	TTC	AAA	CTG	CTG	TTG	TCT	GAG	GAG	CTG	833
Arg	His	Trp	Leu	Phe	Ser	Pro	Phe	Lys	Leu	Leu	Leu	Ser	Glu	Glu	Leu	
		235				240					245					
ACT	AGC	CTG	CTG	GTC	GTG	CAC	TGG	GGT	GGG	CTG	CTG	CTT	GAC	CTC	TCA	881
Thr	Ser	Leu	Leu	Val	Val	His	Trp	Gly	Gly	Leu	Leu	Leu	Asp	Leu	Ser	
250					255					260					265	
GCT	GGT	TTC	CTG	CTC	TTT	TTT	GAT	GTC	TCA	AGA	TCC	ATT	GGC	CTG	TTC	929
Ala	Gly	Phe	Leu	Leu	Phe	Phe	Asp	Val	Ser	Arg	Ser	Ile	Gly	Leu	Phe	
				270				275						280		
TTT	GTG	TCC	TAC	TTC	CAC	TGC	ATG	AAT	TCC	CAG	CTT	TTC	AGC	ATT	GGT	977
Phe	Val	Ser	Tyr	Phe	His	Cys	Met	Asn	Ser	Gln	Leu	Phe	Ser	Ile	Gly	
			285					290					295			
ATG	TTC	TCC	TAC	GTC	ATG	CTG	GCC	AGC	AGC	CCT	CTC	TTC	TGC	TCC	CCT	1025
Met	Phe	Ser	Tyr	Val	Met	Leu	Ala	Ser	Ser	Pro	Leu	Phe	Cys	Ser	Pro	
		300					305					310				
GAG	TGG	CCT	CGG	AAG	CTG	GTG	TCC	TAC	TGC	CCC	CAA	AGG	TTG	CAA	CAA	1073
Glu	Trp	Pro	Arg	Lys	Leu	Val	Ser	Tyr	Cys	Pro	Gln	Arg	Leu	Gln	Gln	
	315					320					325					
CTG	TTG	CCC	CTC	AAG	GCA	GCC	CCT	CAG	CCC	AGT	GTT	TCC	TGT	GTG	TAT	1121
Leu	Leu	Pro	Leu	Lys	Ala	Ala	Pro	Gln	Pro	Ser	Val	Ser	Cys	Val	Tyr	
330					335					340					345	
AAG	AGG	AGC	CGG	GGC	AAA	AGT	GGC	CAG	AAG	CCA	GGG	CTG	CGC	CAT	CAG	1169
Lys	Arg	Ser	Arg	Gly	Lys	Ser	Gly	Gln	Lys	Pro	Gly	Leu	Arg	His	Gln	
				350					355					360		

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CTG Leu	GGA Gly	GCT Ala	GCC Ala 365	TTC Phe	ACC Thr	CTG Leu	CTC Leu	TAC Tyr 370	CTC Leu	CTG Leu	GAG Glu	CAG Gln	CTA Leu 375	TTC Phe	CTG Leu	1217
CCC Pro	TAT Tyr	TCT Ser 380	CAT His	TTT Phe	CTC Leu	ACC Thr	CAG Gln 385	GGC Gly	TAT Tyr	AAC Asn	AAC Asn	TGG Trp 390	ACA Thr	AAT Asn	GGG Gly	1265
CTG Leu	TAT Tyr 395	GGC Gly	TAT Tyr	TCC Ser	TGG Trp	GAC Asp 400	ATG Met	ATG Met	GTG Val	CAC His	TCC Ser 405	CGT Arg	TCC Ser	CAC His	CAG Gln	1313
CAC His 410	GTG Val	AAG Lys	ATC Ile	ACC Thr	TAC Tyr 415	CGT Arg	GAT Asp	GGC Gly	CGC Arg	ACT Thr 420	GGC Gly	GAA Glu	CTG Leu	GGC Gly	TAC Tyr 425	1361
CTT Leu	AAC Asn	CCT Pro	GGG Gly	GTA Val 430	TTT Phe	ACA Thr	CAG Gln	AGT Ser	CGG Arg 435	CGA Arg	TGG Trp	AAG Lys	GAT Asp	CAT His 440	GCA Ala	1409
GAC Asp	ATG Met	CTG Leu	AAG Lys 445	CAA Gln	TAT Tyr	GCC Ala	ACT Thr	TGC Cys 450	CTG Leu	AGC Ser	CGC Arg	CTG Leu	CTT Leu 455	CCC Pro	AAG Lys	1457
TAT Tyr	AAT Asn	GTC Val 460	ACT Thr	GAG Glu	CCC Pro	CAG Gln	ATC Ile 465	TAC Tyr	TTT Phe	GAT Asp	ATT Ile	TGG Trp 470	GTC Val	TCC Ser	ATC Ile	1505
AAT Asn 475	GAC Asp	CGC Arg	TTC Phe	CAG Gln	CAG Gln	AGG Arg 480	ATT Ile	TTT Phe	GAC Asp	CCT Pro	CGT Arg 485	GTG Val	GAC Asp	ATC Ile	GTG Val	1553
CAG Gln 490	GCC Ala	GCT Ala	TGG Trp	TCA Ser	CCC Pro 495	TTT Phe	CAG Gln	CGC Arg	ACA Thr	TCC Ser 500	TGG Trp	GTG Val	CAA Gln	CCA Pro	CTC Leu 505	1601
TTG Leu	ATG Met	GAC Asp	CTG Leu	TCT Ser 510	CCC Pro	TGG Trp	AGG Arg	GCC Ala	AAG Lys 515	TTA Leu	CAG Gln	GAA Glu	ATC Ile	AAG Lys 520	AGC Ser	1649
AGC Ser	CTA Leu	GAC Asp	AAC Asn 525	CAC His	ACT Thr	GAG Glu	GTG Val	GTC Val 530	TTC Phe	ATT Ile	GCA Ala	GAT Asp	TTC Phe 535	CCT Pro	GGA Gly	1697
CTG Leu	CAC His	TTG Leu 540	GAG Glu	AAT Asn	TTT Phe	GTG Val	AGT Ser 545	GAA Glu	GAC Asp	CTG Leu	GGC Gly	AAC Asn 550	ACT Thr	AGC Ser	ATC Ile	1745
CAG Gln 555	CTG Leu	CTG Leu	CAG Gln	GGG Gly	GAA Glu	GTG Val 560	ACT Thr	GTG Val	GAG Glu	CTT Leu	GTG Val 565	GCA Ala	GAA Glu	CAG Gln	AAG Lys	1793
AAC Asn 570	CAG Gln	ACT Thr	CTT Leu	CGA Arg	GAG Glu 575	GGA Gly	GAA Glu	AAA Lys	ATG Met	CAG Gln 580	TTG Leu	CCT Pro	GCT Ala	GGT Gly	GAG Glu 585	1841

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TAC CAT AAG GTG TAT ACG ACA TCA CCT AGC CCT TCT TGC TAC ATG TAC Tyr His Lys Val Tyr Thr Thr Ser Pro Ser Pro Ser Cys Tyr Met Tyr	1889
590 595 600	
GTC TAT GTC AAC ACT ACA GAG CTT GCA CTG GAG CAA GAC CTG GCA TAT Val Tyr Val Asn Thr Thr Glu Leu Ala Leu Glu Gln Asp Leu Ala Tyr	1937
605 610 615	
CTG CAA GAA TTA AAG GAA AAG GTG GAG AAT GGA AGT GAA ACA GGG CCT Leu Gln Glu Leu Lys Glu Lys Val Glu Asn Gly Ser Glu Thr Gly Pro	1985
620 625 630	
CTA CCC CCA GAG CTG CAG CCT CTG TTG GAA GGG GAA GTA AAA GGG GGC Leu Pro Pro Glu Leu Gln Pro Leu Leu Glu Gly Glu Val Lys Gly Gly	2033
635 640 645	
CCT GAG CCA ACA CCT CTG GTT CAG ACC TTT CTT AGA CGC CAA CAA AGG Pro Glu Pro Thr Pro Leu Val Gln Thr Phe Leu Arg Arg Gln Gln Arg	2081
650 655 660 665	
CTC CAG GAG ATT GAA CGC CGG CGA AAT ACT CCT TTC CAT GAG CGA TTC Leu Gln Glu Ile Glu Arg Arg Arg Asn Thr Pro Phe His Glu Arg Phe	2129
670 675 680	
TTC CGC TTC TTG TTG CGA AAG CTC TAT GTC TTT CGC CGC AGC TTC CTG Phe Arg Phe Leu Leu Arg Lys Leu Tyr Val Phe Arg Arg Ser Phe Leu	2177
685 690 695	
ATG ACT TGT ATC TCA CTT CGA AAT CTG ATA TTA GGC CGT CCT TCC CTG Met Thr Cys Ile Ser Leu Arg Asn Leu Ile Leu Gly Arg Pro Ser Leu	2225
700 705 710	
GAG CAG CTG GCC CAG GAG GTG ACT TAT GCA AAC TTG AGA CCC TTT GAG Glu Gln Leu Ala Gln Glu Val Thr Tyr Ala Asn Leu Arg Pro Phe Glu	2273
715 720 725	
GCA GTT GGA GAA CTG AAT CCC TCA AAC ACG GAT TCT TCA CAT TCT AAT Ala Val Gly Glu Leu Asn Pro Ser Asn Thr Asp Ser Ser His Ser Asn	2321
730 735 740 745	
CCT CCT GAG TCA AAT CCT GAT CCT GTC CAC TCA GAG TTC TGAAGGGGGC Pro Pro Glu Ser Asn Pro Asp Pro Val His Ser Glu Phe	2370
750 755	
CAGATGTTGG GTGCAGATGT AGAAGCAGCC AGTCACAGAC CCATTCTATG CAATGGACAT	2430
TTATTTGAAA AAAAAAAAAA AA	2452

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 758 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

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

Met	Ala	Val	Ser	Ala	Gly	Ser	Ala	Arg	Thr	Ser	Pro	Ser	Ser	Asp	Lys
1				5					10					15	
Val	Gln	Lys	Asp	Lys	Ala	Glu	Leu	Ile	Ser	Gly	Pro	Arg	Gln	Asp	Ser
			20					25					30		
Arg	Ile	Gly	Lys	Leu	Leu	Gly	Phe	Glu	Trp	Thr	Asp	Leu	Ser	Ser	Trp
		35					40					45			
Arg	Arg	Leu	Val	Thr	Leu	Leu	Asn	Arg	Pro	Thr	Asp	Pro	Ala	Ser	Leu
	50					55					60				
Ala	Val	Phe	Arg	Phe	Leu	Phe	Gly	Phe	Leu	Met	Val	Leu	Asp	Ile	Pro
65					70					75					80
Gln	Glu	Arg	Gly	Leu	Ser	Ser	Leu	Asp	Arg	Lys	Tyr	Leu	Asp	Gly	Leu
				85					90					95	
Asp	Val	Cys	Arg	Phe	Pro	Leu	Leu	Asp	Ala	Leu	Arg	Pro	Leu	Pro	Leu
			100					105					110		
Asp	Trp	Met	Tyr	Leu	Val	Tyr	Thr	Ile	Met	Phe	Leu	Gly	Ala	Leu	Gly
		115					120					125			
Met	Met	Leu	Gly	Leu	Cys	Tyr	Arg	Ile	Ser	Cys	Val	Leu	Phe	Leu	Leu
	130					135					140				
Pro	Tyr	Trp	Tyr	Val	Phe	Leu	Leu	Asp	Lys	Thr	Ser	Trp	Asn	Asn	His
145					150					155					160
Ser	Tyr	Leu	Tyr	Gly	Leu	Leu	Ala	Phe	Gln	Leu	Thr	Phe	Met	Asp	Ala
				165					170					175	
Asn	His	Tyr	Trp	Ser	Val	Asp	Gly	Leu	Leu	Asn	Ala	His	Arg	Arg	Asn
			180					185					190		
Ala	His	Val	Pro	Leu	Trp	Asn	Tyr	Ala	Val	Leu	Arg	Gly	Gln	Ile	Phe
		195					200					205			
Ile	Val	Tyr	Phe	Ile	Ala	Gly	Val	Lys	Lys	Leu	Asp	Ala	Asp	Trp	Val
	210					215					220				
Glu	Gly	Tyr	Ser	Met	Glu	Tyr	Leu	Ser	Arg	His	Trp	Leu	Phe	Ser	Pro
225					230					235					240
Phe	Lys	Leu	Leu	Leu	Ser	Glu	Glu	Leu	Thr	Ser	Leu	Leu	Val	Val	His
				245					250					255	
Trp	Gly	Gly	Leu	Leu	Leu	Asp	Leu	Ser	Ala	Gly	Phe	Leu	Leu	Phe	Phe
			260					265					270		

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Asp Val Ser Arg Ser Ile Gly Leu Phe Phe Val Ser Tyr Phe His Cys
 275 280 285
 Met Asn Ser Gln Leu Phe Ser Ile Gly Met Phe Ser Tyr Val Met Leu
 290 295 300
 Ala Ser Ser Pro Leu Phe Cys Ser Pro Glu Trp Pro Arg Lys Leu Val
 305 310 315 320
 Ser Tyr Cys Pro Gln Arg Leu Gln Gln Leu Leu Pro Leu Lys Ala Ala
 325 330 335
 Pro Gln Pro Ser Val Ser Cys Val Tyr Lys Arg Ser Arg Gly Lys Ser
 340 345 350
 Gly Gln Lys Pro Gly Leu Arg His Gln Leu Gly Ala Ala Phe Thr Leu
 355 360 365
 Leu Tyr Leu Leu Glu Gln Leu Phe Leu Pro Tyr Ser His Phe Leu Thr
 370 375 380
 Gln Gly Tyr Asn Asn Trp Thr Asn Gly Leu Tyr Gly Tyr Ser Trp Asp
 385 390 395 400
 Met Met Val His Ser Arg Ser His Gln His Val Lys Ile Thr Tyr Arg
 405 410 415
 Asp Gly Arg Thr Gly Glu Leu Gly Tyr Leu Asn Pro Gly Val Phe Thr
 420 425 430
 Gln Ser Arg Arg Trp Lys Asp His Ala Asp Met Leu Lys Gln Tyr Ala
 435 440 445
 Thr Cys Leu Ser Arg Leu Leu Pro Lys Tyr Asn Val Thr Glu Pro Gln
 450 455 460
 Ile Tyr Phe Asp Ile Trp Val Ser Ile Asn Asp Arg Phe Gln Gln Arg
 465 470 475 480
 Ile Phe Asp Pro Arg Val Asp Ile Val Gln Ala Ala Trp Ser Pro Phe
 485 490 495
 Gln Arg Thr Ser Trp Val Gln Pro Leu Leu Met Asp Leu Ser Pro Trp
 500 505 510
 Arg Ala Lys Leu Gln Glu Ile Lys Ser Ser Leu Asp Asn His Thr Glu
 515 520 525
 Val Val Phe Ile Ala Asp Phe Pro Gly Leu His Leu Glu Asn Phe Val
 530 535 540
 Ser Glu Asp Leu Gly Asn Thr Ser Ile Gln Leu Leu Gln Gly Glu Val
 545 550 555 560
 Thr Val Glu Leu Val Ala Glu Gln Lys Asn Gln Thr Leu Arg Glu Gly
 565 570 575

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Glu Lys Met Gln Leu Pro Ala Gly Glu Tyr His Lys Val Tyr Thr Thr
580 585 590

Ser Pro Ser Pro Ser Cys Tyr Met Tyr Val Tyr Val Asn Thr Thr Glu
595 600 605

Leu Ala Leu Glu Gln Asp Leu Ala Tyr Leu Gln Glu Leu Lys Glu Lys
610 615 620

Val Glu Asn Gly Ser Glu Thr Gly Pro Leu Pro Pro Glu Leu Gln Pro
625 630 635 640

Leu Leu Glu Gly Glu Val Lys Gly Gly Pro Glu Pro Thr Pro Leu Val
645 650 655

Gln Thr Phe Leu Arg Arg Gln Gln Arg Leu Gln Glu Ile Glu Arg Arg
660 665 670

Arg Asn Thr Pro Phe His Glu Arg Phe Phe Arg Phe Leu Leu Arg Lys
675 680 685

Leu Tyr Val Phe Arg Arg Ser Phe Leu Met Thr Cys Ile Ser Leu Arg
690 695 700

Asn Leu Ile Leu Gly Arg Pro Ser Leu Glu Gln Leu Ala Gln Glu Val
705 710 715 720

Thr Tyr Ala Asn Leu Arg Pro Phe Glu Ala Val Gly Glu Leu Asn Pro
725 730 735

Ser Asn Thr Asp Ser Ser His Ser Asn Pro Pro Glu Ser Asn Pro Asp
740 745 750

Pro Val His Ser Glu Phe
755

THAT WHICH IS CLAIMED IS:

1. Isolated DNA encoding a vitamin K dependent carboxylase selected from the group consisting of:

5 (a) isolated DNA selected from the group consisting of DNA which encodes a bovine 94,000 dalton vitamin K dependent carboxylase having the sequence of SEQ ID NO: 9, SEQ ID NO: 11 or SEQ ID NO: 13, and DNA having the sequence given herein as SEQ ID NO: 15 and which encodes human
10 vitamin K dependent carboxylase;

(b) isolated DNA which hybridizes to the complementary strand of isolated DNA of (a) above under stringent conditions represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, and 0.1% SDS at 70°C and
15 which encodes a vitamin K dependent carboxylase at least 75% homologous to isolated DNA of (a) above; and

(c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encodes a vitamin
20 K dependent carboxylase.

2. Isolated DNA according to claim 1, which encodes a carboxylase which activates a vitamin K dependent protein selected from the group consisting of Factor VII, Factor
25 IX, Factor X, Protein C, Protein S, and Prothrombin.

3. Isolated DNA according to claim 1 which encodes human vitamin K dependent carboxylase.

30 4. A recombinant DNA molecule comprising cloning vector DNA and a DNA according to claim 1.

5. A recombinant DNA molecule according to claim 4, wherein said vector DNA comprises a vector selected from

the group consisting of plasmids, adenoviruses, and cytomegaloviruses.

6. A recombinant DNA molecule according to claim 4,
5 wherein said vector DNA comprises a baculovirus vector.

7. A host cell containing a recombinant DNA molecule of claim 4 which expresses the encoded carboxylase.

10 8. A host cell according to claim 7, wherein said host cell is a mammalian cell.

9. A host cell according to claim 7, wherein said host cell is selected from the group consisting of baby
15 hamster kidney cells, mouse cells, human embryo cells, and chinese hamster ovary cells.

10. A host cell according to claim 7, wherein said host cell is an insect cell.
20

11. A host cell according to claim 7, wherein said host cell also expresses a vitamin K-dependent protein.

12. A host cell according to claim 7, wherein said
25 host cell also expresses a vitamin K-dependent protein comprising a blood coagulation protein.

13. A host cell according to claim 7, wherein said host cell also expresses a vitamin K dependent protein
30 selected from the group consisting of Factor VII, Factor IX, Factor X, Protein C, Protein S, and Prothrombin.

14. In a method of making a vitamin K dependent protein which comprises culturing a host cell which

expresses a vitamin K dependent protein in the presence of vitamin K, and then harvesting said vitamin K dependent protein from the culture, the improvement comprising:

employing as said host cell a eukaryotic host cell
5 containing a recombinant DNA molecule comprising cloning vector DNA operable in said host cell and a DNA encoding a vitamin K dependent carboxylase selected from the group consisting of:

(a) isolated DNA selected from the group
10 consisting of DNA which encodes a bovine 94,000 dalton vitamin K dependent carboxylase having the sequence of SEQ ID NO: 9, SEQ ID NO: 11 or SEQ ID NO: 13, and DNA having the sequence given herein as SEQ ID NO: 15 and which encodes human vitamin K dependent carboxylase;

15 (b) isolated DNA which hybridizes to the complementary strand of isolated DNA of (a) above under stringent conditions represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, and 0.1% SDS at 70°C and which encodes a vitamin K
20 dependent carboxylase at least 75% homologous to isolated DNA of (a) above; and

(c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which
25 encodes a vitamin K dependent carboxylase;

said host cell expressing increased amounts of said vitamin K dependent protein due to the expression of functional vitamin K dependent carboxylase by said host cell.

30 15. A method according to claim 14, wherein said vitamin K dependent protein comprises a blood coagulation protein.

16. A method according to claim 14, wherein said vitamin K dependent protein is selected from the group consisting of Factor VII, Factor IX, Factor X, Protein C, Protein S, and Prothrombin.

17. A method according to claim 14, wherein said vector DNA comprises a vector selected from the group consisting of plasmids, adenoviruses, and cytomegaloviruses.

18. A method according to claim 14, wherein said vector DNA comprises a baculovirus vector.

19. A method according to claim 14, wherein said host cell is a mammalian cell.

20. A method according to claim 14, wherein said host cell is an insect cell.

21. A method according to claim 14, wherein said vitamin K-dependent carboxylase is bovine vitamin K dependent carboxylase.

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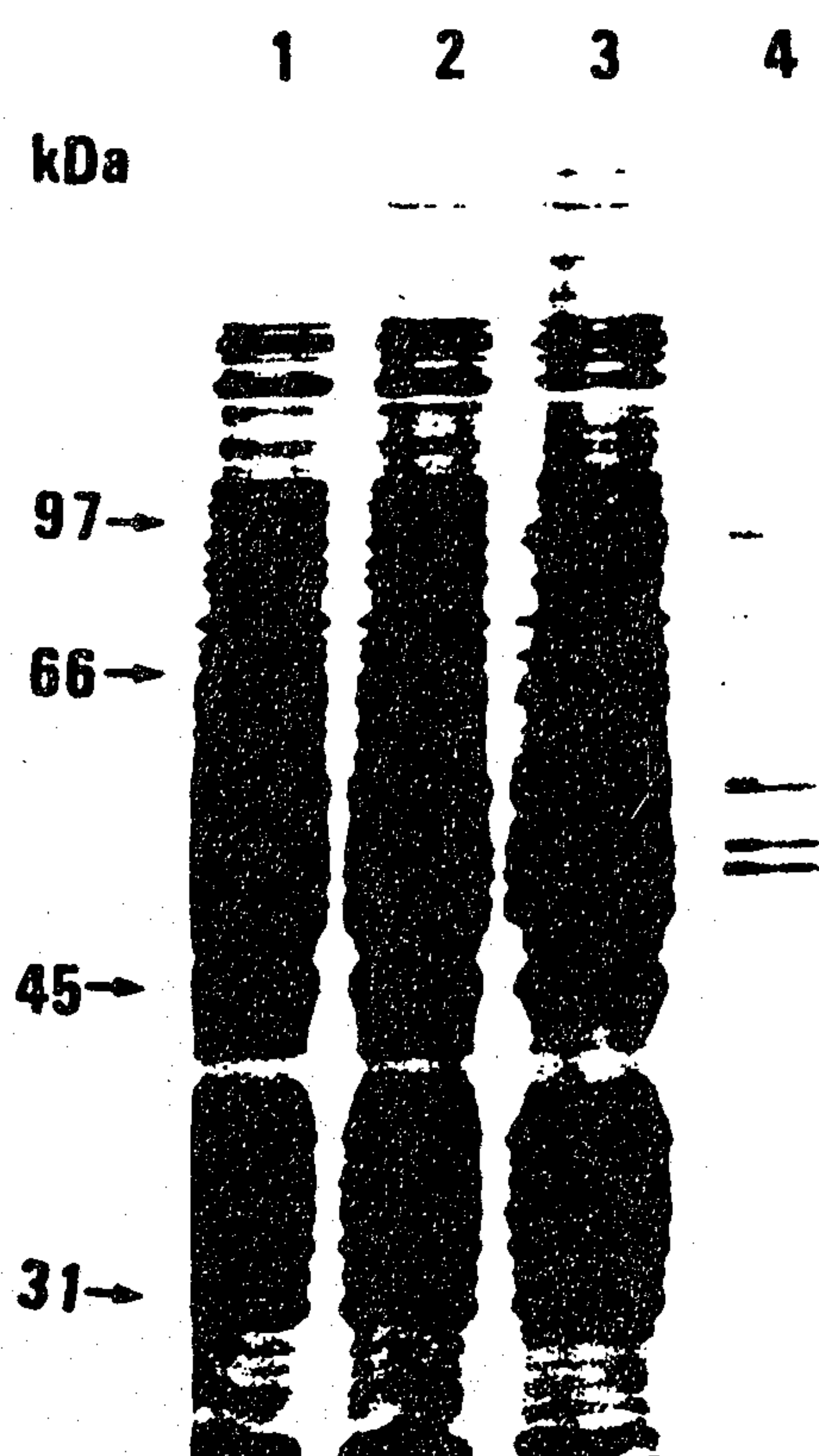


FIGURE 1

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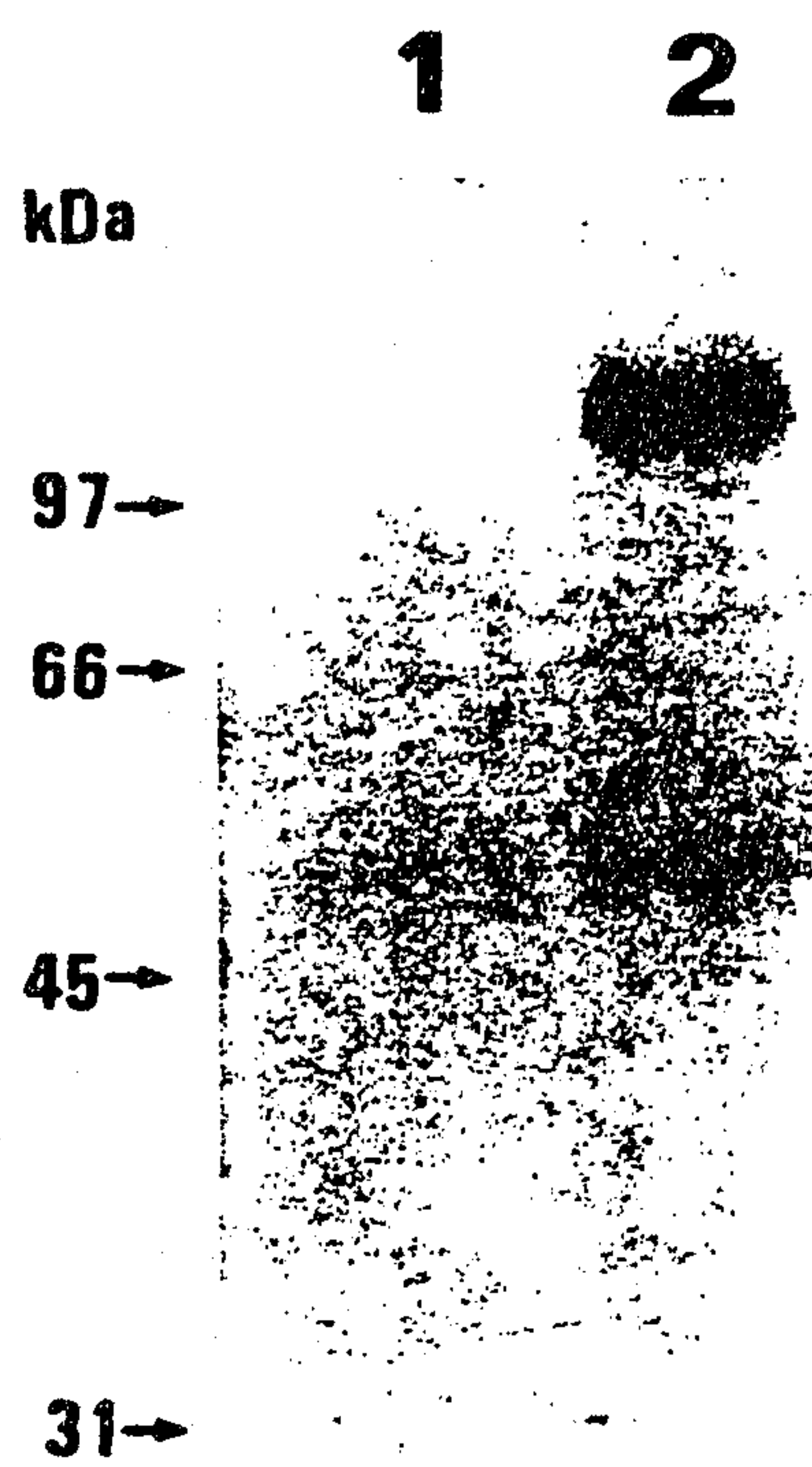


FIGURE 2

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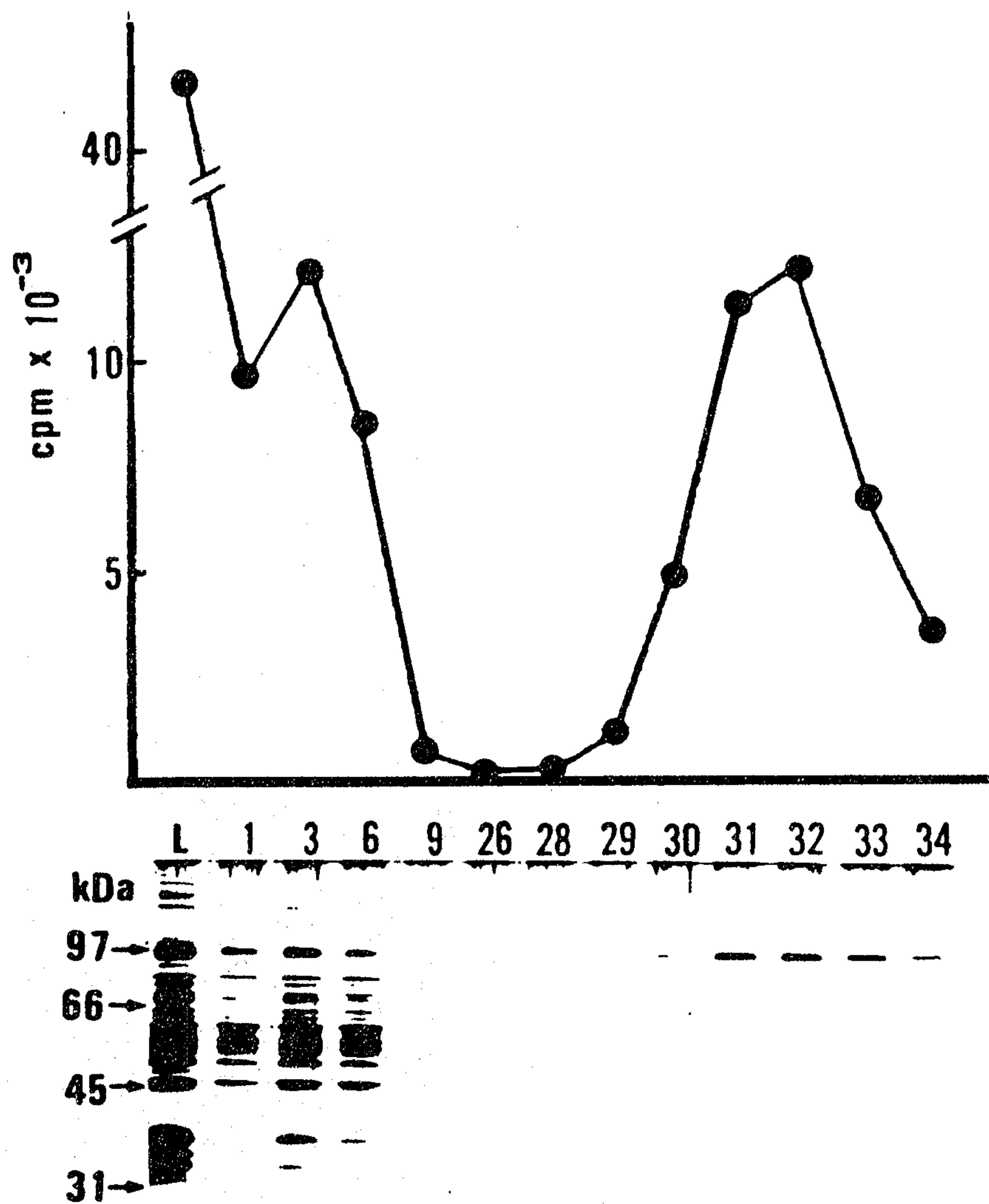


FIGURE 3

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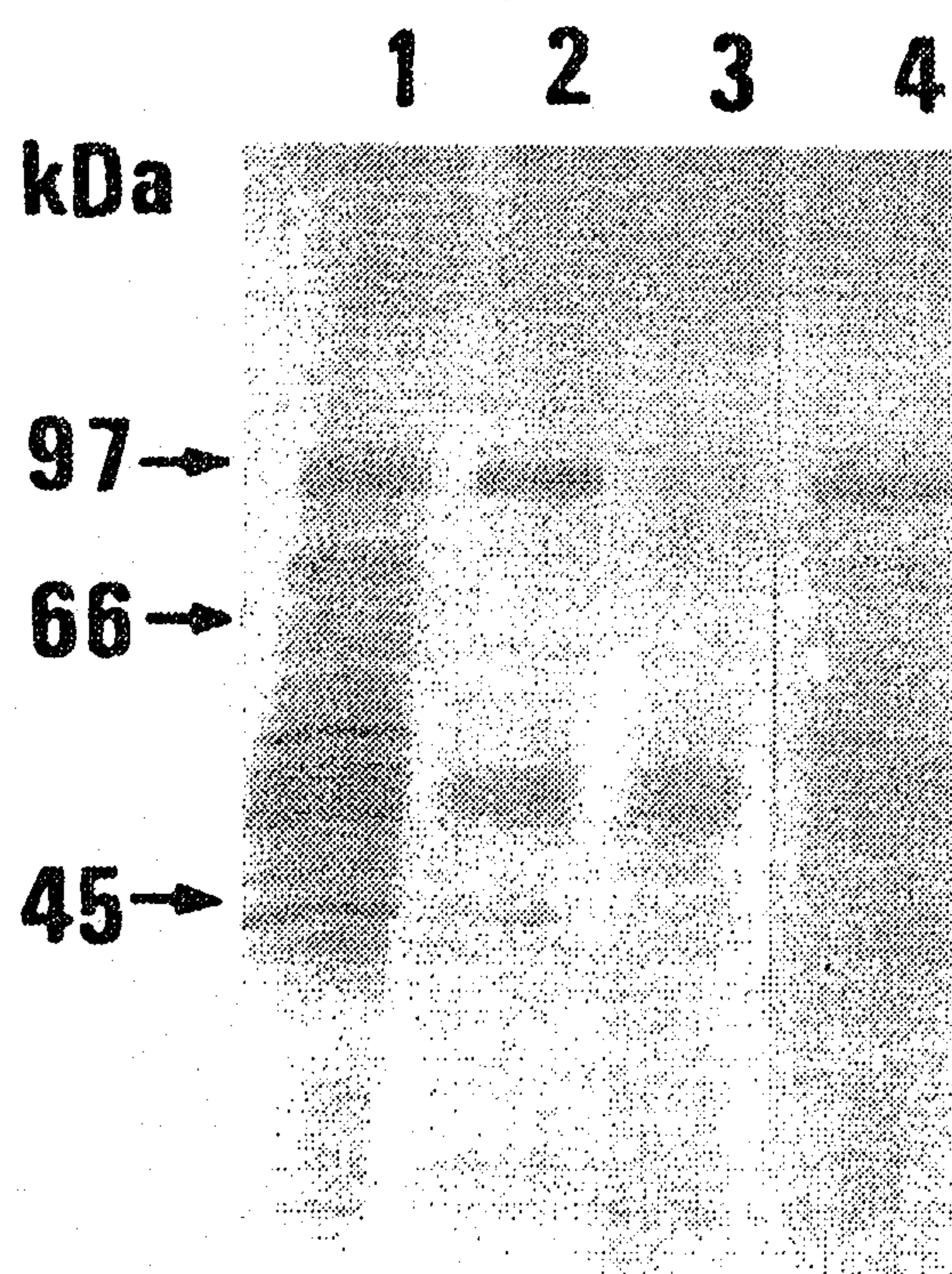


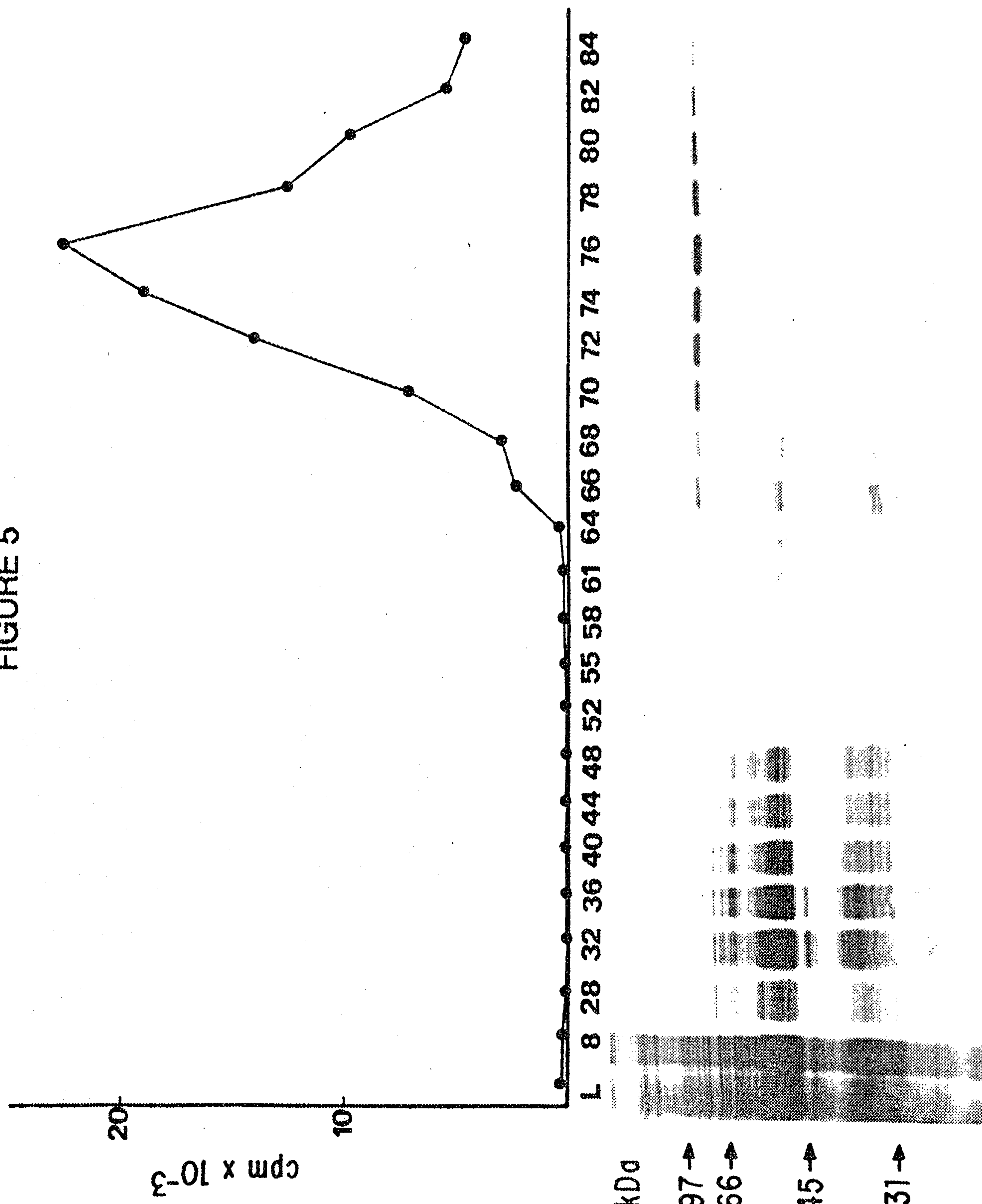
FIGURE 4

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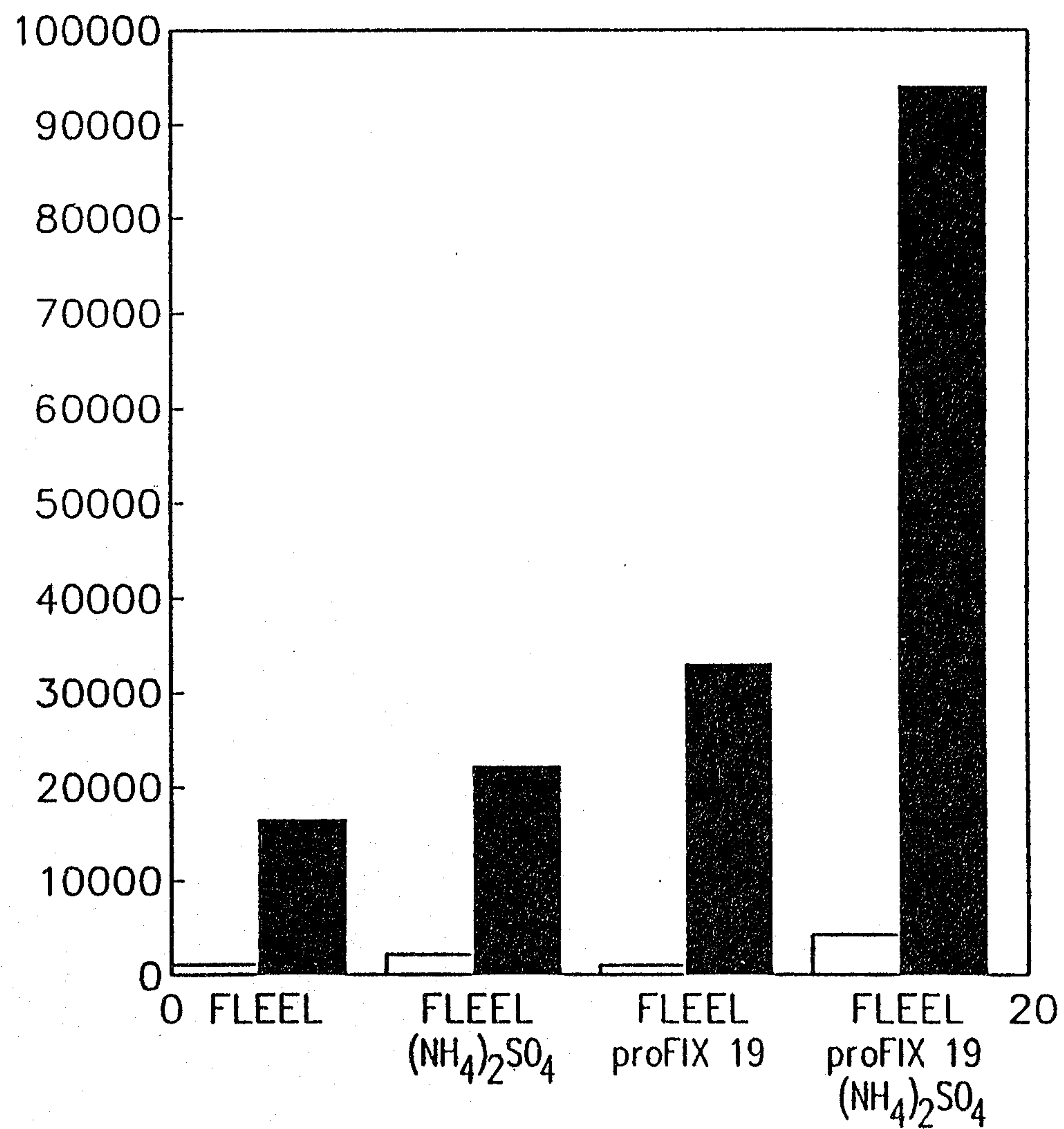
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FIGURE 5



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FIG. 6.

