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(54) Titre : NOUVELLE CYTOKINE DE TYPE FACTEUR DE CROISSANCE TRANSFORMANT- β (TGF- β)
(54) Title: NOVEL TGF- β LIKE CYTOKINE

(57) **Abrégé/Abstract:**

A novel TGF- β like cytokine is described which has been designated pCL13. Polynucleotide molecules encoding pCL13 and biologically active fragments are also described as well as methods of expression and uses of the proteins, fragments and polynucleotide molecules.



Abstract

A novel TGF- β like cytokine is described which has been designated pCL13. Polynucleotide molecules encoding pCL13 and biologically active fragments are also described as well as methods of
5 expression and uses of the proteins, fragments and polynucleotide molecules.

NOVEL TGF- β LIKE CYTOKINE

This invention relates to a novel TGF- β like cytokine and to isolated polynucleotide molecules encoding this protein. Particular applications of the invention may include treatments for wound and fracture healing, treatments and diagnostic assays for cancer, autoimmune and fibrotic diseases.

Macrophages play a central role in chronic inflammatory processes. The importance of these cells derives from the large variety of bioactive molecules that they produce and consequently, their capacity to amplify the inflammatory response. Their central role is also due to their capacity for communication with many other cells. For example, macrophage derived platelet derived growth factor (PDGF) is an important growth factor for both fibroblasts and smooth muscle cells. Another group of proteins of great significance in the relationship of macrophages with various connective tissue cells (e.g. fibroblasts, smooth muscle, endothelium osteoblasts etc) are the TGF- β superfamily cytokines, especially the TGF- β proteins themselves.

The TGF- β superfamily consists of growth and differentiation factors that share substantial structural homology (1). In vertebrates, individual families comprise the TGF- β proteins themselves, the growth and differentiation factors (GDF)(embryonic growth and development), the bone morphogenetic proteins (BMP)(induce cartilage and bone formation), the inhibins and activins (regulate FSH secretion by pituitary), and mullerian inhibitory substance (MIS)(regression of Mullerian duct during male sex differentiation). These proteins share important structural features. Their bioactivity resides in the carboxyterminal region of 100-150 amino acids. Over this region, members of this superfamily share about 30% sequence identity to TGF- β 1 and have 7 conserved cysteine residues. Within individual subgroups of the superfamily, proteins share 70% to 90% identity over the bioactive carboxy terminal domain. All superfamily members are thought to be cleaved at a cluster of basic residues 110 to 140 amino acids from the carboxy terminus of a precursor protein. Processing occurs immediately following a conserved RXXR sequence.

The three human TGF- β proteins share 80% sequence similarity over the bioactive portion of the molecule. The pro peptide (called latency-associated peptide (β 1-LAP)) displays less than 50% similarity between

family members. The β 1-LAP is cleaved from the mature protein, but remains disulphide bonded to it. Separation of the β 1-LAP is necessary to achieve biological activity (2).

The TGF- β proteins have been studied intensively because of their biological importance and therapeutic potential. Their biology and functions are well known and have been extensively reviewed (e.g. 2, 3, 4). In general terms they promote differentiation and differentiated function in a wide variety of cells. They are potent chemotactic factors for macrophages and fibroblasts and generally inhibit cell proliferation, perhaps because of their role in differentiation. In the context of inflammation, TGF- β is a potent stimulator of fibroblast collagen and matrix protein synthesis, promotes angiogenesis, modulates expression of adhesion molecules and inhibits lymphocyte proliferation, production of some lymphokines and NK cell function. This molecule has been of great interest to the pharmaceutical industry mainly, because of its demonstrable capacity to promote wound and fracture healing *in vivo*. TGF- β has also been heavily implicated in the pathogenesis of chronic inflammatory processes and mechanisms. Further, its local production has been used as a surrogate marker e.g. in active fibrotic diseases such as cirrhosis and it therefore has potential in the diagnostic arena.

The present inventors have now isolated a polynucleotide molecule including a novel cytokine gene, clone 13 (CL13), which encodes a dimeric protein (pCL13) that appears to represent the first member of a new class of protein within the TGF- β superfamily.

Thus, in a first aspect, the present invention provides an isolated polynucleotide molecule comprising a nucleotide sequence encoding, or complementary to a sequence encoding, a protein designated pCL13 or a biologically active fragment thereof.

The isolated polynucleotide molecule may comprise a nucleotide sequence the same as that of the CL13 clone described herein or may contain single or multiple nucleotide substitutions and/or deletions and/or additions thereto. The nucleotide substitutions which are envisaged may result in one or more conservative or non-conservative amino acid substitution(s). By conservative substitutions, the intended combinations are - G,A; V,I,L,M; D,E; N,Q; S,T; K,R,H; F,Y,W,H; and P,N α -alkylamino acids. The term "nucleotide sequence" also includes sequences with sufficient homology to

hybridise with the nucleotide sequence under medium or, more preferably, high stringency conditions (5) and to nucleotide sequences encoding functionally equivalent sequences. In addition, the term "nucleotide sequence" includes sequences having at least 70%, more preferably 90%,
5 homology to clone 13 described herein or any portion thereof of > 10 nucleotides in length.

Most preferably, the isolated polynucleotide molecule comprises a nucleotide sequence substantially corresponding to the nucleotide sequence shown in Figure 1 or a portion thereof, or a complementary sequence
10 thereto. The term "portion thereof", in this regard, is to be understood as referring to portions of the nucleotide sequence which encode biologically active protein fragments and also, to portions of the nucleotide sequence, preferably >10 nucleotides in length, which may be used in, or for the production of probes useful for, hybridisation assays.

15 The present invention also further extends to oligonucleotide primers for the above sequences, antisense sequences and homologues of said primers and antisense sequences, complimentary ribozyme sequences, catalytic antibody binding sites and dominant negative mutants of the polynucleotide molecule.

20 In a second aspect, the invention provides a protein designated pCL13, or a biologically active fragment thereof, in substantially pure form.

Preferably, the protein, or biologically active fragment thereof, comprises a monomeric polypeptide having an amino acid sequence substantially corresponding to the amino acid sequence shown in Figure 1 or
25 a fragment thereof.

Biologically active fragments thereof as mentioned in the first and second aspects refers to monomeric pCL13 polypeptides (with or without the propeptide) and other polypeptide or peptide portions (whether monomeric or dimeric) thereof which may consist of sequences which inhibit, mimic or
30 enhance the biological effect of the protein and dominant negative protein mutants, binding proteins including soluble receptors, other protein and/or glycosaminoglycans. The pCL13 propeptide may also represent a biologically active fragment of pCL13.

The protein, or biologically active fragment thereof, according to the
35 second aspect may be purified from natural sources (e.g. lungs, skin etc) or

cell lines, or may be produced recombinantly by any of the methods common in the art (5).

5 In a third aspect, the present invention provides an organism transformed with the polynucleotide molecule of the first aspect of the present invention.

The organisms which may be usefully transformed with the polynucleotide molecule of the first aspect include bacteria such as *E.coli* and *B.subtilis*, eukaryotic cell lines such as CHO, fungi, yeast, non-human animals and plants.

10 Transformed or transgenic, non-human animals may be established to, for example, overexpress CL13, pCL13 or a biologically active fragment thereof or, alternatively, generate antisense or ribozyme RNA molecules to inhibit native CL13 expression.

15 In a fourth aspect, the invention provides an antibody or fragment thereof specific to pCL13 or an antigenic portion thereof. The antibody may be polyclonal or monoclonal and may be produced by any of the methods common in the art.

20 It is also to be understood that the invention relates to kits for diagnostic assays, said kits comprising an antibody according to the fourth aspect of the present invention or nucleotide primers for PCR based assays.

In a fifth aspect the invention provides a protein or antigenic portion thereof, capable of binding to an anti-pCL13 antibody.

25 pCL13 is suitable for *in vivo* and *in vitro* procedures involving both human and animal cells. pCL13 is also suitable for both medical and veterinary use. In particular, pCL13 may be suitable for methods of treatment for any disease or condition beneficially treatable with TGF- β or another member of the TGF- β superfamily.

30 In a further aspect, the present invention provides a method of treatment to assist wound and/or fracture healing and/or ischaemic injury, comprising administering (for example, orally, topically, intravenously or subcutaneously) to a subject a preparation comprising a protein, or biologically active fragment thereof, according to the second or fifth aspects of the present invention, optionally in admixture with a suitable pharmaceutically acceptable carrier.

35 The protein, or biologically active fragment thereof, according to the second or fifth aspects may also be useful for one or more of the following:

- (i) Immunosuppression and anti-inflammatory effects for conditions such as autoimmune diseases or transplantation;
- (ii) Down regulation of leukocyte extravasation and motility in infective or inflammatory processes; and
- 5 (iii) Treatment of tumours through promotion of differentiation and antiproliferation action.

Such uses may be achieved by administration of the protein, or a biologically active fragment thereof, to a subject, or by gene therapy using all or part of the polynucleotide molecule of the first aspect. Such gene therapy
10 may be used to, for example, establish overexpression of CL13, or pCL13 or a biologically active fragment thereof in the host cell or, alternatively, to generate antisense or ribozyme RNA molecules to inhibit native CL13 expression.

It is also possible that inhibiting the action of pCL13 may provide
15 treatment of fibrotic/fibroproliferative disorders such as rheumatoid arthritis, atherosclerosis, pulmonary fibrosis, scleroderma, liver cirrhosis and keloids, and inhibition of tumour immunosuppression associated with conditions such as tumours, infections (especially viral) and chronic inflammatory diseases. These treatments may be achieved by using:

20 fragments or peptides of the pCL13 protein that inhibit receptor binding;

binding proteins for pCL13 including soluble receptors for this molecule, glycosaminoglycans, and other molecules which may inhibit or destabilise receptor ligand interaction;

25 antibodies directed at pCL13 or its receptor;

antisense or ribozyme strategies in which expression or stability of the pCL13 gene product is disturbed;

dominant negative mutants of the CL13 gene which, when expressed in a host cell, will destabilise or affect the activity of pCL13. (As the pCL13
30 protein is a dimer, a second gene product which has been modified may bind to the native pCL13 to form a heterodimer). Thus, an appropriately modified pCL13 variant may essentially render the pCL13 inactive through mechanisms such as enhanced degradation, aberrant intracellular trafficking and inhibition of export from the cell and inhibition of bioactivity.)

The invention thus further resides in a heterodimeric protein comprising a monomeric polypeptide of pCL13 together with a monomeric polypeptide of another protein from the TGF- β superfamily.

pCL13 or biologically active fragments thereof may be formulated
5 into standard pharmaceutical compositions suitable for the administration of proteins. Suitable formulations can be found, for example, in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Company, Easton, PA.

The dosage levels of pCL13 or biologically active fragments thereof
10 may be comparable to those useful for other members of the TGF- β superfamily. These levels are well understood in the art, and the precise dosage can be adjusted according to the condition of the subject, the mode of administration, and the judgement of the attending physician.

Possible diagnostic applications include diagnosis of cancer,
15 inflammatory and fibrotic disorders such as rheumatoid arthritis, cirrhosis and atherosclerosis in which enhanced synthesis of this gene may be present.

To facilitate the abovementioned applications for pCL13, it will be necessary to produce the protein in large quantities. However, extensive
20 studies with other protein members of the TGF- β superfamily, has revealed a number of difficulties in achieving expression in commercial amounts. For instance, expression in simple prokaryotic systems are largely unsuitable since members of the superfamily are cysteine knot dimeric proteins having a complex pattern of disulphide bond linkages.

The current strategy for expression of TGF- β superfamily proteins is
25 therefore to express the whole protein from a suitable DNA construct transfected into mammalian cells. However, this strategy necessitates treatment of the culture supernatant to separate processed (cleaved) bioactive mature protein from the propeptide and unprocessed (uncleaved)
30 material. This creates additional costs and difficulties because some of the expressed material is non-productive as typically 30%-50% of the secreted material will not be appropriately cleaved. The additional chromatographic procedure also generates extra losses of protein and incurs additional cost and time.

Previous efforts to express the mature bioactive portion of these proteins alone, has been unsuccessful, indicating that the propeptide is essential for achieving expression and secretion.

The present inventors, however, have been unexpectedly able to achieve expression and secretion of pCL13 without expressing the leader or propeptide, using transfected mammalian cell cultures.

Thus, in a still further aspect, the present invention provides a method for producing a protein designated pCL13 or a biologically active fragment thereof, comprising transforming a suitable host organism with a polynucleotide molecule comprising a nucleotide sequence encoding pCL13 or a biologically active fragment thereof, wherein said polynucleotide molecule is constitutively or inducibly expressed in said host organism.

Preferably, the nucleotide sequence encoding the pCL13 or a biologically active fragment thereof, does not comprise sequence encoding the leader or propeptide of pCL13.

In place of sequences encoding the native leader or propeptide, it may be preferable to include within the polynucleotide molecule sequences encoding a heterologous leader (e.g. the follicle stimulating hormone (FSH) leader sequence) to assist expression.

Suitable host organisms may be any of those mentioned above in respect to the third aspect of the present invention. However, preferred organisms include mammalian cell lines, yeast (e.g. *Pichia* and *Saccharomyces*) and non-human animals.

Expression of only the mature bioactive portion of pCL13 thereby provides the following advantages:

- (i) Higher levels of expression; and
- (ii) No necessity to purify from propeptide and unprocessed full length CL13 protein.

Further, since it is not necessary to express the whole protein, it is possible and simple to add amino-terminal epitope tags (e.g. FLAG and/or HIS) that can significantly assist with the purification and visualisation of recombinant protein.

Also, the capacity to express the mature bioactive portion of pCL13 in mammalian cells, indicates that it will also be able to be readily expressed in yeast strains, such as the *Pichia pastoris* which is capable of secreting

disulphide linked proteins. Production of protein in yeast is much cheaper and easier than production by mammalian cells.

The invention will now be further described by way of the following non-limiting examples and with reference to the
5 accompanying figures.

In accordance with one aspect of the present invention there is provided an isolated polynucleotide molecule obtainable from human chromosome 19p13.1, wherein said polynucleotide molecule comprises a nucleotide sequence encoding a protein that belongs to the TGF- β
10 superfamily having a conserved RXXR site, 7 conserved cysteine residues and, in mature processed form, is dimerized, wherein the encoded protein is capable of: (i) being expressed by the human neonatal fibroblast line CCD34Lu in increased levels in response to IGF-1, PDGF BB, TGF- β or TNF- α ; (ii) being expressed by macrophages
15 in increased levels in response to GM-CSF, M-CSF, IL-2 or TNF- α ; and (iii) being expressed by human umbilical vein endothelial cells in increased levels in response to ECGF, and comprises the partial amino acid sequence of amino acids 263 to 308 of Figure 1.

In accordance with another aspect of the present invention there
20 is provided an isolated polynucleotide molecule comprising a nucleotide sequence shown in Figure 1, or a nucleotide sequence complementary thereto.

In accordance with yet another aspect of the present invention there is provided an isolated polynucleotide molecule comprising a
25 nucleotide sequence having at least 70% identity to the nucleotide sequence shown in Figure 1, and which encodes a protein that belongs to the TGF- β superfamily and has a conserved RXXR site and 7 conserved cysteine residues, and wherein the encoded protein is capable of: (i) being expressed by the human neonatal fibroblast line
30 CCD34Lu in increased levels in response to IGF-1, PDGF BB, TGF- β or TNF- α ; (ii) being expressed by macrophages in increased levels in response to GM-CSF, M-CSF, IL-2 or TNF- α ; and (iii) being expressed

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by human umbilical vein endothelial cells in increased levels in response to ECGF, or a mature bioactive portion thereof.

In accordance with still yet another aspect of the present invention there is provided an isolated polynucleotide molecule comprising a nucleotide sequence having at least 90% identity to the nucleotide sequence shown in Figure 1, and which encodes a protein that belongs to the TGF- β superfamily and has a conserved RXXR site and 7 conserved cysteine residues, and wherein the encoded protein is capable of: (i) being expressed by the human neonatal fibroblast line CCD34Lu in increased levels in response to IGF-1, PDGF BB, TGF- β or TNF- α ; (ii) being expressed by macrophages in increased levels in response to GM-CSF, M-CSF, IL-2 or TNF- α ; and (iii) being expressed by human umbilical vein endothelial cells in increased levels in response to ECGF, or a mature bioactive portion thereof.

In accordance with still yet another aspect of the present invention there is provided an isolated polynucleotide encoding a protein that belongs to the TGF- β superfamily having a conserved RXXR site and 7 conserved cysteine residues, the isolated polynucleotide comprising a sequence selected from: a) the sequence of the polynucleotide as set out in Figure 1; and b) the sequence of a polynucleotide that hybridizes to a complement of the sequence set out in Figure 1 under highly stringent hybridization conditions comprising hybridization in 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 μ g/ml denatured salmon sperm DNA at 68°C overnight, followed by washing in a solution comprising 0.1 X SSPE and 0.1% SDS at 68°C.

Brief Description of the Figures

Figure 1 provides the nucleotide sequence and putative amino acid sequence of clone 13 encoding pCL13.

Figure 2 shows CL13 expression in macrophage cultures.

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Panel A. 15 µg total RNA was loaded per lane. Macrophage treatments were: lane 1, no treatment; lane 2, 1,000 U IFN γ overnight; lane 3, 1 µM retinoic acid overnight; lane 4, 1 µM retinoic acid overnight followed by 10 µg/mL LPS for 3 hours; lane 5, 10 µg/mL LPS for 3 hours.

Panel B. 20 µg total RNA was loaded per lane. Macrophage treatments were: lane 1, 1 µM retinoic acid for 3 days followed by 10 µg/mL LPS for 3 hours; lane 2, 1 µM retinoic acid overnight followed by 50 nM PMA for 3 hours; lane 3, 50 nM PMA for 3 hours; lane 4, untreated macrophages.

Figure 3 shows a northern blot analysis of clone 13 expression from macrophages treated with cytokines. All treatments were 3 hours. Lane 1, untreated macrophages; lane 2, 50 nM PMA; lane 3, 50 U/mL GM-CSF; lane 4, 100 U/mL M-CSF; lane 5, 100 U/mL IL1- β ; lane 6, 10ng/mL TGF- β ; lane 7, 10 U/mL PDGF-BB; lane 8, 50 U/mL IL-2; lane 9, 100 U/mL TNF- α ; lane 10, 50 U/mL IL-6.

Figure 4 shows a northern blot analysis of the expression of clone 13 in U937. 20 µg total RNA was loaded per lane on a 1.2% agarose denaturing formaldehyde gel. Lane 1, no treatment; lane 2, 1 µM retinoic acid for 3 days; lanes 3, 4, 5, 6, 7, 1 µM retinoic acid for 3 days followed by 160 nM PMA for 20 min. 1 h, 2 h, 3 h and 12 h respectively; lane 8, 160 nM PMA for 3 h. Probes were labelled with ^{32}P . The blot was hybridized at 65°C and subjected to post hybridization washes and autoradiography.

Figure 5 provides a multiple sequence alignment of the carboxy terminal halves of pCL13 and other TGF- β superfamily members.

Figure 6 provides the nucleotide sequence and putative amino acid sequence for clone 13 in construct C13LB. The coding region for the bioactive portion of pCL13 commences with nucleotide 625.

Figure 7 provides the nucleotide sequence and putative amino acid sequence for clone 13 in construct FFC13S. The predicted bioactive portion of pCL13 commences with amino acid 92.

Figure 8 provides the nucleotide sequences and putative amino acid sequence for clone 13 in construct C13SA. The coding region for the bioactive portion of pCL13 commences with nucleotide 136.

Figure 9 shows a Western blot of purified recombinant pCL13 (FFC13S construct) visualised with anti-FLAG antibody.

Figure 10 provides a graph of the results obtained from glycosaminoglycan analysis in non-transfected (K1) and CL13 (FFC13S construct) transfected (P4N, 15 and 24) CHO cells using dimethyl-methylene blue (DMB) assay.

Figure 11 provides a graph of results obtained from collagen production assays of non-transfected (K1) and CL13 (FFC13S construct) transfected (P4N, 15 and 24) CHO cells.

Figure 12 provides graphs of results obtained from glycosaminoglycan production analysis in 3T3 (Figure 12A) and CCD (Figure 12B) cells following addition of various concentrations of pCL13 (expressed from construct C13SA).

Figure 13 provides graphical results obtained from collagen production analysis in CCD cells following addition of various concentrations of pCL13 (expressed from construct C13SA).

Figure 14 provides graphs of results showing growth factor activity under limiting serum conditions of pCL13 (expressed from construct C13SA) against TGF β in human baby foreskin fibroblasts (BFF) (Figure 14A) and 3T3 cells (Figure 14B).

Figure 15 provides graphs of results showing growth factor activity in the presence of serum of pCL13 (expressed from construct C13SA) and TGF β in BFF and 3T3 cells.

Figure 16 provides graphs of results showing the effect of pCL13 (expressed from construct C13SA) of pCL13, TGF β and IFN α 2b on the proliferation of U937 human monocytic cells (Figure 16A) and mono Mac 6 human monocytic cells (Figure 16B).

Figure 17 provides graphical results of an analysis of differing pCL13 (expressed from construct C13SA) concentrations on TNF- α production in human culture derived macrophages.

The largest open reading frame codes for a high cysteine containing protein with a signal peptide. It bears strong homology to members of the

TGF- β superfamily (including TGF- β itself) when analysed using the *fasta* program on the ANGIS facility (opt scores 180-250). Extensive multiple sequence alignment using the CLUSTAL V program on GCG has been undertaken with the CL13 translated amino acid sequence (pCL13) and most members of this superfamily (see Figure 2).

pCL13 is a dimeric protein with a conserved RXXR site that is likely to be involved in cleavage of a large pro-peptide with the encoded polypeptide decreasing from a predicted monomeric mass of about 34kDa to 13kDa. pCL13 has a potential glycosylation sites in its pro-peptide, but none in the mature protein, suggesting that glycosylation may be for intracellular targeting as it is in TGF- β .

In this superfamily the bio-activity resides in the carboxy terminal half of the molecule. There is strong conservation in this region between all superfamily members, especially in 7 of the cysteine residues. The full alignment data unequivocally demonstrates that pCL13 belongs to the TGF- β superfamily. In this superfamily within family identity is of the order of 70-80%. pCL13 does not display identity of this degree to any of the individual families and therefore appears to represent an entirely new and separate category within the TGF- β superfamily.

The full nucleotide sequence and putative amino acid sequence for clone 13 (CL13) is provided at Figure 1.

EXAMPLE 2: CL13 Gene Expression and Analysis of Biological Activity

Extensive studies of regulation of CL13 gene expression have been undertaken using the ^{32}P labelled clone insert and the results are summarised at Table 1. Some examples are also illustrated in Figures 2, 3 and 4. The results indicate that the 1.2 kb transcript was present at very low levels in untreated U937 and culture derived macrophages. Expression increased markedly with phorbol 12-myristate 13-acetate (PMA), but was not upregulated by LPS or interferon- γ (IFN- γ). Clone 13 was expressed strongly in macrophages treated with GM-CSF, M-CSF, IL2 or TNF- α and to a lesser extent with TGF- β , PDGF-BB or IL-6. There was also increased expression of CL13 mRNA in a human neonatal fibroblast cell line (CCD34Lu) in response to IGF-1, PDGF BB, TGF- β or

TNF- α and in human umbilical vein endothelial cells grown with ECGF. No expression of this gene was found in either resting or activated B or T lymphocytes/cell lines.

We can deduce reasonable hypotheses about the nature of the biological role of this protein on the basis of its expression and the general characteristics of the superfamily. CL13 expression could be induced in culture derived macrophages (MAC) by a variety of activation agents including cytokines and PMA but not LPS. Its expression was also induced in fibroblasts by activation and could not be induced at all in lymphocytes. As the endothelial cells tested were grown in the presence of ECGS, it is not possible to conclude whether expression is absent under resting conditions.

It may be of particular significance that TGF- β induces expression of CL13 in both fibroblasts and MAC. It is possible that some of the functions ascribed to TGF- β may be due to an autocrine or paracrine induction of TGF- β by CL13.

Many of the proteins in this TGF- β superfamily act on mesenchymal cells and it is anticipated that this will be true for pCL13. It is also thought that pCL13 may enhance the effector function of these cells, perhaps in a manner similar to TGF- β itself.

Lymphocytes and macrophages are intimately related in biological function. The fact that lymphocytes do not appear able to express CL13, but MAC express it in large amounts suggests the possibility that the lymphocyte may also represent a target for pCL13.

In summary, pCL13's properties and pattern of expression suggest that there may be some similarities to TGF- β . However, whilst it belongs to this superfamily, it can be said with some certainty, on the basis of sequence comparison, that pCL13 is one of a new class of proteins within this superfamily and is not an undescribed TGF- β protein (e.g. TGF- β 6).

TABLE 1
SUMMARY OF NORTHERN BLOT ANALYSIS OF CLONE 13[#]

	<u>TREATMENT</u>	<u>1.2 kb mRNA</u>
Monocytoid cell lines: HL60, KG1	untreated	-
	RA or PMA	-
	RA/PMA	+
Monocytoid cell line: U937	untreated	+
	IFNg or LPS or both	+
	RA alone or with LPS	++
	PMA	+++
	RA/PMA (3 h)	++++
	RA /PMA (12 h)	+++++
	TGFb	++
	PMA/IL4	++
Macrophages ¹ (peripheral blood derived)	untreated	-
	RA	+
	PMA	+++
	RA /PMA	++++
	LPS or IFN-g	-
	IFN-g followed by IL2	+
	GM CSF	+
	IL 6 or IL2 or PDGF BB or TGF b	++
	M CSF or IL1 b or TNFa	+++
B cell lines ² , T cell lines, peripheral blood T cells	with or without PMA	-
Fibroblasts (CCD 34 Lu)	nil	-
	cytokines ³	+
Replicating endothelial cells ⁴	with or without cytokines ⁵	+

[#]Standardisation of the blots was achieved by probing with an oligonucleotide for 28S rRNA; All cell lines are human : **1.** Macrophages are serum-free. **2.** B cell lines were Sultan. Daudi, RPMI and U266. **3.** Cytokines were IGF1, PDGF BB, TGFb and TNFa for 3 hrs. **4.** HUVEC was grown with 20% FCS & growth factor (ECGF). **5.** Cytokines were IFNg, TNFa, IL1b and IL2 for 3h.

EXAMPLE 3: Expression of Recombinant pCL13 and Antibody Generation**1. Prokaryotic expression of CL13**

5 This has been undertaken using the pGEX vector which generates a glutathione-S-transferase fusion protein. Material of the correct molecular weight was synthesised but was denatured and insoluble and hence unsuitable for purification. As a consequence, no further work was done with this vector because of the difficulties that are likely to be involved.

10 2. Eukaryotic expression of CL13**General Approach**

A number of DNA constructs based on the CL13 have been made. To some of these constructs the DNA sequence for the FLAG epitope has been added. This epitope codes for the 8 amino acid peptide (N-Asp-Tyr-Lys-Asp-
15 Asp-Asp-Asp-Lys-C) which codes for an enterokinase cleavage site is recognised by two commercially available monoclonal antibodies. A protein containing this marker peptide can then be affinity purified using these antibodies. Additionally the protein can also be detected using Western blotting or other antibody based assays. Addition of this small hydrophilic
20 peptide of the amino terminal region of the construct would not be expected to influence the bioactivity of the whole protein. However, if desired, enterokinase can be used to selectively cleave the FLAG peptide from the construction, without affecting the rest of the molecule.

Prediction of the signal sequence cleavage site of any protein is only
25 75-80% accurate. For this reason in some constructions it was necessary to use the follicle stimulating hormone (FSH) leader sequence. It is known to function in the eukaryotic cell to be used for transfection and its precise cleavage site is known. This was important to ensure that the FLAG peptide remained attached to the propeptide and was not removed with signal
30 sequence cleavage.

The following DNA constructs were made:

1. CL13: Unmodified full C13 sequence (Figure 1).
(CL13 leader sequence-Sequence for CL13 propeptide-Sequence for mature bioactive CL13 peptide).
- 35 2. C13LB: Full length CL13 with FLAG (Figure 6).

(FSH Leader sequence-FLAG-CL-13 propeptide-Sequence for mature bioactive CL13 peptide).

3. FFC13S: Bioactive CL13 with FLAG (Figure 7)

(FSH Leader sequence-FLAG sequence-about 40 amino acids propeptide-Sequence for mature bioactive CL13 peptide).

4. C13SA: Bioactive CL13 with FLAG (Figure 8)

(FSH leader - FLAG sequence - PKA - Mature bioactive CL13). PKA is the recognition sequence for protein kinase A to allow *in vitro* phosphorylation.

These constructs were cloned into two different mammalian cell expression vectors. These are the pCEP4 vector which is a semipermanent expression vector or the pCEP4 vector from which the EBNA gene sequence has been deleted to allow it to permanently integrate into the genome of the mammalian cell into which it is transfected. This allows for the development of a permanent cell line secreting this protein. The constructions have all been transferred into CHO and COS cells and either semipermanent or permanent cell lines bearing the transfectant established with the use of hygromycin to kill non transfectant bearing cells. Protein production and purification have been undertaken to date only in construction numbers 2, 3 and 4 (dominantly 3 and 4), bioactive CL13 with FLAG.

Cell Culture

Both COS and CHO cells are grown in Ham's F12 medium with 5% foetal calf serum (FCS) and 400ug/ml hygromycin (only in semipermanent cell lines). At confluence, medium is removed and replaced with HamF12 containing no serum or other supplements. The conditioned medium is removed after 3 days and used for purification of recombinant FLAG-CL13. The cells are then passaged and once more placed in serum containing medium.

Quantification

A dot blot assay has been established for quantification of recombinant FLAG containing proteins - either in culture supernatant or in purified form. Protein from culture supernatants (10-100ul) is deposited onto nitrocellulose using a dot-blot apparatus. The membrane is then

reacted with monoclonal anti-FLAG antibody and then biotinylated rabbit anti mouse IgG. This is then visualised by enhanced chemiluminescence on autoradiographic film. A standard curve is generated using a protein bacterial alkaline phosphatase (BAP) that has been engineered so that it contains 1 copy of the FLAG epitope at its amino terminus (Mr 50-55 kDa). The sensitivity of this assay is about 20ng of BAP.

When this assay was used to analyse the production of FLAG-CL13 it was found that cultures produced between about 25 and 400ng of recombinant protein per ml of culture supernatant. The best expression is seen with constructs 3 and 4.

Purification

Recombinant protein containing medium is incubated with sepharose beads to which anti-FLAG antibody has been conjugated. Approximately 1 ml of beads is used per 100 ml of conditioned medium. The sepharose and medium are incubated for 18hrs at 3deg C then beads are pelleted and poured into a minicolumn. They are then washed extensively with PBS and the recombinant protein is released with FLAG peptide. This is a very gentle but efficient procedure and ensures that the bioactivity of the recombinant protein is not damaged. The FLAG peptide is removed using gel filtration chromatography. The beads are then stripped with pH 3.5 glycine buffer and can then be re-used.

Figure 9 shows a Western blot of purified pCL13 protein from C13LB and C13SA constructs. The purified material was electrophoresed using SDS PAGE on a 15% gel under reducing and non reducing conditions prior to Western blotting and visualisation using monoclonal anti-FLAG antibody. The constructs migrate at molecular weights slightly higher than predicted, something that seems to be a function of the amino acids in the FLAG sequence and has been previously reported with the use of this epitope tag. However, there is the expected change in molecular weight associated with the use of reducing conditions indicating that the material is in the dimeric conformation.

The fact that these dimeric proteins are secreted into the medium also indicates that they are folded correctly as improperly folded and aggregated proteins expressed in eukaryotic cells are not secreted. The two constructs (FFC13SC and C13SC) which encode the bioactive protein alone,

both appear to be expressed at much higher levels than the native CL13 sequence which has only been modified to contain a FLAG epitope (C13LB). This is exemplified in Figure 9 which compares relative protein expression from constructs C13SA and C13LB.

5

EXAMPLE 4: Effect of pCL13 on Fibroblast Function

TGF- β stimulates fibroblast differentiated function and inhibits replication. In order to compare the function of pCL13 with TGF- β , the effect of pCL13 on fibroblast functions may be examined as follows.

10

a. Collagen and Glycosaminoglycan production

Neonatal lung fibroblasts (CCD34LU) can be grown to confluence and the growth medium replaced with DMEM containing 0.1% BSA. The cells can then be stimulated with recombinant pCL13 or TGF- β (10ng/ml) as a positive control. The culture supernatants can then be collected 18 hours later and assayed for total collagen and glycosaminoglycans (GAG). Collagen synthesis can be measured using a microtitre plate colorimetric assay developed in this laboratory which depends on the binding of total collagen to the dye sirius red (18). Total sulphated GAG can be measured with a colorimetric assay adapted in this laboratory for microtitre plate format and which has already been used for the *in vitro* determination of fibroblasts GAG synthesis (9,10). This assay is based on the metachromatic shift in absorption maximum for the cationic dye dimethyl-methylene blue consequent on binding the polyionic moieties of GAG (9,10).

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b. Fibroblast replication

TGF- β is known to have a variable effect on *in vitro* fibroblast proliferation that probably depends on the balance between its capacity to down-regulate the PDGF receptor and the its induction of fibroblast PDGF synthesis. To determine whether pCL13 also modifies replication, a growth factor assay will be undertaken with CCD34Lu essentially as previously described (11, 12, 13)). These cells are sparsely plated at a concentration of about 1000 cells/ well (96 well plate). pCL13 protein or TGF- β (100ng/ml) (positive control) will be either added alone or in combination with a known

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35

fibroblast growth factor present within fetal calf serum. Growth factor activity can be determined by ^3H -thymidine incorporation.

c. Collagenase activity

5 It would be expected that pCL13 inhibits the induction of collagenase activity. To test this, neonatal lung fibroblasts (CCD34LU) can be grown to confluence then the growth medium replaced with DMEM containing 0.1% BSA. The cells can then be stimulated with recombinant PMA to induce synthesis of collagenase in either the presence or absence of
10 pCL13 or TGF- β (50ng/ml - positive control). The supernatants can then be assayed for collagenase activity using our adaptation (14) of an assay (15) that is based on the degradation of 20ug of purified type I collagen that has been coated onto a microtitre plate. The undigested collagen is visualized by staining with sirius red and quantified photometrically.

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EXAMPLE 5: Effect of pCL13 on Macrophage Function

The effects of TGF-b on macrophages are complex and in some instances apparently paradoxical. In general terms TGF-b has been
20 considered to be a potent macrophage chemotactic agent, a down-regulator of macrophage activation and a promoter of differentiation (3,4). To test the effect of pCL13 on macrophages, culture derived macrophages (MAC) will be used as the major cell source and will be grown free of serum in Iscove's Modified Dulbecco's Medium, using methods established by our laboratory
25 (15,16). As replicating cells become non adherent, it is possible to utilise both adherent, and undamaged non-adherent MAC for study.

a. Chemotaxis

This may be examined using a standard Boyden chamber chemotaxis
30 assay as previously performed (17). TGF- β (1pg/ml) will be used as the positive control for chemotaxis, and its response will be compared with that of pCL13.

b. Monocytoid cell differentiation

35 Both PMA and retinoic acid (RA) are potent inducers of CL13 mRNA. Both PMA, RA (as well as TGF- β) are known to induce the *in vitro*

differentiation of the primitive human monocytoid cell lines U937 and HL60 as well as bone marrow monocyte precursors. To examine the role of pCL13 in this process, the U937 and HL60 cell lines can be grown in the presence of TGF- β , pCL13 (with or without additional RA). Their differentiation will be
5 monitored by morphology, increased adherence and inhibition of replication (^3H -thymidine incorporation).

It has been previously demonstrated that human MAC grow in serum free medium, and their differentiation from monocytes to macrophages in vitro can be monitored by the expression of surface CD71, the transferrin
10 receptor (13,15). This is not seen on the surface of monocytes but is found on most MAC by day 7 of culture. Cells will be grown with TGF-b or pCL13 or interferon gamma then stained with fluoresceinated CD71 antibody and examined flow cytometrically on day 3 of culture (13). Promotion of differentiation will be associated with earlier expression of this surface
15 antigen.

c. Cytokine production

TGF- β has been reported to inhibit LPS induced production of TNF- α and IL-1. Further, as TGF- β induces pCL13 expression in a number of
20 situations, it is possible that some of the functions ascribed to TGF- β may be contributed to by pCL13. This can be examined using the above bioassays in which both TGF- β and pCL13 are active. The fibroblasts will be stimulated by TGF- β in the presence of blocking pCL13 antibody and pCL13 in the presence of a blocking TGF- β antibody. If autocrine pathways are in
25 operation, the function in question should be reduced or inhibited by the blocking antibody. Antisense oligonucleotide inhibition experiments can also be undertaken.

EXAMPLE 6: Effect of pCL13 on Endothelial Cells

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Like TGF- β , pCL13 may modify endothelial expression of adhesion molecules with subsequent downregulation of adhesion of neutrophils, monocytes or lymphocytes. Additionally pCL13 may modify angiogenesis and endothelial mediator production. This may be investigated by
35 investigating the effect of pCL13 on:

- (i) Leukocyte adherence to resting and cytokine activated vascular endothelium;
- (ii) Endothelial production of cytokines such as IL-8, MCP-1, IL-1, IL-6, and endothelin;
- 5 (iii) Endothelial prostanoid synthesis;
- (iv) Endothelial procoagulant activity; and
- (v) Angiogenesis (in vitro and vivo).

EXAMPLE 7: Effect of pCL13 on Lymphocyte Function

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Like TGF- β , pCL13 may act as an immunosuppressive agent. This can be investigated by determining the effect of pCL13 on:

- (i) T and B cell proliferation;
- (ii) Immunoglobulin synthesis;
- 15 (iii) LAK cell and NK cell activity; and
- (iv) Production in vitro of cytokines (protein and/or mRNA) such as IL-2, IFN-g, IL-4, IL-5, IL-10.

EXAMPLE 8: Effect of pCL13 on Tumor Cell Proliferation

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pCL13 may like TGF- β inhibit tumor cell replication and promote tumor differentiation. This can be investigated by determining the effect of pCL13 on:

- (i) In vitro investigation of the proliferation of a wide range of tumour cell lines available through the ATCC; and
- 25 (ii) Observing change in tumor phenotype towards a more differentiated form (e.g. change from non-adherent to adherent phenotype).

EXAMPLE 9: Effect of pCL13 on Glycosaminoglycan Production by Non-transfected and transfected CHO cells.

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The effect of pCL13 on glycosaminoglycan production was investigated using non-transfected and transfected CHO cells. Figure 10 shows glycosaminoglycan analysis in the non-transfected (KI) and CL13 transfected (P4N, 15 and 24) CHO cells using dimethyl-methylene blue (DMB) assay (9, 10). P4N, 15 and 24 produce increasing amounts of pCL13

35

respectively. Cells were cultured in DMEM/F-12 medium containing 5% FCS for 5 days. Cells were then changed to FCS-free DMEM for 24 hours. To 100 µl cell culture medium 100 µl DMB dye was added and the absorbance was read at 492 nm immediately. The results represent the mean
5 +/- SD of triplicate wells.

EXAMPLE 10: Effect of pCL13 on Collagen Production by Non-transfected and Transfected CHO Cells

10 The effect of pCL13 on collagen production was investigated. Figure 11 shows the effect of pCL13 on collagen production by non-transfected (K1) and CL13 transfected (P4N, 15 and 24) CHO cells. P4N, 15 and 24 produce increasing amounts of pCL13 respectively. Cells were cultured in DMEM/F-12 medium containing 5% FCS for 5 days. Cells were then
15 changed to FCS-free DMEM for 24 hours. The amount of collagen produced by these cells was determined using a Biodot apparatus. Culture supernatant (50 µl) was placed on nitrocellulose membrane. The membrane was washed in 100 µl PBS and dried. Collagen retained in the nitrocellulose membrane was stained with 0.1% Sirius red dye (18). Individual spots were
20 cut out and eluted with 0.1 N NaOH and absorbance was read at 550 nm. The results represent the mean +/- SD of triplicate wells.

Examples 11 to 16 described hereinafter were conducted with the C13SA construct or pCL13 produced from the C13SA construct. As
25 described above, the C13SA construct varies from CL13 in that it includes no propeptide encoding sequences.

EXAMPLE 11: Effects of pCL13 on matrix protein production

30 a. Glycosaminoglycan production

Figure 12 shows the effect of pCL13 on ³⁵S-labelled-proteoglycan production in 3T3 (mouse fibroblasts) and neonatal human lung fibroblasts (CCD 34 Lu) after 24 hour incubation. Confluent cells were changed to RPMI culture medium containing 0.1% BSA and 50 µg/ml ascorbic acid for 24
35 hours. Cells were then incubated with different pCL13 concentrations in the presence of 10 µCi/ml [³⁵S] sulphate for 24 hours. At the end of the

incubation period medium was removed and protease inhibitors were added. Proteoglycans present in the extracellular matrix were extracted using 4M guanidine hydrochloride containing protease inhibitors for one hour at 4°C. Total proteoglycan production in the medium and the cell fraction was
5 determined using Sephadex G-25 chromatography columns (19). The results represent the mean +/-SD of triplicate wells.

In 3T3 cells, after 24 hour incubation period, pCL13 caused a dose dependent increase in the proteoglycan production. A 92% increase was observed at 25ng/ml pCL13 concentrations and 60% increase was seen at 6.7
10 and 2.2 ng/ml pCL13 concentration. In comparison TGF- β at 20ng/ml elevated proteoglycan production by 95%. In CCD 34Lu cells, pCL13 at 50ng/ml caused 23% increase in the proteoglycan production and 6% increase at 25ng/ml pCL13 concentration. In comparison TGF- β at 10ng/ml elevated proteoglycan production by 36%.

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b. Collagen production

Figure 13 shows the effect of pCL13 on collagen type 1 production in neonatal lung fibroblasts (CCD 34 Lu). Confluent cells were changed to DMEM culture medium containing 0.1% BSA and 50 μ g/ml ascorbic acid for
20 24 hours. Cells were then incubated with different pCL13 concentrations in the presence of 50 μ g/ml b-aminopropionitrile for 24 hours. At the end of incubation period the amount of collagen present in the medium was determined using an ELISA. Briefly, supernatants from treated and non-treated fibroblasts as well as type 1 collagen standards were incubated for 72
25 hours at 4°C in 96-well microtitre plates (NUNC). At the end of incubation period plates were washed, blocked with 4% bovine serum albumin in phosphate buffered saline, incubated with collagen type 1 monoclonal antibody (Sigma). The plates were then rewashed and biotinylated mouse IgG was added and followed by streptavidin complex. After the addition of
30 substrate, plates were read at 490/405 nm on a plate reader. The results represent the mean +/-SD of triplicate wells. pCL13 at 50ng/ml caused 140% increase in the collagen production, 190% increase at 25ng/ml and 11% at 5ng/ml concentration. In comparison TGF- β at 10ng/ml elevated collagen production by 34% after 24 hours. The relatively poor TGF- β response has
35 occurred because TGF- β requires 48-78 hours to achieve maximal effect.

EXAMPLE 12: Effect of pCL13 on fibroblast replication*a. Growth under limiting serum conditions*

The growth factor activity of pCL13 and transforming growth factor beta (TGF β) on growth-arrested BFF (human baby foreskin fibroblasts) and 3T3 (mouse fibroblasts) cells was determined. The cytokines were added to BFF and 3T3 cells in 0.2% foetal bovine serum (FBS) media to determine whether they were true growth factors which could stimulate a resting cell to progress through the cell cycle and undergo division. The growth factor assay was performed as previously described (11, 12). In brief, the cells were plated at 1.2×10^3 cells/well in 200mL of growth-arresting medium (0.2% FBS) for 72h. The media was then replaced with fresh 0.2% FBS media with or without cytokines and 0.5mCi/well of [3-H] Thymidine for a further 72h. The cells were then harvested with an automated cell harvester and the thymidine uptake into proliferating cells was counted on a liquid scintillation analyser. The controls included 0.2% FBS media only, 10% FBS media only (normal growth media), and pCL13 diluent (0.1% CHAPS) in 0.2% FBS media.

The results shown in Figure 14 indicate that pCL13 appears to have true growth factor activity on both human BFF. TGF β appears to be inhibitory for BFF cells. Neither pCL13 nor TGF β exhibit growth factor activity on 3T3 under the conditions of this assay.

b. Growth in the presence of serum

The growth factor activity of pCL13 and transforming growth factor beta (TGF β) on growth-arrested BFF (human baby foreskin fibroblasts) and 3T3 (mouse fibroblasts) cells was determined. The cytokines were added to BFF and 3T3 cells in 2% foetal bovine serum (FBS) media to determine whether they were growth enhancing substances which could enhance the rate at which the cells moved through the cell cycle. The growth factor assay was performed as previously described (11, 12). In brief, the cells were plated at 1.2×10^3 cells/well in 200ml of growth-arresting medium (0.2% FBS) for 72h. The media was then replaced with fresh 2% FBS media with or without cytokines and 0.5mCi/well of [3-H] Thymidine for a further 72h. The cells were then harvested with an automated cell harvester and the thymidine uptake into proliferating cells was counted on a liquid

scintillation analyser. The controls included 2% FBS media only, 10% FBS media only (normal growth media), and pCL13 diluent (0.1% CHAPS) in 0.2% FBS media.

5 The results (Figure 15) show that pCL13 had a growth-enhancing effect on human BFF and murine 3T3 cells. TGF β appears to be inhibitory for BFF cells but to have growth-enhancing activity at low concentration on 3T3 cells.

10 **EXAMPLE 13: Effects of pCL13 on replication of human monocytoid cell lines**

pCL13 was compared with transforming growth factor beta (TGF β) and interferon alpha 2b (IFN α 2b), for their antiproliferative effect on the cell line U937 (a human monocyte-like histiocytic lymphoma) and Mono Mac 6
15 (a monoclastic leukemia cell line). The cells were plated at 3×10^4 cells/well in 200mL of 10% FBS (foetal bovine serum) medium with or without cytokines. For the final 6h of a 48h incubation period, the wells were pulsed with 0.5mCi/well of [3-h] Thymidine. The cells were then harvested with an automated cell harvester and the thymidine uptake into proliferating cells
20 were counted on a liquid scintillation analyser. The controls include 10% FBS medium alone and pCL13 diluent in 10% FBS medium.

The results (Figure 16) indicate that two batches of pCL13, B447 and B448A, at concentrations of 10 and 100ng/ml have a small antiproliferative effect on two human cell lines of monocytic origin. This contrasts with the
25 stronger antiproliferative effects of TGF β (2 and 20ng/ml) and IFN α 2b (10^3 and 10^5 U/ml) on U937 and Mono Mac 6 cells.

EXAMPLE 14: Effects of pCL13 on macrophage production of TNF

30 The data in Figure 17 shows the effect of different pCL13 concentration on LPS stimulated TNF- α production from human culture derived macrophages. Monocytes were purified by elutriation from buffy coats and cultured in Iscove's medium containing 0.1% BSA (13, 16). On day 5, cells were incubated with different pCL13 concentrations in the
35 presence of 10 μ g/ml LPS in the Iscove's medium for 24 hours. At the end of incubation period medium was removed and the amount of TNF- α present

was determined using a sandwich ELISA (Genzyme). The results show that pCL13 caused inhibition of LPS induced TNF- α production. A 47% inhibition was observed at 20ng/ml pCL13 and a 27% inhibition was seen at 7ng/ml of pCL13. In comparison TGF- β only brought about 10% reduction at 20ng/ml.

EXAMPLE 15: Effects of pCL13 on tumor cytotoxicity

The direct effect of pCL13 and TGF β on tumour target cells (5637 bladder carcinoma and MDA-MB-231 breast adenocarcinoma) and the effect of pCL13 and TGF β on monocyte-mediated killing of tumor cells was examined by measuring the release of radiolabelled DNA from lysed tumour target cells. The cytotoxicity assay was performed as previously described (20). Tumour target cells (labelled while in the exponential growth phase with 20 μ Ci of [^{3} H] Thymidine/ 1×10^6 cells for 24h) were added to the monocytes (effectors) at an effector:target (E:T) ratios of 10:1 for 72 h. The cells were then centrifuged and the supernatants counted in scintillation fluid on a liquid scintillation analyser. The controls included untreated tumour cells, untreated tumour cells co-cultured with monocytes and cytokine diluent alone. TGF β and pCL13 were incubated with monocytes for 48h.

The results are shown at Figure 18. Neither pCL13 nor TGF β had a direct cytotoxic effect on the 5637 or MDA-MB-231 tumour lines. However pCL13 enhanced the ability of monocytes to kill 5637 cells. pCL13 also enhanced the monocyte-mediated killing of T24 (bladder carcinoma), J82 (bladder carcinoma), T47D (breast ductal carcinoma) and JCPL (ovarian carcinoma)(data not shown).

EXAMPLE 16: In vivo Effects of pCL13

Rats (Fisher F343) were injected subcutaneously on their backs with 0.1ml of three concentrations of pCL13, a negative saline control and TGF β . The injections were widely separated and each animal was administered with the whole panel of 5 injections. The amounts of pCL13 injected were 60ng, 30ng and 2ng. The dose of TGF β administered was 10ng. The animals were then sacrificed at intervals commencing at 3 hours and up to 2 weeks

following administration. Three animals were used for each time point, and following sacrifice the areas in which material had been administered was excised, formalin fixed, mounted, then stained with haematoxylin and eosin. The material was then evaluated microscopically.

5

a. Macroscopic Changes

There was no macroscopic difference between the biopsies in any of the animals, under any of the various conditions other than at the two week time point. At the two week time point however, the biopsies, only of the areas with the two highest doses of pCL13, showed obvious macroscopic differences in the area between the muscle and skin. This area seemed somewhat expanded and had a white glistening appearance, suggestive of excess matrix protein deposition.

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15 b. Microscopic Evaluation

No differences were seen on histological sections at the three hour time points. However at the day one (24 hour) time point the areas in which the two highest concentrations of pCL13 had been administered demonstrated a mononuclear cell infiltrate which was somewhat patchy in character and was present dominantly in the subcutaneous tissue (Figure 19A). No similar changes were observed in either the negative saline control (Figure 19B) or TGF β at a dose of 10ng/ml. The infiltrate seemed to be present maximally at days one and two and be markedly diminished or absent from day four onwards. These findings suggest that pCL13 was chemotactic for macrophages and or lymphocytes.

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This study was not undertaken in such a manner as to be able to supply good quantitative data on the amount of collagen that was present in the areas where the two substances were administered. However in conjunction with the macroscopic appearance, it appears likely that the amount of collagen was increased in the samples containing the two highest doses of clone 13, at least at the two week time point.

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EXAMPLE 17: Clone 13 Variants

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Re-screening was undertaken using a fetal lung cDNA library using a portion of the coding sequence of clone 13 as a probe. This was undertaken

in order to determine the existence of clone 13 variants. Using this approach a number of additional clones (a1, b2, h1, b1, d2, dd2, f1 and u2) were obtained and the sequence of these clones is illustrated in Figure 20A which shows the nucleotide sequence and Figure 20B which shows a portion of the translated open reading frame. It also compares the sequences with that of the original clone 13 sequence (C13). From Figure 20B, it can be seen that the translated coding region of these clone 13 variants displays only minor differences. These occur at amino acids 9, 48 and 202. These are all in the propeptide region and are likely to represent genetic differences between the individuals whose RNA was used to prepare the cDNA library. However, at the DNA level, there is substantial variation dominantly in the 5' untranslated region, but to a lesser extent in the 3' untranslated region. Whilst these variants may well be important in areas such as transcriptional regulation they are untranslated and hence cannot affect bioactivity.

Some of the clones isolated and displayed in Figure 20A (e.g. b2 and h1) even though they have very long 5' untranslated region, still do not represent the complete coding sequence. This can be ascertained as when the 5' untranslated region is used as a probe, on northern blots, hybridisation to a band of approximately 7kb is demonstrable. The reasons for this marked length variation are unclear but could include alternate splicing of an untranslated exon, the use of alternate transcriptional start sites or even gene duplication.

EXAMPLE 18: Expression of clone is using a yeast eukaryotic system

The bioactive region of clone 13, modified at its amino terminus so as to contain a number of additional marker epitopes (construct C13SA/5H - Figure 21) was cloned into the pPIC9 plasmid. This plasmid was then used to transform the yeast *Pichia pastoris* according to the manufacturers instructions (Invitrogen Corp.). Yeast, successfully transformed by this plasmid were selected on the basis of methanol sensitivity. Colonies of yeast were then grown for two days, in suspension as per the manufacturers instructions. Culture medium was collected and an aliquot subjected to SDS-PAGE followed by western blotting. pCL13 containing bands were visualised using the anti-FLAG M2 antibody using standard procedures. Electrophoresis was carried out under both reducing and non-reducing

conditions. It can be seen that large amounts of the protein are produced which are easily detectable with unconcentrated yeast culture medium, indicating secretion of the protein in an appropriate manner (Figure 22). The molecular weight approximates that expected on the basis of the amino acid composition and the doubling of the molecular weight under non-reducing conditions (Figure 22) indicates that the protein is, as expected, a disulphide bonded dimer. This is the correct structural configuration and indicates that the protein has been processed and secreted by the yeast organism in an appropriate manner.

10 The capacity to express this complex dimeric, protein with a high disulphide bond content in yeast systems is highly advantageous as it dramatically lowers the cost of production per unit quantity of protein and makes it far more suitable as a biopharmaceutical compared with material produced by mammalian cells.

15 Whilst this work has been undertaken with the yeast *Pichia pastoris*, it is quite likely that similar secretion will occur with a range of yeast organisms transduced with an appropriate yeast expression vector. As the bioactive region being expressed does not contain potential n-glycosylation sites, the hyperglycosylation, that sometimes occurs with mammalian
20 proteins expressed by yeast strains, is not an issue.

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It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

15

CLAIMS:

1. An isolated polynucleotide molecule obtainable from human chromosome 19p13.1, wherein said polynucleotide molecule comprises a nucleotide sequence encoding a protein that belongs to the TGF- β superfamily having a conserved RXXR site, 7 conserved cysteine residues and, in mature processed form, is dimerized,
wherein the encoded protein is capable of:
 - (i) being expressed by the human neonatal fibroblast line CCD34Lu in increased levels in response to IGF-1, PDGF BB, TGF- β or TNF- α ;
 - (ii) being expressed by macrophages in increased levels in response to GM-CSF, M-CSF, IL-2 or TNF- α ; and
 - (iii) being expressed by human umbilical vein endothelial cells in increased levels in response to ECGF, and comprises the partial amino acid sequence of amino acids 263 to 308 of Figure 1.
2. The polynucleotide molecule of claim 1, wherein the nucleotide sequence does not comprise sequence encoding a native leader or propeptide.
3. The polynucleotide molecule of claim 2, wherein said nucleotide sequence is operably linked to a nucleotide sequence encoding a heterologous leader.
4. The polynucleotide molecule of claim 3, wherein said heterologous leader is the follicle stimulating hormone (FSH) leader.
5. The polynucleotide molecule of any one of claims 1 to 4, wherein the nucleotide sequence is operably linked to a nucleotide sequence encoding an epitope tag.

6. The polynucleotide molecule of claim 5, wherein the epitope tag is FLAG.

7. The polynucleotide molecule of any one of claims 1 to 6, wherein the
5 polynucleotide molecule is DNA.

8. A vector comprising the DNA molecule of claim 7 operably linked to a promoter.

10 9. A vector comprising the DNA molecule of claim 7, the DNA molecule being operably linked in opposite orientation to a promoter such that expression proceeds 5' to the 3' terminus to produce antisense RNA.

10. The vector of claim 9, wherein said DNA molecule comprises or is
15 linked to a nucleotide sequence encoding a ribozyme domain.

11. A protein encoded by the polynucleotide molecule of any one of claims 1 to 7, wherein the protein is in substantially pure form.

20 12. The polynucleotide molecule of claim 1, wherein the nucleotide sequence comprises a variant nucleotide sequence selected from a1 and f1 as shown in Figure 20A.

25 13. The polynucleotide molecule of claim 1 or 12, wherein the protein comprises a polypeptide having an amino acid sequence selected from a1 and f1 as shown in Figure 20B.

14. The polynucleotide molecule of claim 12 or 13, wherein the polynucleotide molecule is DNA.

30

15. A vector comprising the DNA molecule as defined in claim 14 operably linked to a promoter.

16. A protein encoded by the polynucleotide molecule as defined in any one of claims 12 to 14, wherein the protein is in substantially pure form.

17. The protein of claim 16, wherein the protein comprises a monomeric polypeptide having an amino acid sequence selected from a1 and f1 as shown in Figure 20B.

18. The protein of claim 11 or 16, wherein the protein is the mature protein.

19. An antibody or fragment thereof which specifically binds to the protein of any one of claims 11 and 16 to 18.

20. A cell transformed with the polynucleotide molecule of any one of claims 1 to 7 and 14 or the vector of any one of claims 8 to 10 and 15.

21. The cell of claim 20 selected from eukaryotic cell lines and yeast.

22. A method of producing a protein comprising transforming a non-human host organism with the polynucleotide molecule of any one of claims 1 to 7 and 14, wherein said polynucleotide molecule is constitutively or inducibly expressed in said host organism.

23. The method of claim 22, wherein said host organism is selected from eukaryotic cell lines and yeast.

24. The method of claim 23, wherein said host organism is a yeast.

25. The method of claim 24, wherein said yeast is *Pichia pastoris*.

26. The use of the protein of any one of claims 11 and 16 to 18 in the preparation of a medicament for the treatment of a disease or condition

selected from wound and/or fracture healing, ischaemic injury, cancer, and autoimmune diseases.

27. A method for diagnosing a disease or condition in a subject, said
5 disease or condition being selected from cancer, inflammatory and fibrotic diseases, comprising detecting the presence or activity of the protein according to any one of claims 11 and 16 to 18 in said subject, and wherein detection of the presence or activity of said protein indicates the presence of cancer or an inflammatory or fibrotic disease in the subject.

10

28. A kit for use in a method according to claim 27, said kit comprising the protein of any one of claims 11 and 16 to 18, and the antibody or fragment thereof of claim 19.

15 29. A gene therapy agent comprising the polynucleotide molecule of any one of claims 1 to 7 and 14 or the vector of any one of claims 8 to 10 and 15.

30. An isolated polynucleotide encoding a protein that belongs to the TGF- β superfamily having a conserved RXXR site and 7 conserved cysteine
20 residues, the isolated polynucleotide comprising a sequence selected from:
a) the sequence of the polynucleotide as set out in Figure 1; and
b) the sequence of a polynucleotide that hybridizes to a complement of the sequence set out in Figure 1 under highly stringent hybridization conditions comprising hybridization in
25 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 μ g/ml denatured salmon sperm DNA at 68°C overnight, followed by washing in a solution comprising 0.1X SSPE and 0.1% SDS at 68°C.

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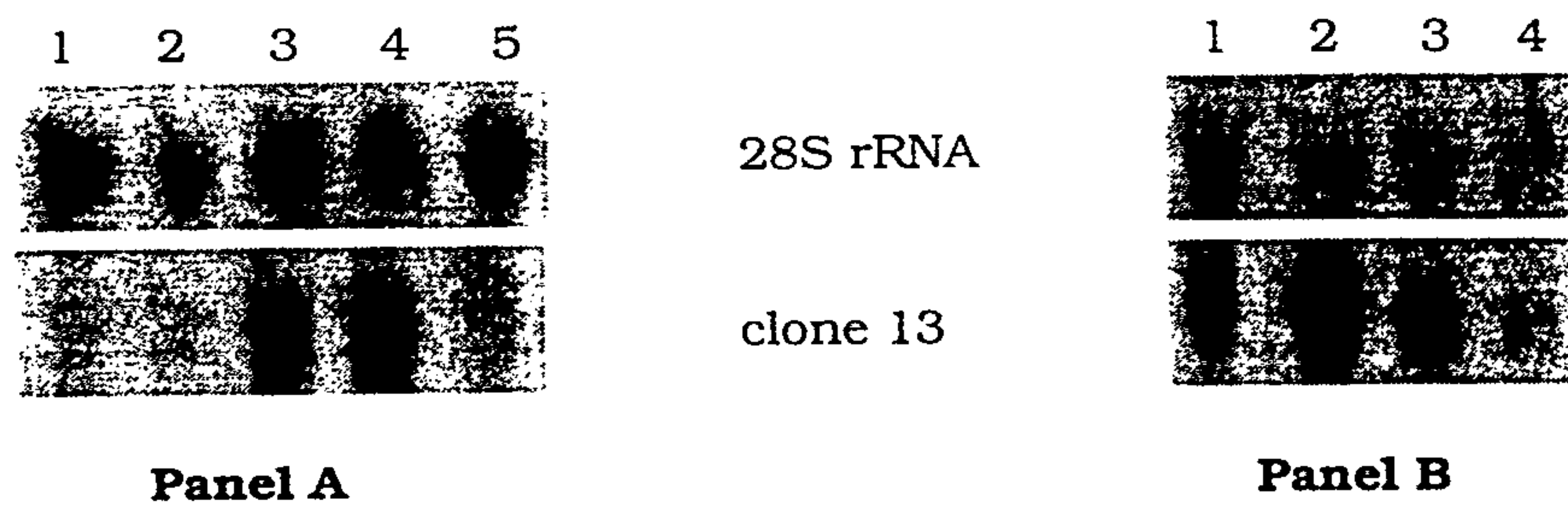
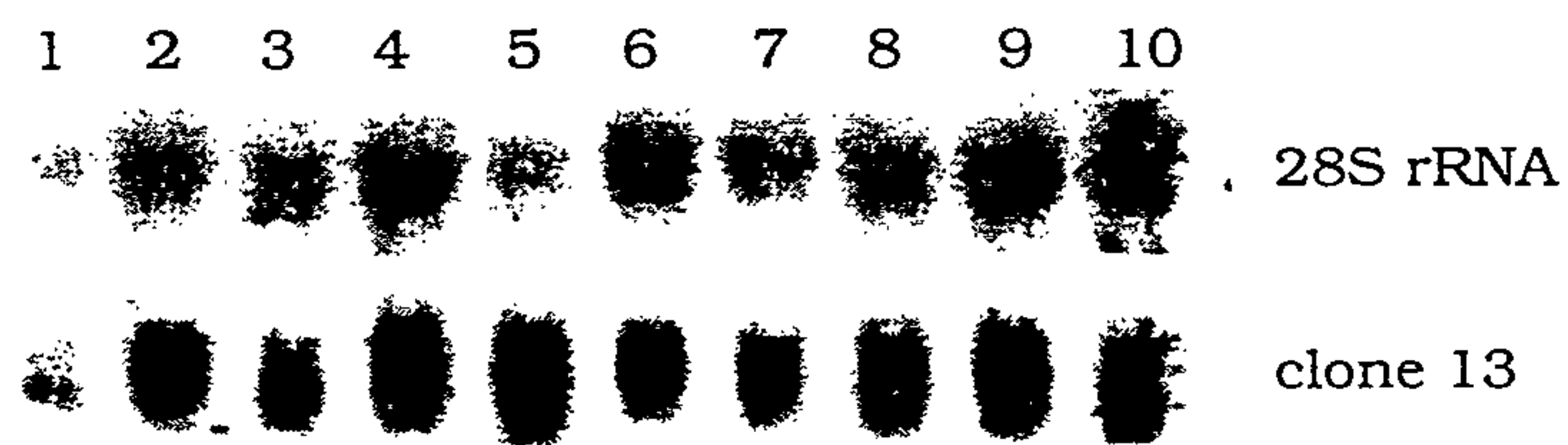
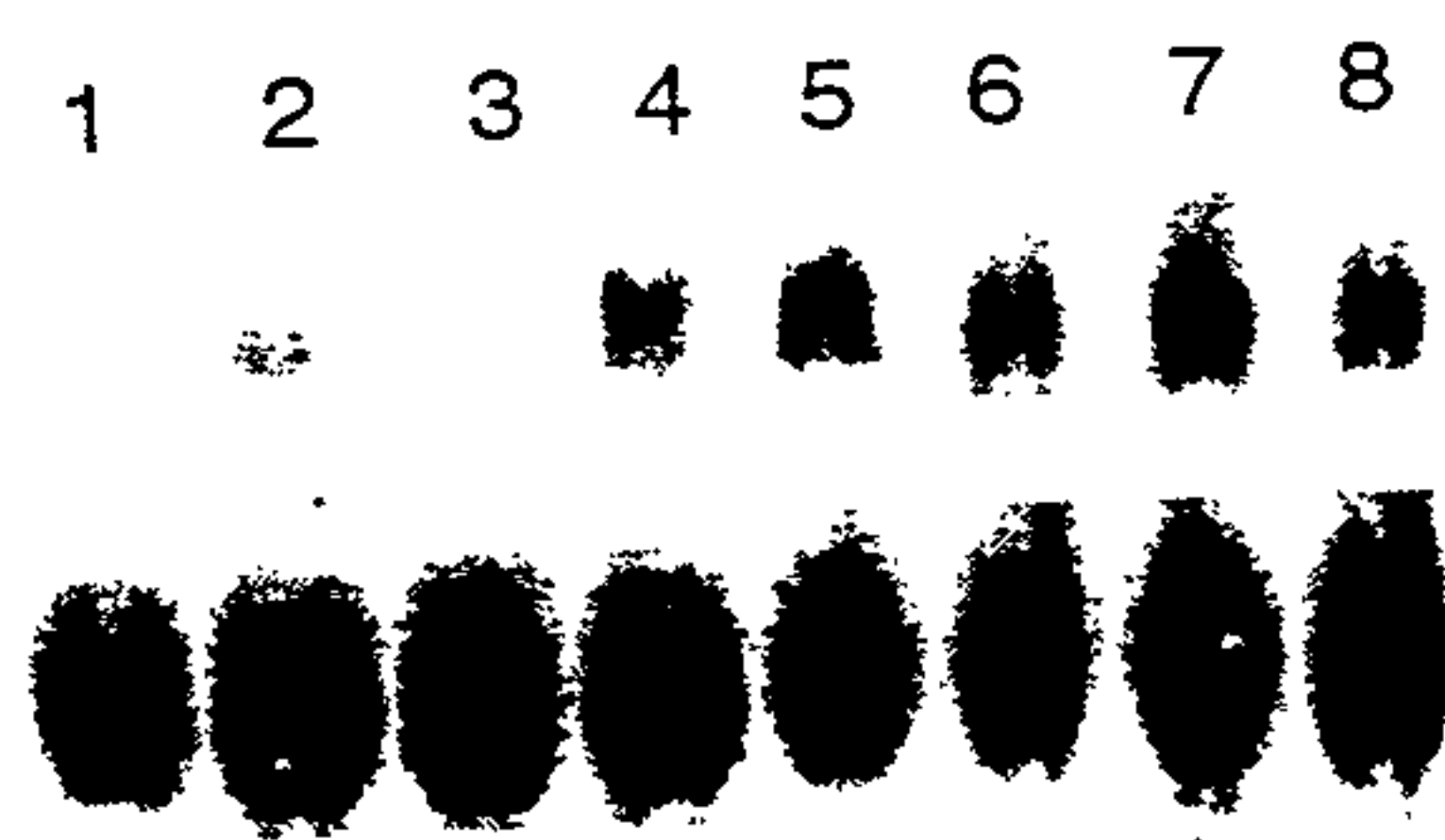
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1	GCGGCCGCTGCACAGCC	ATG CCC GGG CAA GAA CTC AGG ACG CTG AAT GGC TCT CAG ATG CTC	62
1		M P G Q E L R T L N G S Q M L	15
63	CTG GTG TTG CTG GTG CTC TCG TGG CTG CCG CAT GGG GGC GCC CTG TCT CTG GCC GAG GCG	122	
16	L V L L V L S W L P H G G A L S L A E A	35	
123	AGC CGC GCA AGT TTC CCG GGA CCC TCA GAG TTG CAC ACC GAA GAC TCC AGA TTC CGA GAG	182	
36	S R A S F P G P S E L H T E D S R F R E	55	
183	TTG CGG AAA CGC TAC GAG GAC CTG CTA ACC AGG CTG CGG GCC AAC CAG AGC TGG GAA GAT	242	
56	L R K R Y E D L L T R L R A N Q S W E D	75	
243	TCG AAC ACC GAC CTC GTC CCG GCC CCT GCA GTC CGG ATA CTC ACG CCA GAA GTG CGG CTG	302	
76	S N T D L V P A P A V R I L T P E V R L	95	
303	GGA TCC GGC GGC CAC CTG CAC CTG CGT ATC TCT CGG GCC GCC CTT CCC GAG GGG CTC CCC	362	
96	G S G G H L H L R I S R A A L P E G L P	115	
363	GAG GCC TCC CGC CTT CAC CGG GCT CTG TTC CGG CTG TCC CCG ACG GCG TCA AGG TCG TGG	422	
116	E A S R L H R A L F R L S P T A S R S W	135	
423	GAC GTG ACA CGA CCT CTG CGG CGT CAG CTC AGC CTT GCA AGA CCC CAG GCG CCC GCG CTG	482	
136	D V T R P L R R Q L S L A R P Q A P A L	155	
483	CAC CTG CGA CTG TCG CCG CCG CCG TCG CAG TCG GAC CAA CTG CTG GCA GAA TCT TCG TCC	542	
156	H L R L S P P P S Q S D Q L L A E S S S	175	
543	GCA CGG CCC CAG CTG GAG TTG CAC TTG CGG CCG CAA GCC GCC AGG GGG CGC CGC AGA GCG	602	
176	A R P Q L E L H L R P Q A A R G R R R A	195	
	↓		
603	CGT GCG CGC AAC GGG GAC CAC TGT CCG CTC GGG CCC GGG CGT TGC TGC CGT CTG CAC ACG	662	
196	R A R N G D H C P L G P G R C C R L H T	215	
663	GTC CGC GCG TCG CTG GAA GAC CTG GGC TGG GCC GAT TGG GTG CTG TCG CCA CGG GAG GTG	722	
216	V R A S L E D L G W A D W V L S P R E V	235	
723	CAA GTG ACC ATG TGC ATC GGC GCG TGC CCG AGC CAG TTC CGG GCG GCA AAC ATG CAC GCG	782	
236	Q V T M C I G A C P S Q F R A A N M H A	255	
783	CAG ATC AAG ACG AGC CTG CAC CGC CTG AAG CCC GAC ACG GTG CCA GCG CCC TGC TGC GTG	842	
256	Q I K T S L H R L K P D T V P A P C C V	275	
843	CCC GCC AGC TAC AAT CCC ATG GTG CTC ATT CAA AAG ACC GAC ACC GGG GTG TCG CTC CAG	902	
276	P A S Y N P M V L I Q K T D T G V S L Q	295	
903	ACC TAT GAT GAC TTG TTA GCC AAA GAC TGC CAC TGC ATA TGA GCAGTCCTGGTCCTTCCACTGTGC	968	
296	T Y D D L L A K D C H C I	309	
969	ACCTGCGCGGGGGAGGCGACCTCAGTTGTCCTGCCCTGTGGA	1037	
1038	ATT CCT GCC CAA ACA GCT GTA TTT ATA TAA GTCTGTTATTTATTATTAATTTATTGGGGTGACCTTCTTG	1107	
1108	GGGACTCGGGGGCTGGTCTG ATG GAA CTG TGT ATT TAT TTA AAA CTC TGG TGA TAAAAATAAAGCTGT	1175	
1176	CTGAAGTGTAAAAA	1202	

↓ Processing site following nucleotide 605
 • Stop codon

FIGURE 1

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FIGURE 2FIGURE 3FIGURE 4

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CEMP2_HUM	CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECFFPLADHLNSTINHAIVQTLVN
CEMP3_HUM	CARRYLKVDFA-DIGWSEWIIISPKSFDAYYCAGQFFMPKSLKPSNHATTIQSIVR
CEMP4_HUM	CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTINHAIVQTLVN
CEMP5_HUM	CKKHELYVSFR-DLGWQDWIIAPEGYAIFYCDGECSEFPLNAHMAINHAIVQTLVH
CEMP6_HUM	CRKHELYVSFQ-DLGWQDWIIAPKGYAANYCDGECSEFPLNAHMAINHAIVQTLVH
CEMP7_HUM	CKKHELYVSFR-DLGWQDWIIAPEGYAIFYCEGECAPFLNSYMAINHAIVQTLVH
CGDF3_MOUS	CHRHQLFINFQ-DLGWHKWVIAPKGFMANYCHGECPFSTMTYLNSSNYAFMQALMH
CGDF9_MOUS	CELHDFSLSFS-QLKWDNWIVAPHSYNPSYCKGDCPSAVSHRYGSPVHTIMQNMITY
CHSOP1_5	CKKHELYVSFR-DLGWQDWIIAPEGYA-----FPLNSYMAINHAIVQTLVH
CIHBA_HUM	CCKKQFFVSFK-DIGWNDWIIAPSGYHANYCEGECPSHLAGTSGSSLSFHSSTVINHYRM
CMIS_HUM	CALRELSVDLRAERS-----VLIPEYQANNCQGVCGWPQSDRNPRYGNHVLLLLKM
CPEP13	CCRLHIVRASLEDLGWADWVLSPREVQVIMCIGACP---SQFRAANMHAQIKTSLH
CTGF1_HUM	CCVRQLYIDFRKDLGWK-WIHEPKGYHANFCLGPCPYIWS---LDTQYSKVLALYN
CTGF2_HUM	CCLRPLYIDFRKDLGWK-WIHEPKGYANANFCAGACPYLWS---SDTQHSRVLSLYN
CTGF3_HUM	CCVRPLYIDFRQDLGWK-WIHEPKGYANANFCAGACPYLRS---ADTTHSTVLGLYN
CTGF4_CHIC	CCVRPLYIDFRKDLQWK-WIHEPKGYMANANFCMGPCPYIWS---ADTQYTKVLALYN
CTGF5_X	CCVKPLYINFRKDLGWE-----ANYCLGNCPYIWS---MDTQYSKVLSLYN
CVG1_X	CKKRHLVVEFK-DVGWQNWVIAPOGYMANANYCYGECPYPLTEILNGSNHAILQTLVH
	*
CEMP2_HUM	SVNSK--IPKACCVPTELSAISMLYLDENKWLKQNYQDMVEGCGCR
CEMP3_HUM	AVGVVPGIPEPCCVPEKMSSLSILFFDENKNWVLKVYFNMIVESCACR
CEMP4_HUM	SVNSS--IPKACCVPTELSAISMLYLDYDKWLKQNYQDMVEGCGCR
CEMP5_HUM	LMFPLH-VPKPCCAPTKLNALISVLYFDDSSNVILKKYRNMVVRACGCH
CEMP6_HUM	LMNPEY-VPKPCCAPTKLNALISVLYFDDSSNVILKKYRNMVVRACGCH
CEMP7_HUM	FINPET-VPKPCCAPTQLNALISVLYFDDSSNVILKKYRNMVVRACGCH
CGDF3_MOUS	MADP-K-VPKAVCVPTKLSPI SMLYQDSDKNVILRHVEDMVADECGCG
CGDF9_MOUS	E-KLDPSVPSPCVPGKYSPLSVLTTEPDGSTAYKEYEDMMATSCCTR
CHSOP1_5	FINPET-VPKPCCAPTQLNALISV-----ILKKYRNMVVRACGCH
CIHBA_HUM	RGHSPFANLKSOCVPTKLRPM SMLYDDGQNIKKQIQNMIVEEGGCS
CMIS_HUM	QARGAALARPPCCVPTAYAG-KLLISLSEERISAHVFNMVATECGCR
CPEP13	RLKPDV-VPAPCCVPASYNPM-VLIQKTDITGVSLQTYDDLLAKOCHCI
CTGF1_HUM	QHNPASAA-PCCVPQALEPLPIVYY-VGRKPKVEQLSNMIVRSCKCS
CTGF2_HUM	TINPEASAS-PCCVQDLEPLTILYY-IGKTPKIEQLSNMIVKSKCS
CTGF3_HUM	TINPEASAS-PCCVQDLEPLTILYY-VGRTPKVEQLSNMIVKSKCS
CTGF4_CHIC	QHNPASAA-PCCVPQILDPLPIIYY-VGRNVRVEQLSNMIVRACKCS
CTGF5_X	QNNPGASIS-PCCVP-----YY-VGRTPKVEQLSNMIVRSCNCS
CVG1_X	SIEPED-IPLPCCVPTKMSPI SMLFYDNNENWVLRHYENMAVDECGCR

FIGURE 5

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1	AAGCTT ATG CCC GGG CAA GAA CTC AGG ACG CTG AAT GGC TCT CAG ATG CTC CTG GTG TTG	60
1	M P G Q E L R T L N G S Q M L L V L	18
61	CTG GTG CTC TCG TGG CTG CCG CAT GGG GGC GCC CTG TCT CTG GCC GAG GCG AGC CGC GCA	120
19	L V L S W L P H G G A L S L A E A S R A	38
121	AGT TTC CCG GGA CCC TCA GAG TTG CAC ACC GAA GAC TCC AGA TTC CGA GAG TTG CGG AAA	180
39	S F P G P S E L H T E D S R F R E L R K	58
181	CGC TAC GAG GAC CTG CTA ACC AGG CTG CGG GCC AAC CAG AGC TGG GAA GAT TCG AAC ACC	240
59	R Y E D L L T R L R A N Q S W E D S N T	78
241	GAC CTC GTC CCG GCC CCT GCA GTC CGG ATA CTC ACG CCA GAA GTG CGG CTG GGA TCC GGC	300
79	D L V P A P A V R I L T P E V R L G S G	98
301	GGC CAC CTG CAC CTG CGT ATC TCT CGG GCC GCC CTT CCC GAG GGG CTC CCC GAG GCC TCC	360
99	G H L H L R I S R A A L P E G L P E A S	118
361	CGC CTT CAC CGG GCT CTG TTC CGG CTG TCC CCG ACG GCG TCA AGG TCG TGG GAC GTG ACA	420
119	R L H R A L F R L S P T A S R S W D V T	138
421	CGA CCT CTG CGG CGT CAG CTC AGC CTT GCA AGA CCC CAG GCG CCC GCG CTG CAC CTG CGA	480
139	R P L R R Q L S L A R P Q A P A L H L R	158
481	CTG TCG CCG CCG CCG TCG CAG TCG GAC CAA CTG CTG GCA GAA TCT TCG TCC GCA CGG CCC	540
159	L S P P P S Q S D Q L L A E S S S A R P	178
541	CAG CTG GAG TTG CAC TTG CGG CCG CAA GCC GCC AGG GGG CGC CGC AGA GCG CGT GAA TTC	600
179	Q L E L H L R P Q A A R G R R R A R E F	198
601	GAC TAC AAG GAC GAC GAT GAC AAG GCG CGC AAC GGG GAC CAC TGT CCG CTC GGG CCC GGG	660
199	<u>D Y K D D D D K</u> A R N G D H C P L G P G	218
661	CGT TGC TGC CGT CTG CAC ACG GTC CGC GCG TCG CTG GAA GAC CTG GGC TGG GCC GAT TGG	720
219	R C C R L H T V R A S L E D L G W A D W	238
721	GTG CTG TCG CCA CGG GAG GTG CAA GTG ACC ATG TGC ATC GGC GCG TGC CCG AGC CAG TTC	780
239	V L S P R E V Q V T M C I G A C P S Q F	258
781	CGG GCG GCA AAC ATG CAC GCG CAG ATC AAG ACG AGC CTG CAC CGC CTG AAG CCC GAC ACG	840
259	R A A N M H A Q I K T S L H R L K P D T	278
841	GTG CCA GCG CCC TGC TGC GTG CCC GCC AGC TAC AAT CCC ATG GTG CTC ATT CAA AAG ACC	900
279	V P A P C C V P A S Y N P M V L I Q K T	298
901	GAC ACC GGG GTG TCG CTC CAG ACC TAT GAT GAC TTG TTA GCC AAA GAC TGC CAC TGC ATA	960
299	D T G V S L Q T Y D D L L A K D C H C I	318
961	TGA CTCGAG	969
319	.	319

↓ = processing site
underline = FLAG epitope that is fused to amino terminus of bioactive region of clone 13
• = stop codon

FIGURE 6

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```

1 GCTAGCGCC ATG GAT TAC TAC AGA AAA TAT GCA GCT ATC TTT CTG GTC ACA TTG TCG GTG 60
1      M   D   Y   Y   R   K   Y   A   A   I   F   L   V   T   L   S   V   17
      ↓
61 TTT CTG CAT GTT CTC CAT TCC GCT CCT GAT GAA TTC GAC TAC AAG GAC GAC GAT GAC AAG 120
18 F   L   H   V   L   H   S   A   P   D   E   F   D   Y   K   D   D   D   D   K 37

121 CGT CAG CTC AGC CTT GCA AGA CCC CAG GCG CCC GCG CTG CAC CTG CGA CTG TCG CCG CCG 180
38 R   Q   L   S   L   A   R   P   Q   A   P   A   L   H   L   R   L   S   P   P   57

181 CCG TCG CAG TCG GAC CAA CTG CTG GCA GAA TCT TCG TCC GCA CGG CCC CAG CTG GAG TTG 240
58 P   S   Q   S   D   Q   L   L   A   E   S   S   S   A   R   P   Q   L   E   L   77
      ▼
241 CAC TTG CGG CCG CAA GCC GCC AGG GGG CGC CGC AGA GCG CGT GCG CGC AAC GGG GAC CAC 300
78 H   L   R   P   Q   A   A   R   G   R   R   R   A   R   A   R   N   G   D   H   97

301 TGT CCG CTC GGG CCC GGG CGT TGC TGC CGT CTG CAC ACG GTC CGC GCG TCG CTG GAA GAC 360
98 C   P   L   G   P   G   R   C   C   R   L   H   T   V   R   A   S   L   E   D   117

361 CTG GGC TGG GCC GAT TGG GTG CTG TCG CCA CGG GAG GTG CAA GTG ACC ATG TGC ATC GGC 420
118 L   G   W   A   D   W   V   L   S   P   R   E   V   Q   V   T   M   C   I   G   137

421 GCG TGC CCG AGC CAG TTC CGG GCG GCA AAC ATG CAC GCG CAG ATC AAG ACG AGC CTG CAC 480
138 A   C   P   S   Q   F   R   A   A   N   M   H   A   Q   I   K   T   S   L   H   157

481 CGC CTG AAG CCC GAC ACG GTG CCA GCG CCC TGC TGC GTG CCC GCC AGC TAC AAT CCC ATG 540
158 R   L   K   P   D   T   V   P   A   P   C   C   V   P   A   S   Y   N   P   M   177

541 GTG CTC ATT CAA AAG ACC GAC ACC GGG GTG TCG CTC CAG ACC TAT GAT GAC TTG TTA GCC 600
178 V   L   I   Q   K   T   D   T   G   V   S   L   Q   T   Y   D   D   L   L   A   197

601 AAA GAC TGC CAC TGC ATA TGA CTCGAG 627
198 K   D   C   H   C   I   • 204

```

underline = FLAG epitope
 ↓ = signal sequence cleavage site following first 24 amino acids representing the FSH leader sequence
 • = stop codon
 ▼ = predicted processing site is NOT used and the protein secreted commences after the signal sequence at amino acid 25

FIGURE 7

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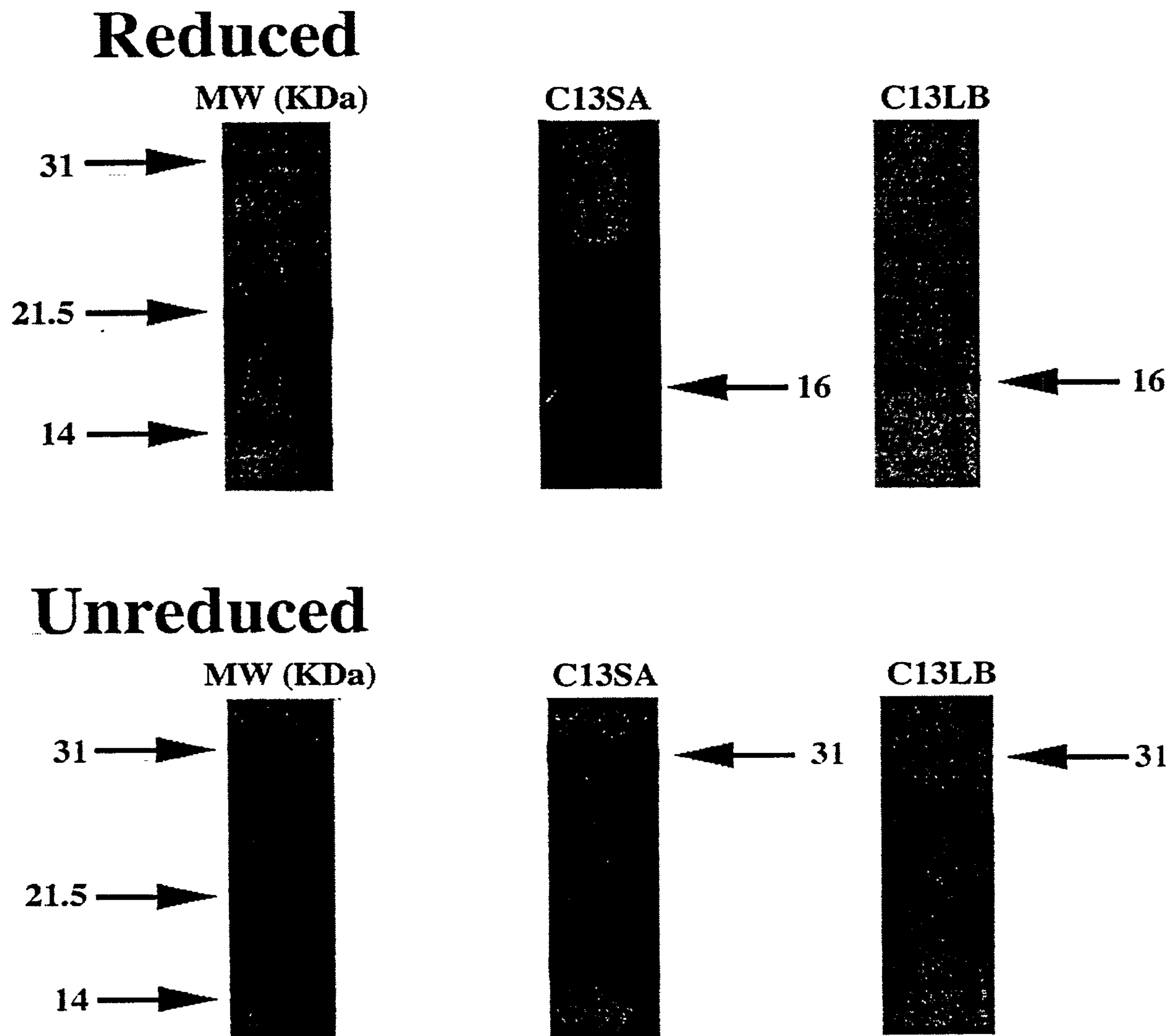
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 1 M D Y Y R K Y A A I F L V T L S V 17
 ↓
 61 TTT CTG CAT GTT CTC CAT TCC GCT CCT GAT GAA TTC GAC TAC AAG GAC GAC GAC GAC AAG 120
 18 F L H V L H S A P D E F D Y K D D D D K 37
 121 [CTC CGC GCC TCC GTG] GCG CGC AAC GGG GAC CAC TGT CCG CTC GGG CCC GGG CGT TGC TGC 180
 38 [L R A S V] A R N G D H C P L G P G R C C 57
 181 CGT CTG CAC ACG GTC CGC GCG TCG CTG GAA GAC CTG GGC TGG GCC GAT TGG GTG CTG TCG 240
 58 R L H T V R A S L E D L G W A D W V L S 77
 241 CCA CGG GAG GTG CAA GTG ACC ATG TGC ATC GGC GCG TGC CCG AGC CAG TTC CGG GCG GCA 300
 78 P R E V Q V T M C I G A C P S Q F R A A 97
 301 AAC ATG CAC GCG CAG ATC AAG ACG AGC CTG CAC CGC CTG AAG CCC GAC ACG GTG CCA GCG 360
 98 N M H A Q I K T S L H R L K P D T V P A 117
 361 CCC TGC TGC GTG CCC GCC AGC TAC AAT CCC ATG GTG CTC ATT CAA AAG ACC GAC ACC GGG 420
 118 P C C V P A S Y N P M V L I Q K T D T G 137
 421 GTG TCG CTC CAG ACC TAT GAT GAC TTG TTA GCC AAA GAC TGC CAC TGC ATA TGA CTCGAG 480
 138 V S L Q T Y D D L L A K D C H C I • 155

underline = FLAG epitope
 [] = PKA site
 ↓ = signal sequence cleavage site following first 24 amino acids representing
 the FSH leader sequence
 • = stop codon

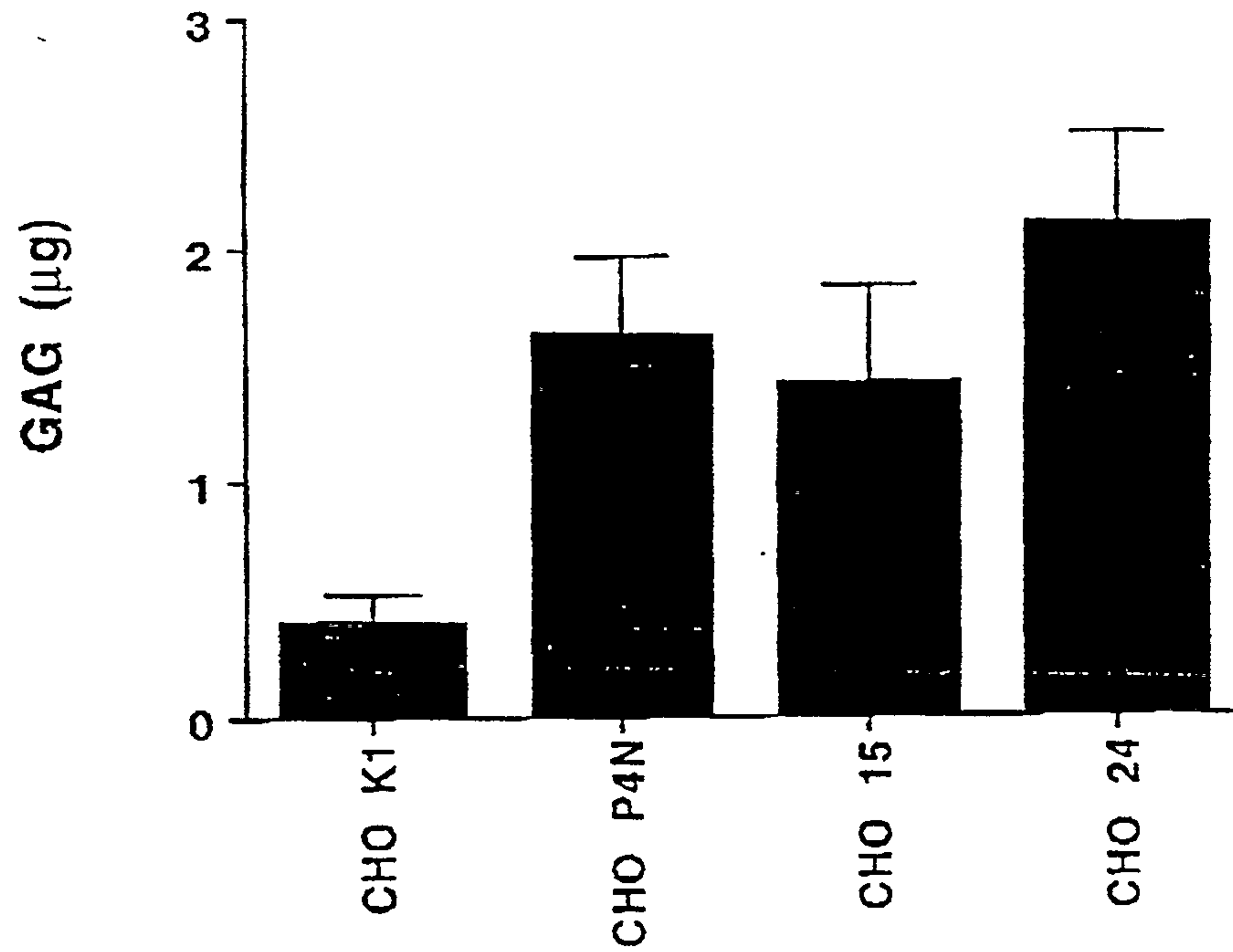
FIGURE 8

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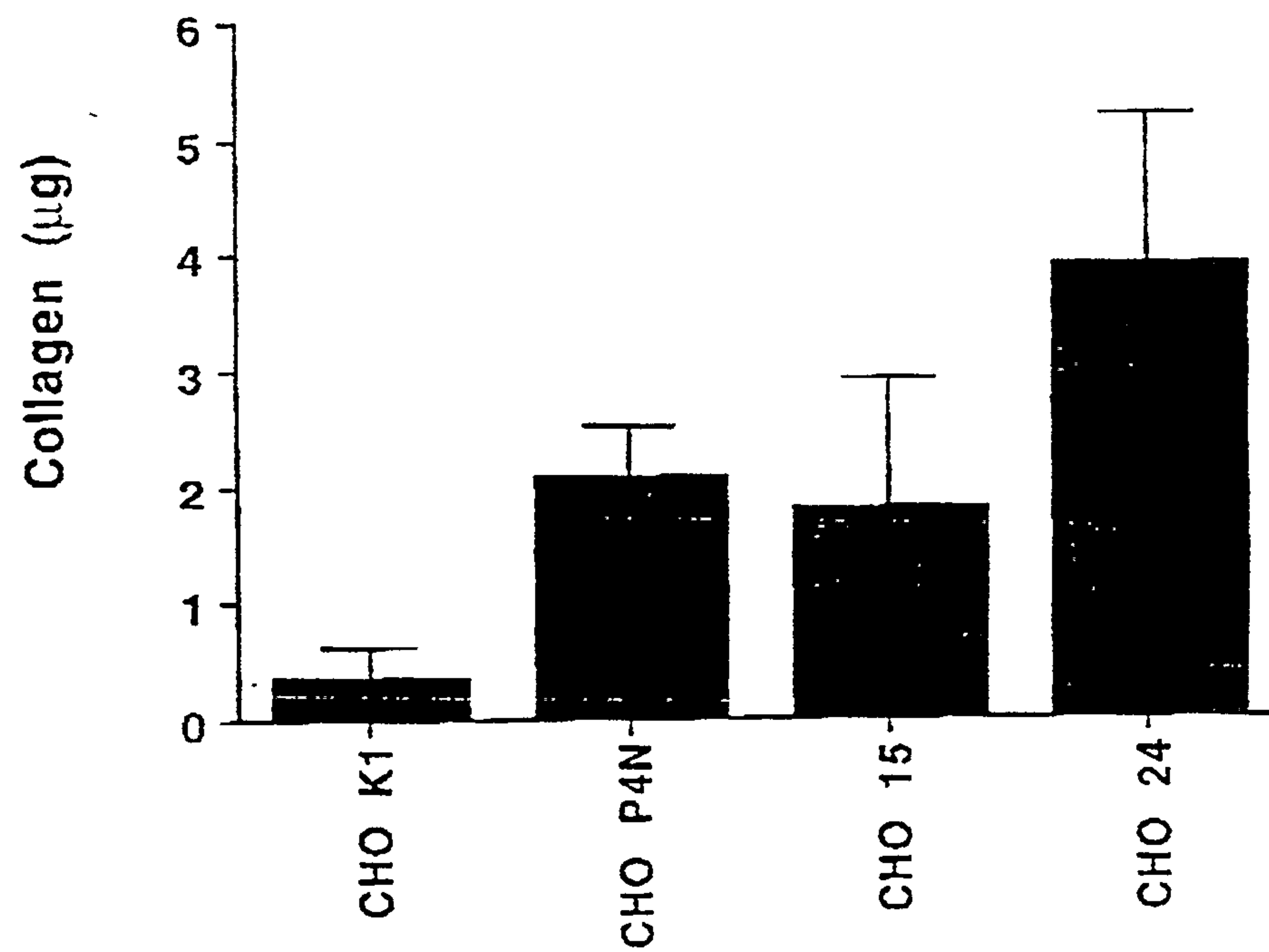
Figure

**FIGURE 9**

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FIGURE 10

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**FIGURE 11**

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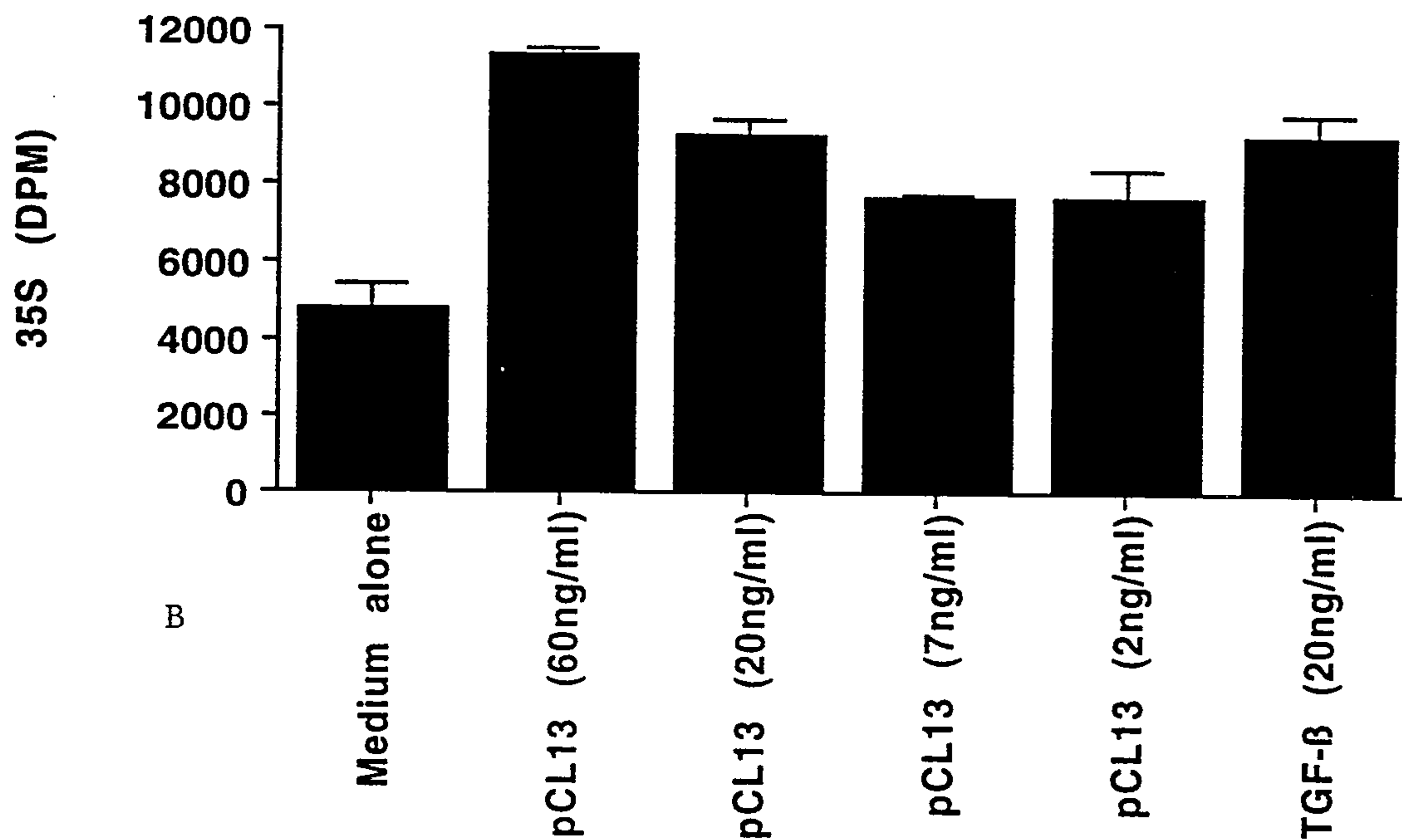
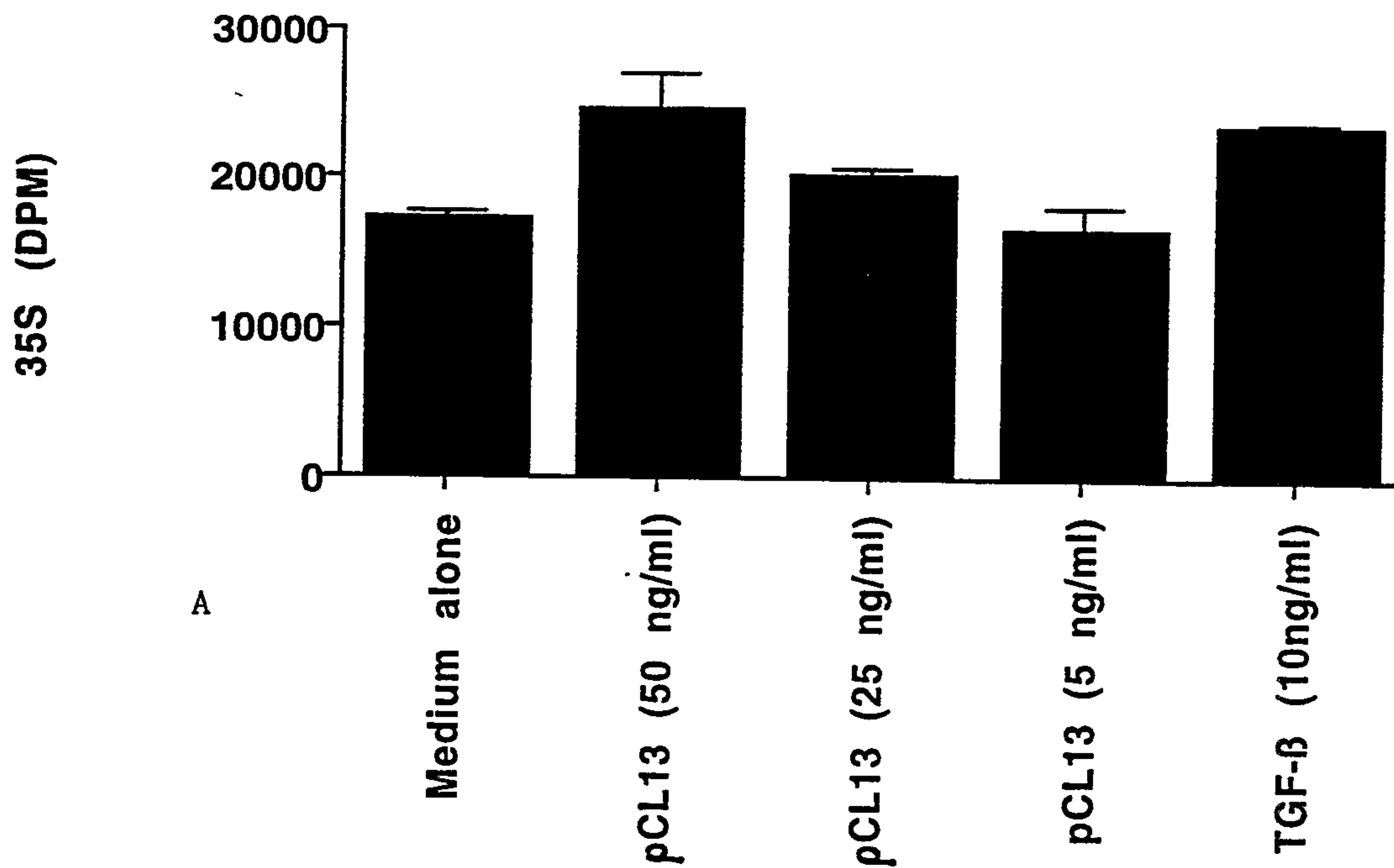


FIGURE 12

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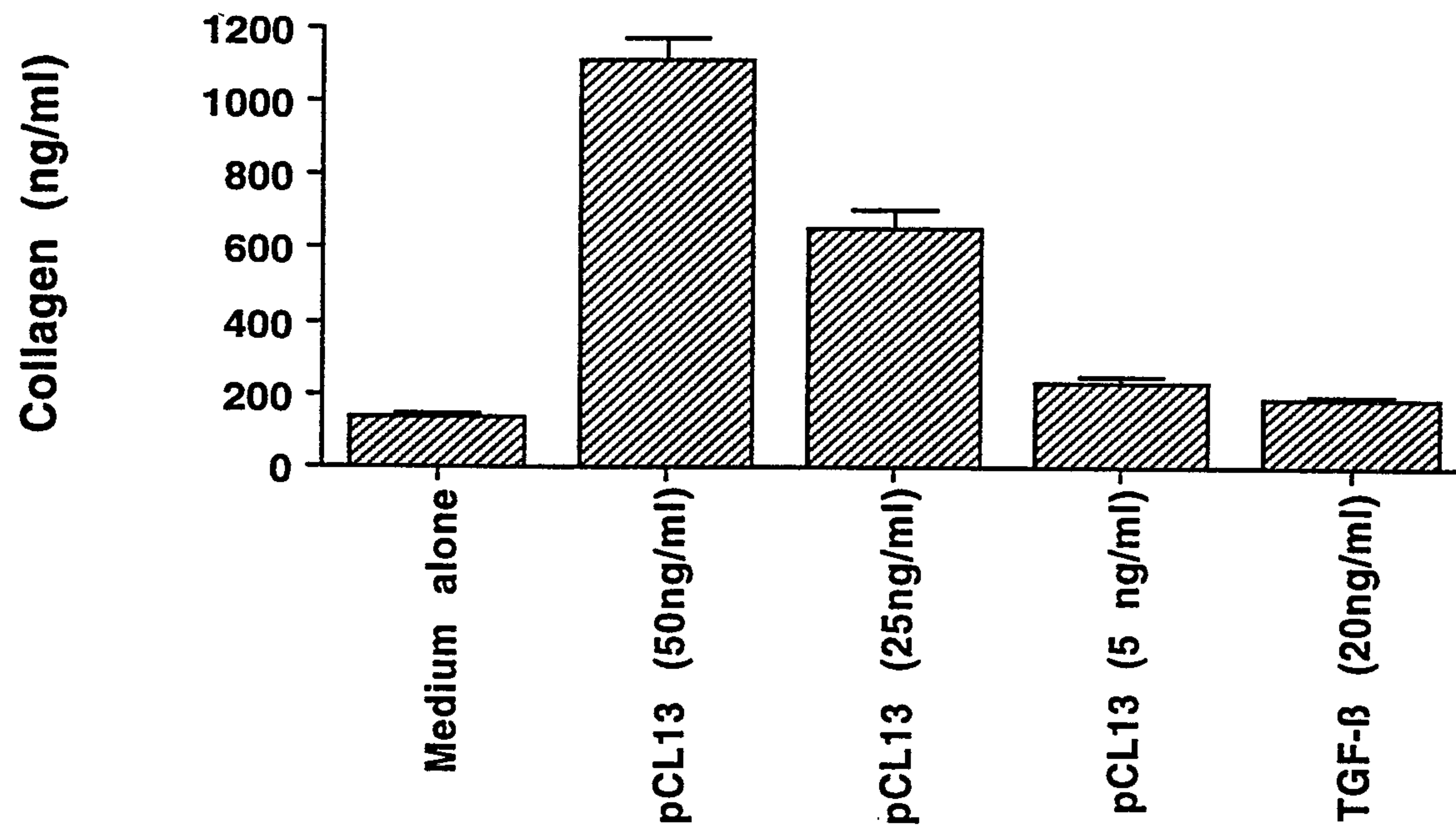


FIGURE 13

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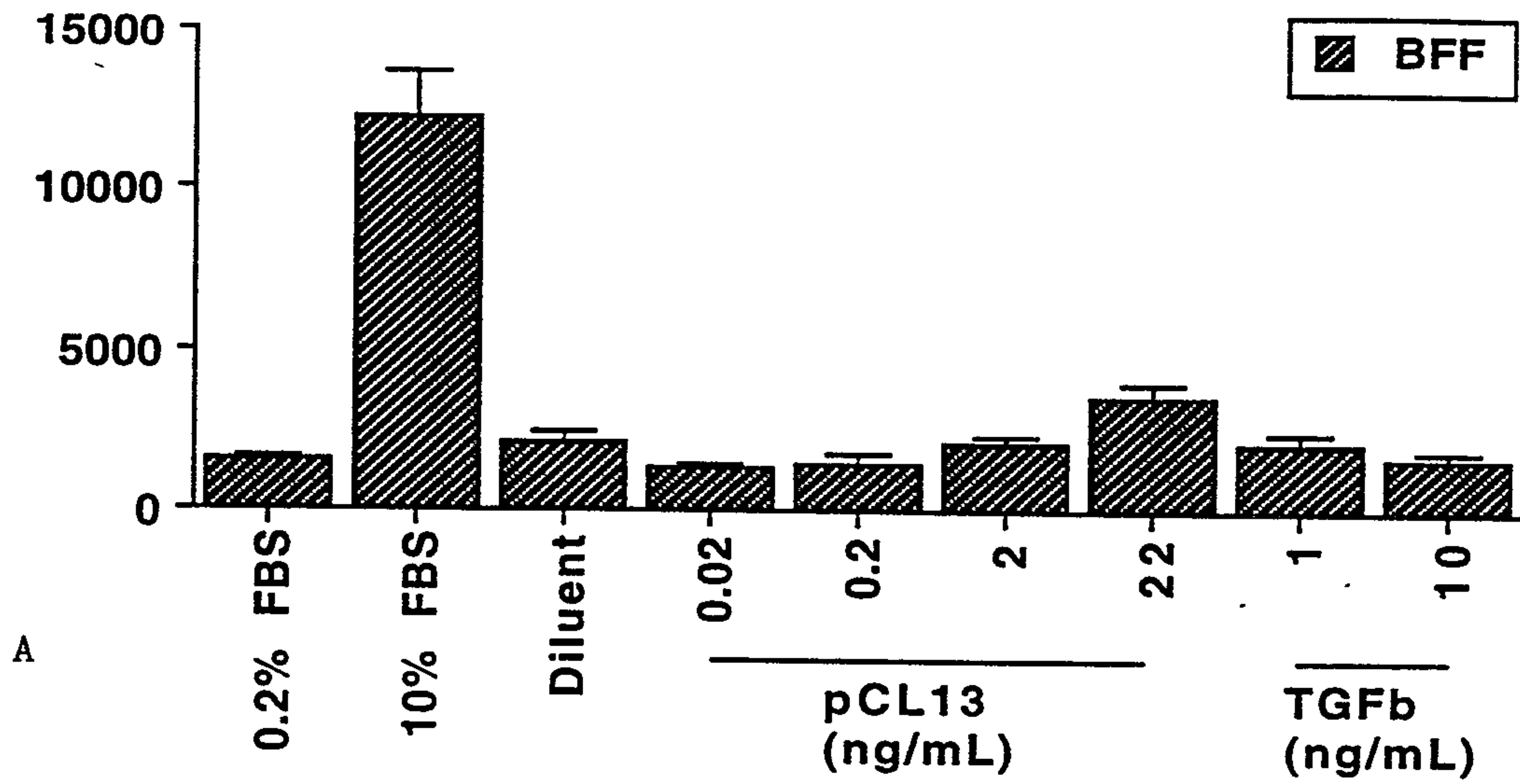
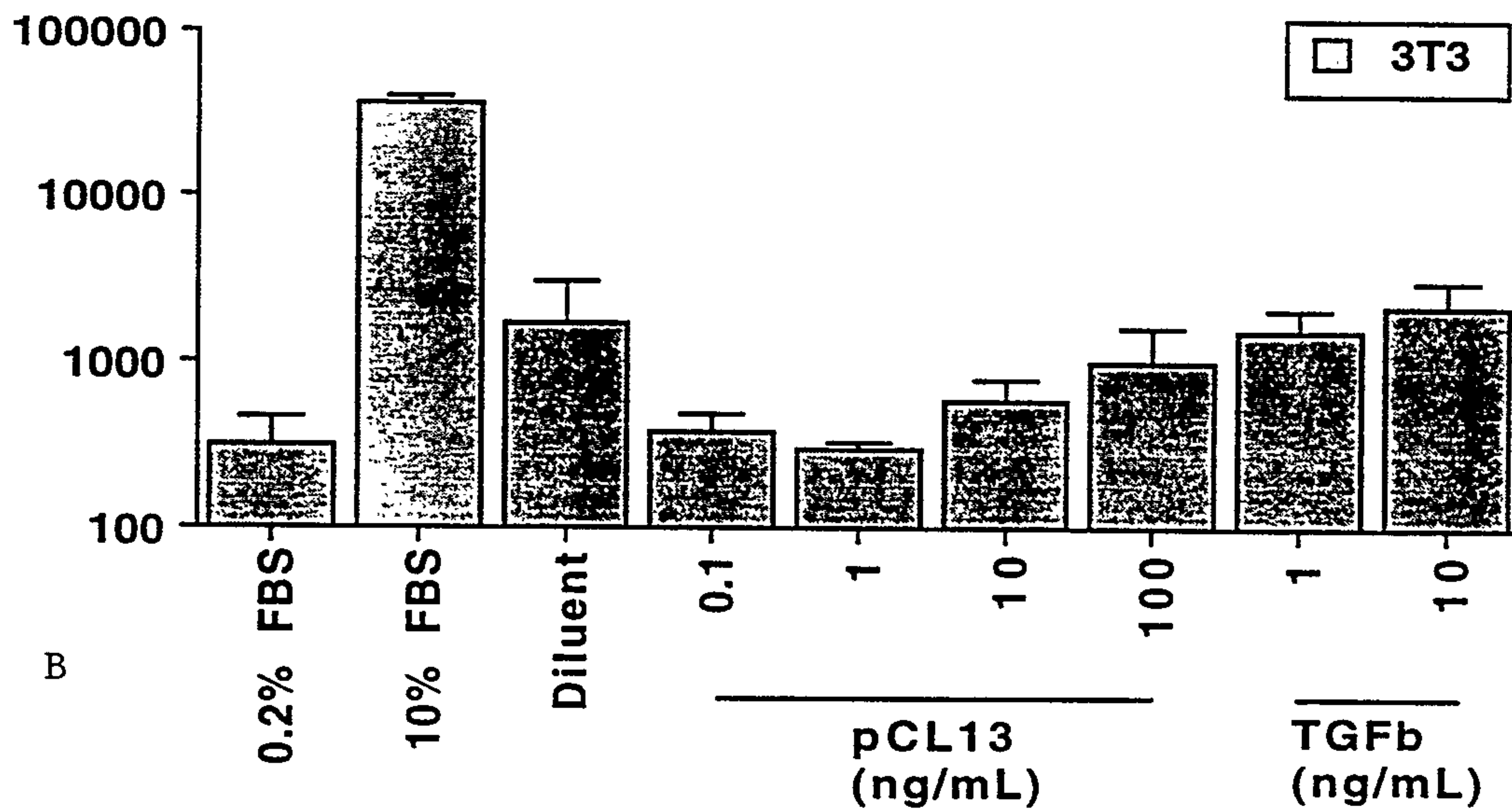
thymidine
uptake (dpm)log thymidine
uptake (dpm)

FIGURE 14

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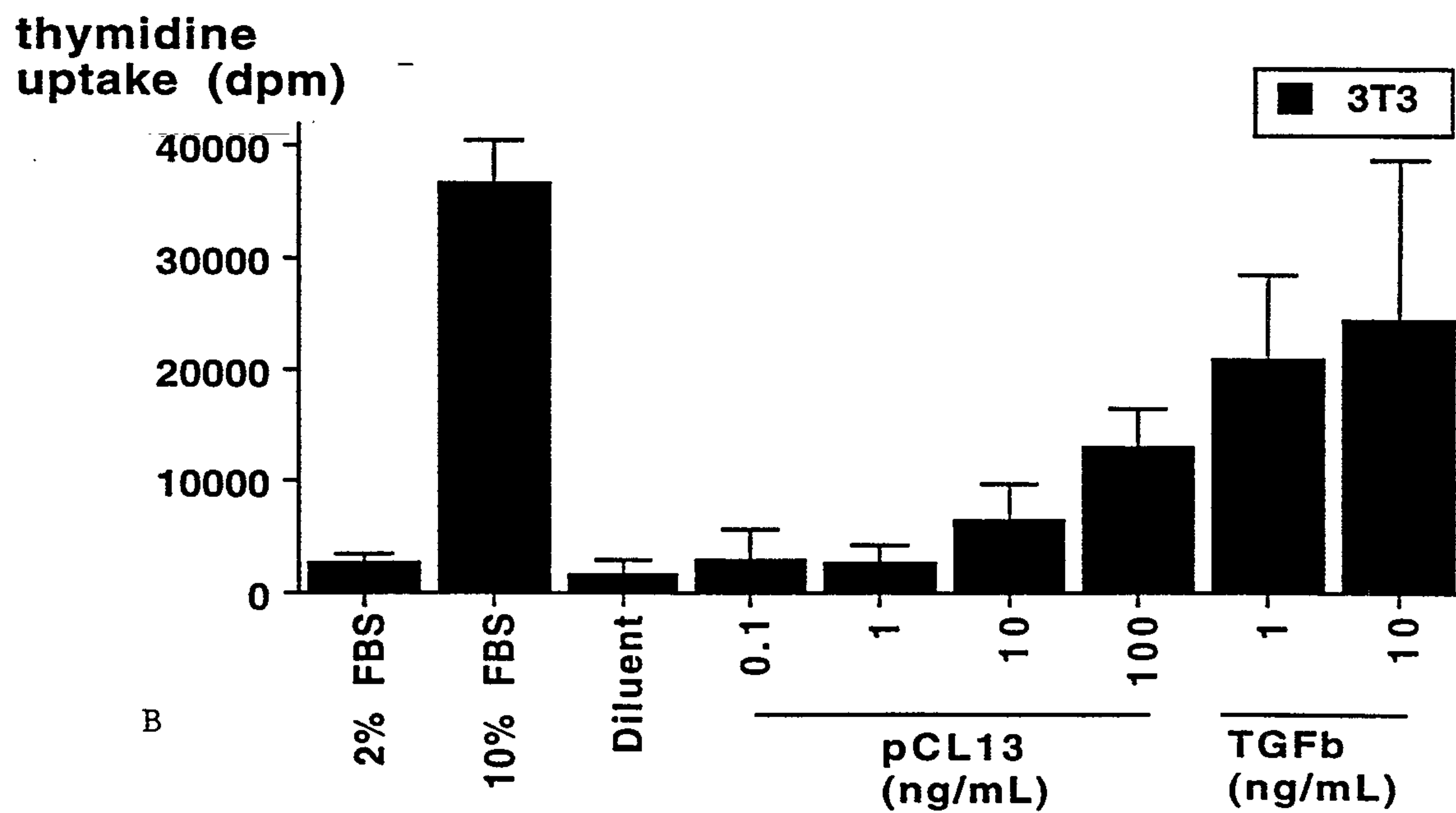
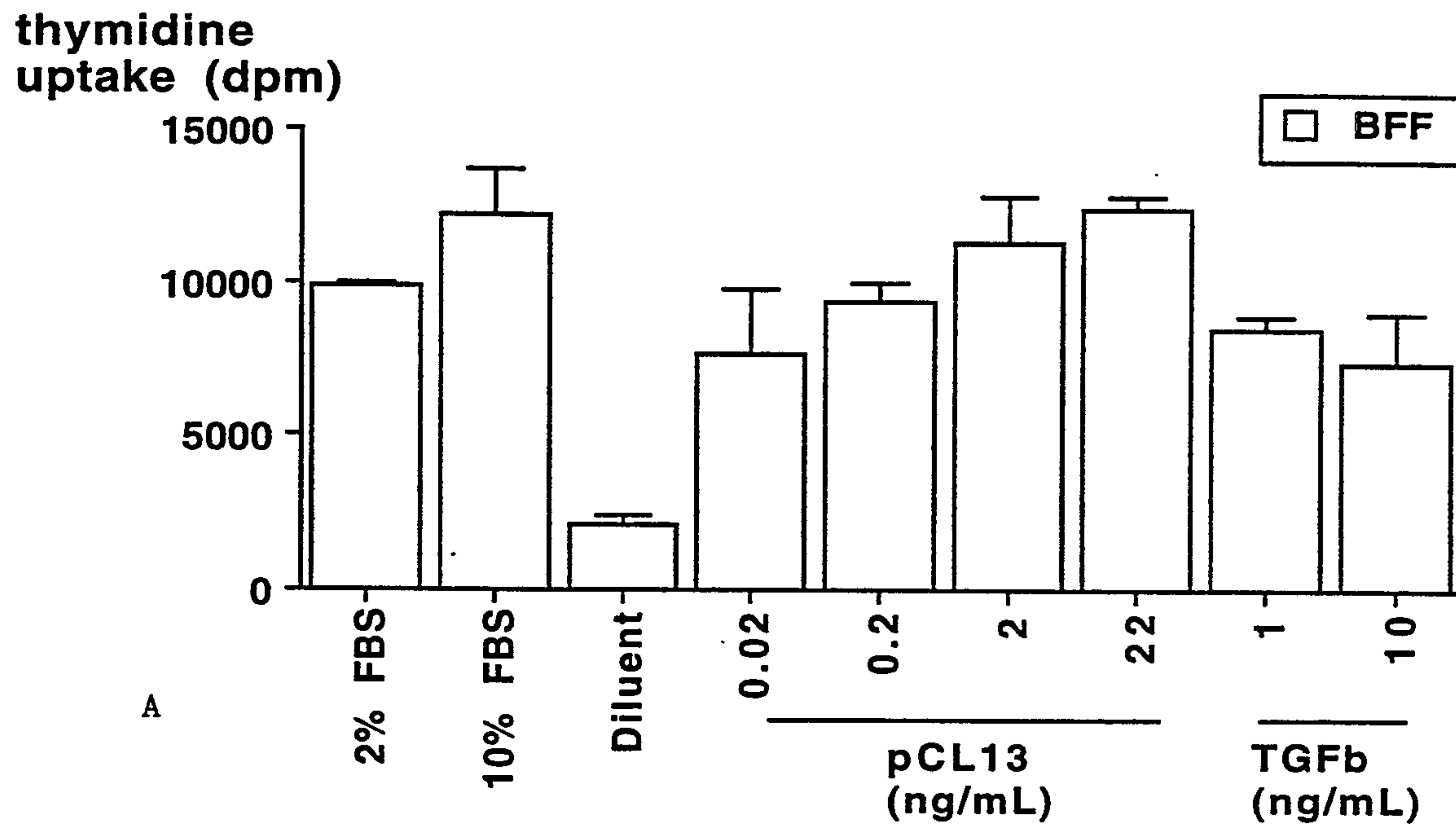


FIGURE 15

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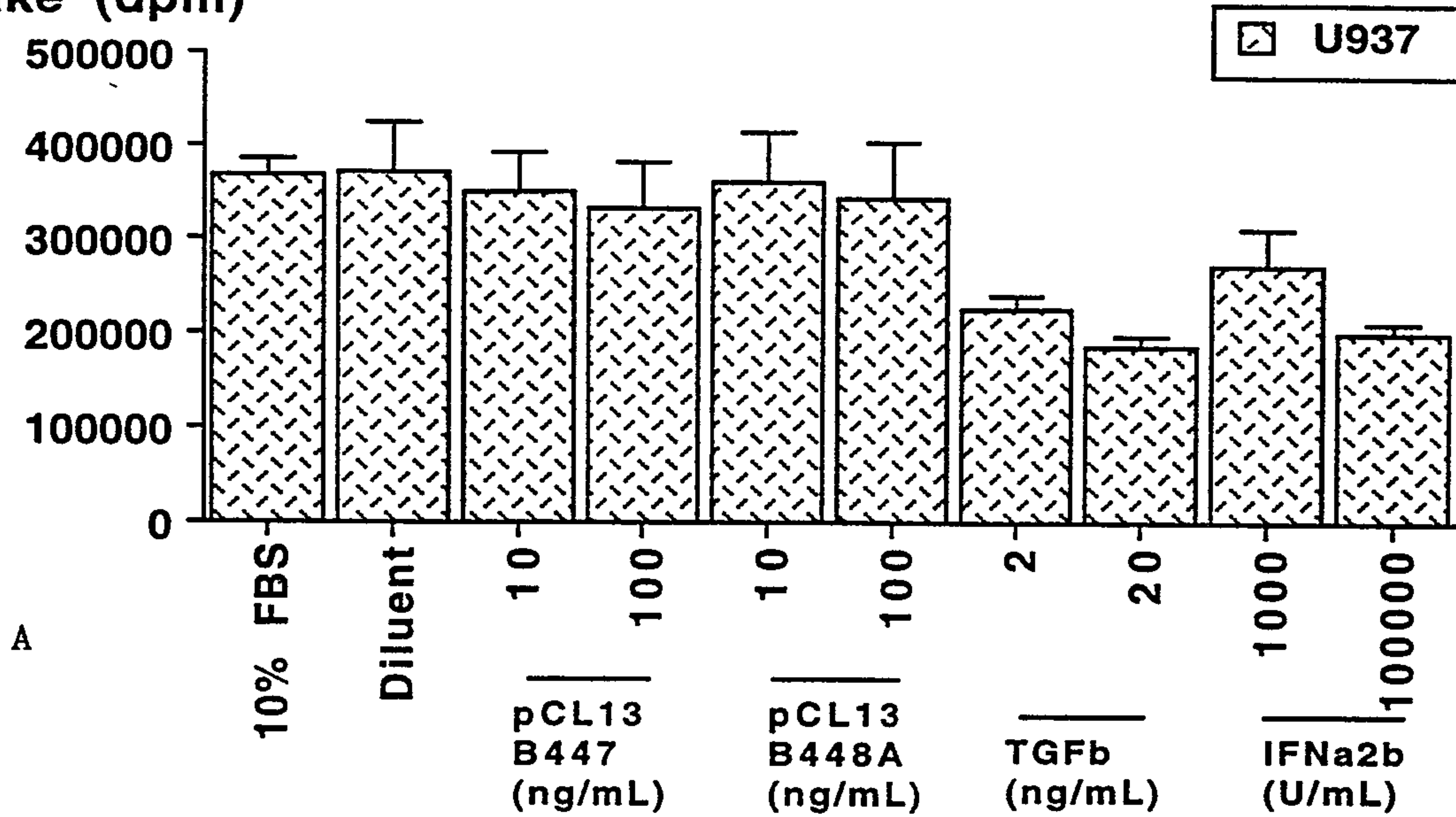
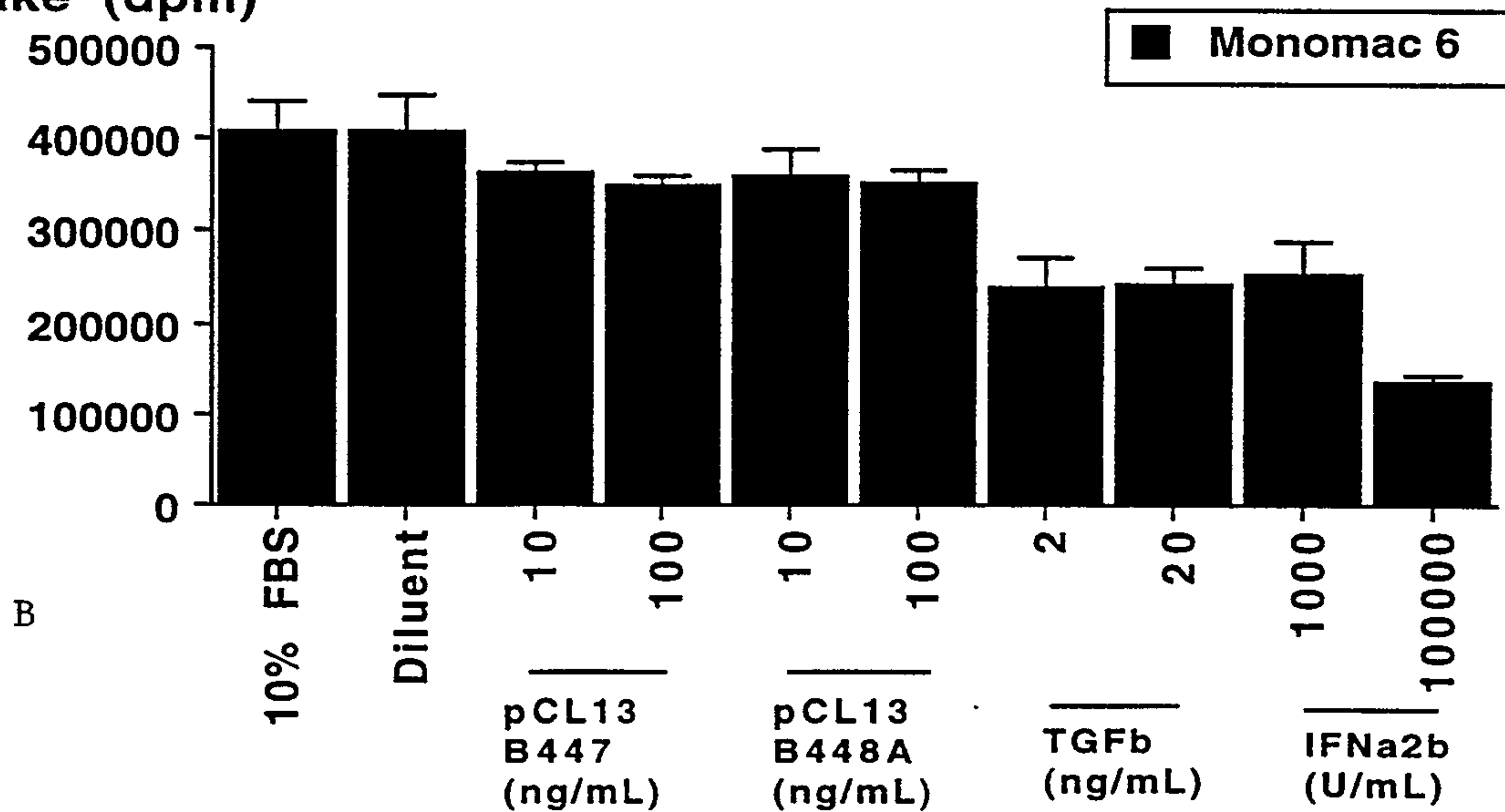
thymidine
uptake (dpm)thymidine
uptake (dpm)

FIGURE 16

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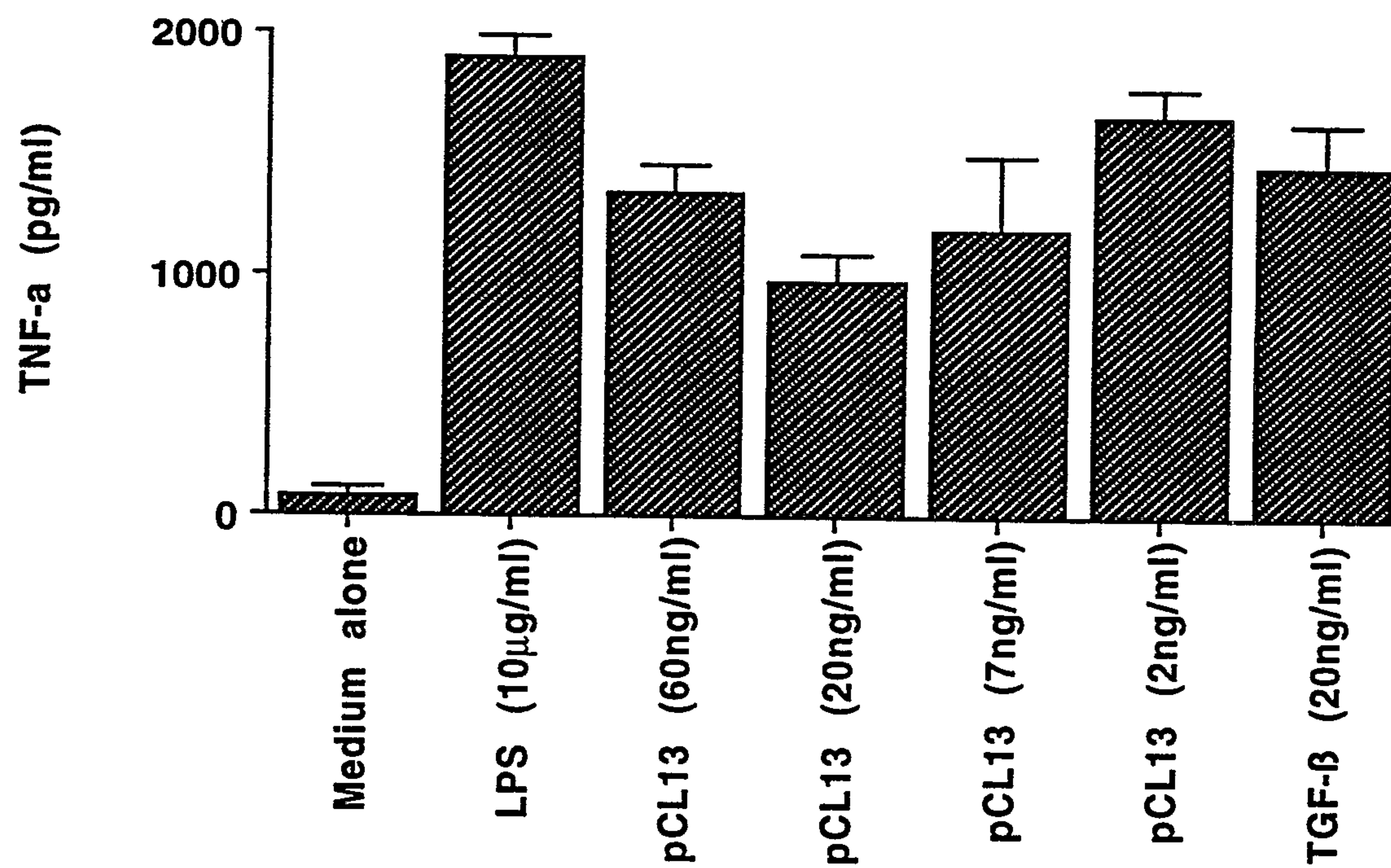


FIGURE 17

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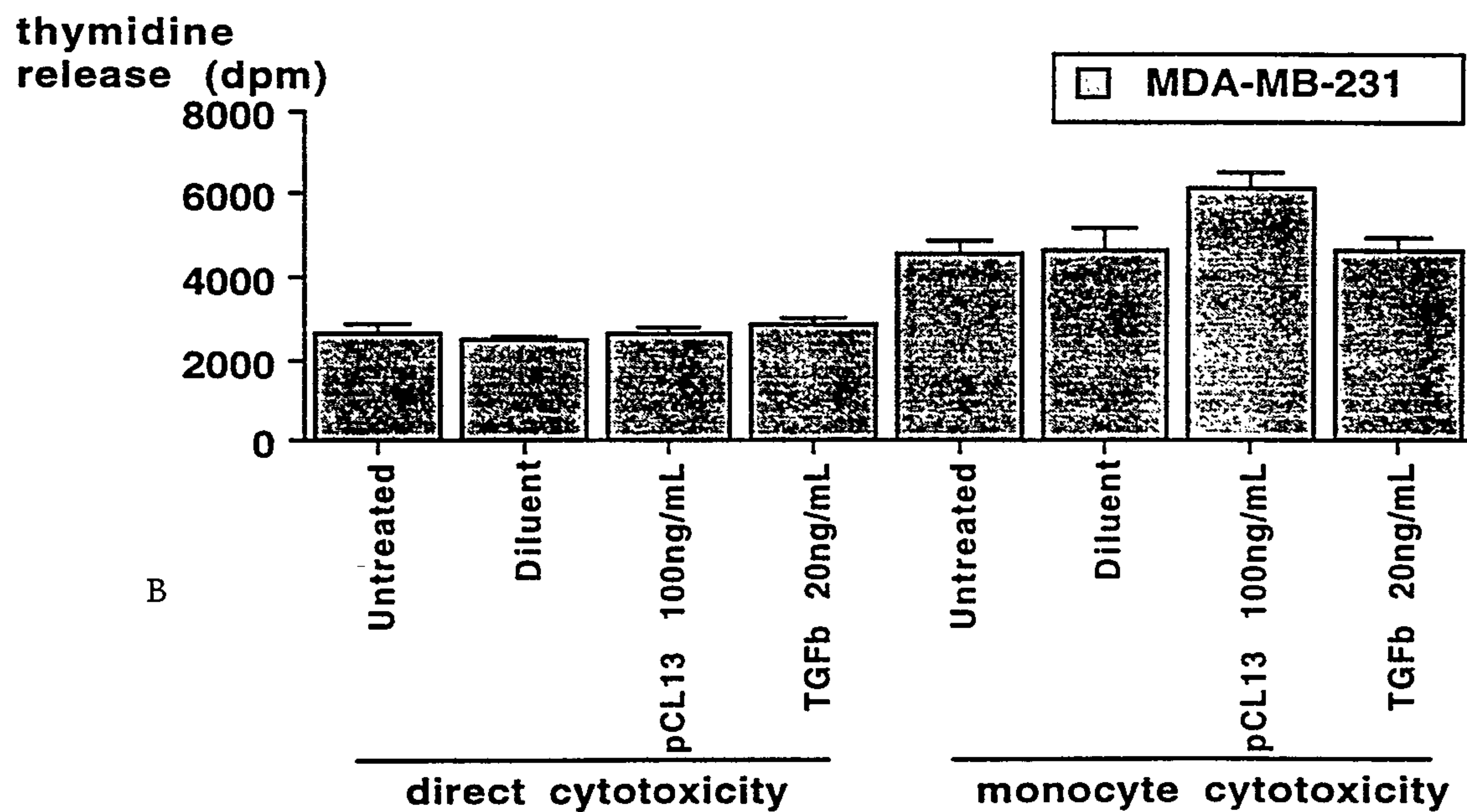
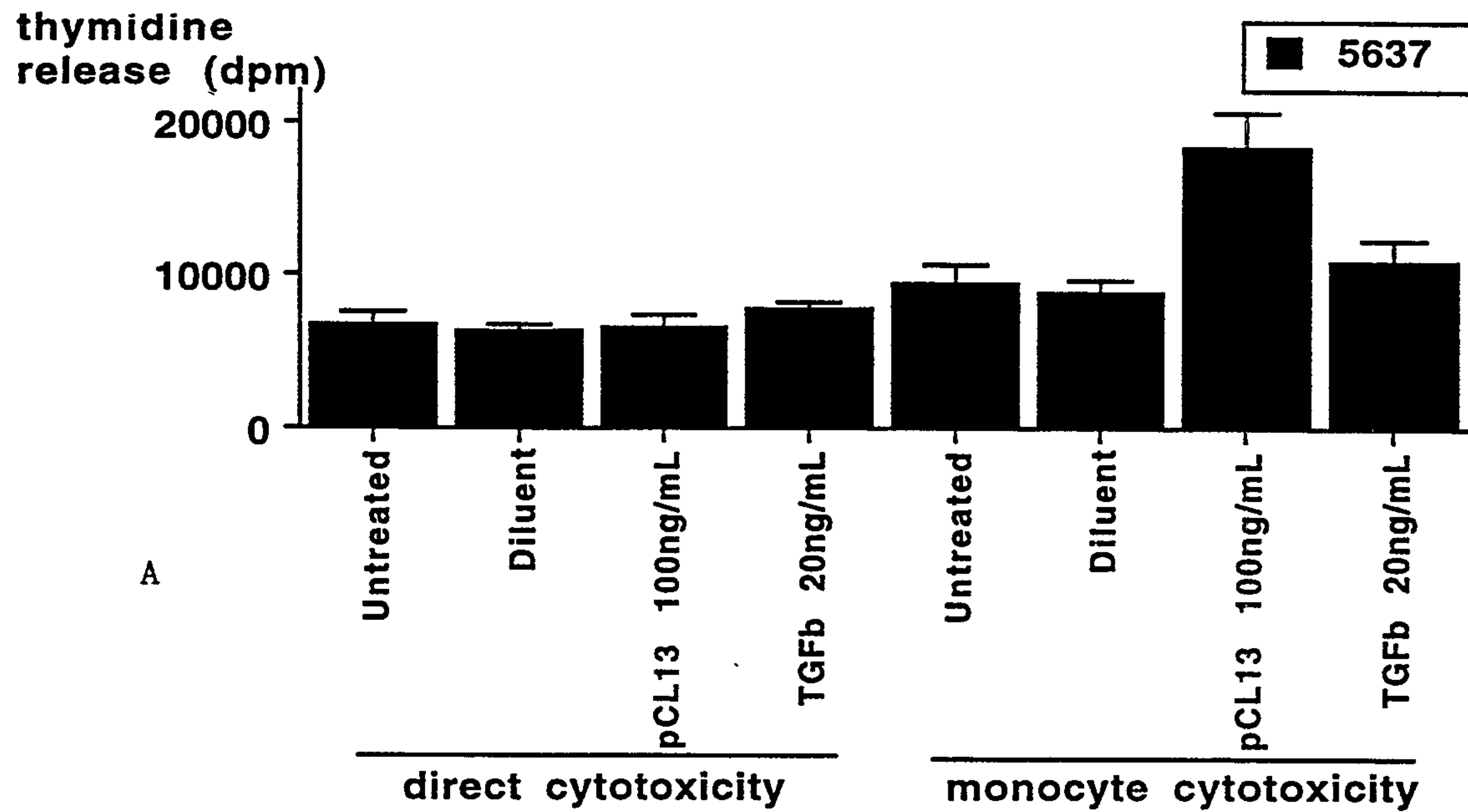


FIGURE 18

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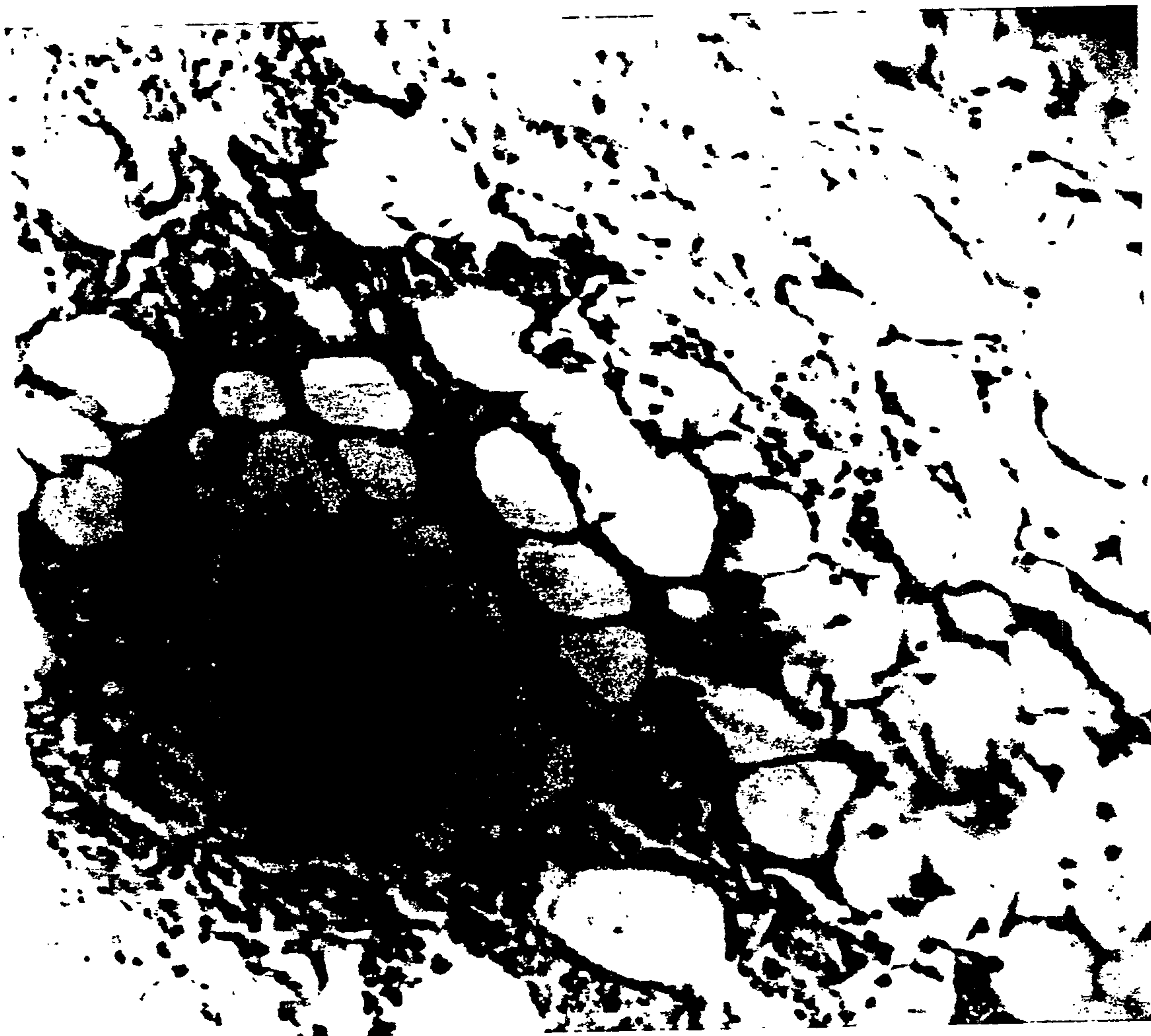


FIGURE 19A

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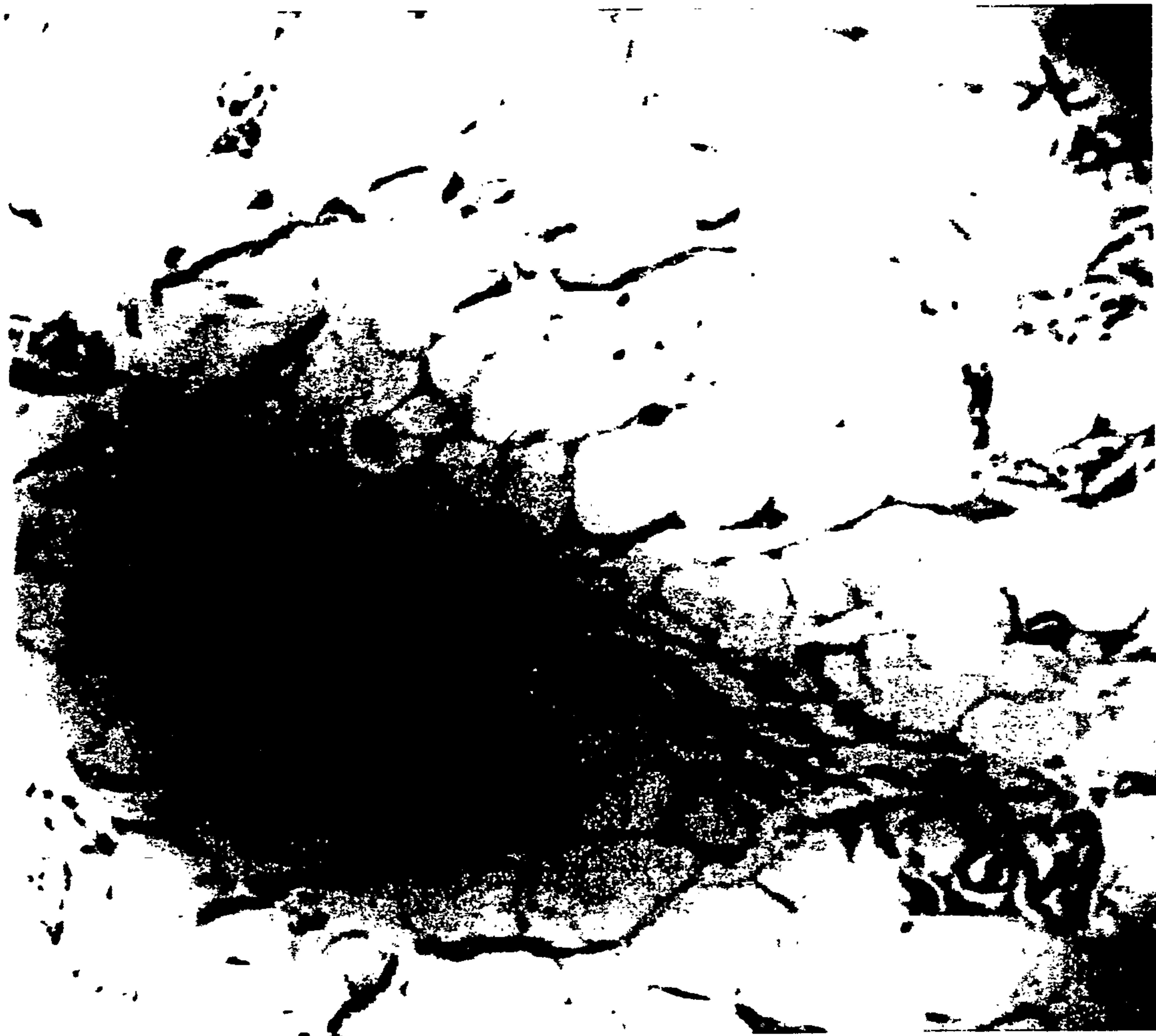


FIGURE 19B

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	10	20	30	40	
1	A C T C T T G G C G T C A G A T G A T C C A C G T G C C T C G G C C T C C C A A				b2
1	A C T C T T G A C G T C A G G T G A T C C A C G T G C C T C G G C C T C C C A A				h1
1	G C - - - - -				u2
1	G C - - - - -				f1
1	G C - - - - -				C13
1	G C - - - - -				a1
1	G C - - - - -				b1
1	G C - - - - -				d2
1	G C - - - - -				dd2
	50	60	70	80	
41	A G T G C T G G G A T T A C A G G C G A G A G C C A C C G T G C C C G G C G C A				b2
41	A G T G C T G G G A T T A C A G G C G A G A A C C A C C G T G C C C G G C G C A				h1
3	- - - - -				u2
3	- - - - -				f1
3	- - - - -				C13
3	- - - - -				a1
3	- - - - -				b1
3	- - - - -				d2
3	- - - - -				dd2
	90	100	110	120	
81	G A A T T C T T T T T T T T G G A G A T G A G G T A T T G C C A T C T T G C C C				b2
81	G A A T T C T T T T T T T T G G A G A T G A G G T A T T G C C A T C T T G C C C				h1
3	- - - - -				u2
3	- - - - -				f1
3	- - - - -				C13
3	- - - - -				a1
3	- - - - -				b1
3	- - - - -				d2
3	- - - - -				dd2
	130	140	150	160	
121	A G A C T T G T C T C G A A C T C C T G G G C T C A A A C A A T C C A C C C A C				b2
121	A G A C T T G T C T C G A A C T C C T G G G C T C A A G C A A T C C A C C C A C				h1
3	- - - - -				u2
3	- - - - -				f1
3	- - - - -				C13
3	- - - - -				a1
3	- - - - -				b1
3	- - - - -				d2
3	- - - - -				dd2
	170	180	190	200	
161	C T C G G C C T C C C A A G G T G C T G A G A T T A C T G A C A T A A G C C A C				b2
161	C T C G G C C T C C C A A G T G C T G A G A T T A C T G A C A T A A G C C A C				h1
3	- - - - -				u2
3	- - - - -				f1
3	- - - - -				C13
3	- - - - -				a1
3	- - - - -				b1
3	- - - - -				d2
3	- - - - -				dd2

FIGURE 20A (Cont'd)

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	210	220	230	240	
201	C A T G C C T G G C C C C C A G A A T T A T G A A T C C T G T G A G G A T G G C				b2
201	C A T G C C T G C C C C C C A G A A T T A T G A A T C C T G T G A G G A T G G C				h1
3	-				u2
3	-				f1
3	-				C13
3	-				a1
3	-				b1
3	-				d2
3	-				dd2
	250	260	270	280	
241	T T C A A G G T G A G C G C T G A G C C A G A C A A A A G G A T G G G G T T T G				b2
241	T T C A A G G T G A G C G C T G A G C C A G A C A A A A G G A T G G G G T T T G				h1
3	-				u2
3	-				f1
3	-				C13
3	-				a1
3	-				b1
3	-				d2
3	-				dd2
	290	300	310	320	
281	G G A G C A C C C T G C T T A G A C T G G A A A G A T A A T G T T G G A G A A G				b2
281	G G A G C A C C C T G C T T A G A C T G G A A A G A T A A T G T T G G A G A A G				h1
3	-				u2
3	-				f1
3	-				C13
3	-				a1
3	-				b1
3	-				d2
3	-				dd2
	330	340	350	360	
321	A C T T C C T G G A A G A G G G G C T T T T T G C G T A G A G T T T T G A A G A				b2
321	A C T T C C T G G A A G A G G G G C T T T T T G C G T A G A G T T T T G A A G A				h1
3	-				u2
3	-				f1
3	-				C13
3	-				a1
3	-				b1
3	-				d2
3	-				dd2
	370	380	390	400	
361	A T G A G T A G G A G T T C T C C A G A G G A G G A T G A G T A A C T G C A A T				b2
361	A T G A G T A G G A G T T C T C C A G A G G A G G A T G A G T A A C T G C A A T				h1
3	-				u2
3	-				f1
3	-				C13
3	-				a1
3	-				b1
3	-				d2
3	-				dd2

FIGURE 20A (Cont'd)

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		410	420	430	440	
401	A A C A C C C A G T T T A T C A A G T G C C T C C T A T G T G T C T G G C C C T					b2
401	A A C A C C C A G T T T A T C A A G T G C C T C C T A T G T G T C T G G C C C T					h1
3	- - - - -					u2
3	- - - - -					f1
3	- - - - -					C13
3	- - - - -					a1
3	- - - - -					b1
3	- - - - -					d2
3	- - - - -					dd2
		450	460	470	480	
441	G T G C T T T A C C C C T C A T T T G A C C A C C T C T C C A G T G A G A G T C					b2
441	G T G C T T T A C C C C T C A T T T G A C C A C C T C T C C A G T G A G A G T C					h1
3	- - - - -					u2
3	- - - - -					f1
3	- - - - -					C13
3	- - - - -					a1
3	- - - - -					b1
3	- - - - -					d2
3	- - - - -					dd2
		490	500	510	520	
481	T C A G T C C T T T T T T T C C T G G T G A G G A A A C A G G C A T G G C A G A					b2
481	T C A G T C C T T T T T T T C C T G G T G A G G A A A C A G G C A T G G C A G A					h1
3	- - - - -					u2
3	- - - - -					f1
3	- - - - -					C13
3	- - - - -					a1
3	- - - - -					b1
3	- - - - -					d2
3	- - - - -					dd2
		530	540	550	560	
521	G A G G C A T G A C A C A T C A A G G T T G C C C T T C C T G G C T C C A T C T					b2
521	G A G G C A T G A C A C A T C A A G G T T G C C C T T C C T G G C T C C A T C T					h1
3	- - - - -					u2
3	- - - - -					f1
3	- - - - -					C13
3	- - - - -					a1
3	- - - - -					b1
3	- - - - -					d2
3	- - - - -					dd2
		570	580	590	600	
561	A G C C C G T T C T C C T C T G C T T C C T T T G T T T T T C A C C A T C T T T					b2
561	A G C C C G T T C T C C T C T G C T T C C T T T G T T T T T C A C C A T C T T T					h1
3	- - - - -					u2
3	- - - - -					f1
3	- - - - -					C13
3	- - - - -					a1
3	- - - - -					b1
3	- - - - -					d2
3	- - - - -					dd2

FIGURE 20A (Cont'd)

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		22/34			
		610	620	630	640
601	A G C C T T T G A C C C C A A C C A A A A G A G A A G A G A G G A A A T C C C	b2			
601	A G C C T T T G A C C C C A A C C A A A A G A G A A G A G A G G A A A T C C C	h1			
3	- - - - -	u2			
3	- - - - -	f1			
3	- - - - -	C13			
3	- - - - -	a1			
3	- - - - -	b1			
3	- - - - -	d2			
3	- - - - -	dd2			
		650	660	670	680
641	A T G G G C A T A G A C A G C C A C C T C T T A A A C T C T T G T C T G G A A T	b2			
641	A T G G G C A T A G A C A G C C A C C T C T T A A A C T C T T G T C T G G A A T	h1			
3	- - - - -	u2			
3	- - - - -	f1			
3	- - - - -	C13			
3	- - - - -	a1			
3	- - - - -	b1			
3	- - - - -	d2			
3	- - - - -	dd2			
		690	700	710	720
681	T T T T C A C A T A G T A A C A A T G T C T T T T T T T C C T C C A A A A A G A	b2			
681	T T T T C A C A T A G T A A C A A T G T C T T T T T T T C C T C C A A A A A G A	h1			
3	- - - - -	u2			
3	- - - - -	f1			
3	- - - - -	C13			
3	- - - - -	a1			
3	- - - - -	b1			
3	- - - - -	d2			
3	- - - - -	dd2			
		730	740	750	760
721	C T C C C A G G C T G G A A T G G T G T C C T C A T A T C G A G G A A G A G G A	b2			
721	C T C C C A G G C T G G A A T G G T G T C C T C A T A T C G A G G A A G A G G A	h1			
3	- - - - -	u2			
3	- - - - -	f1			
3	- - - - -	C13			
3	- - - - -	a1			
3	- - - - -	b1			
3	- - - - -	d2			
3	- - - - -	dd2			
		770	780	790	800
761	T A C T G A G G C C C A G A A A T G T G C C C T A G C T T T A C T A G G A G C G	b2			
761	T A C T G A G G C C C A G A A A T G T G C C C T A G C T T T A C T A G G A G C G	h1			
3	- G C T G A G G C C C A G A A A T G T G C C C T A G C T T T A C T A G G A G C G	u2			
3	- G C - - - - -	f1			
3	- - - - -	C13			
3	- - - - -	a1			
3	- - - - -	b1			
3	- - - - -	d2			
3	- - - - -	dd2			

FIGURE 20A (Cont'd)

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		810		820		830		840		
801		CCCCCACCTAAAGATCCTCCCCCTAAATACACCCCCCAGAC								b2
801		CCCCCACCTAAAGATCCTCCCCCTAAATACACCCCCCAGAC								h1
42		CCCCCACCTAAAGATCCTCCCCCTAAATACACCCCCCAGAC								u2
5		-----								f1
3		-----								C13
3		-----								a1
3		-----								b1
3		-----								d2
3		-----								dd2
		850		860		870		880		
841		CCCGCCCAAGCTGTGGTCATTGGAGTGTTTACTCTGCAGGC								b2
841		CCCGCCCAAGCTGTGGTCATTGGAGTGTTTACTCTGCAGAC								h1
82		CCCGCCCAAGCTGTGGTCATTGGAGTGTTTACTCTGCAGGC								u2
5		-----								f1
3		-----								C13
3		-----								a1
3		-----								b1
3		-----								d2
3		-----								dd2
		890		900		910		920		
881		AGGGGGAGGAGGGCGGGACTGAGCAGGCGGAGACGGACAA								b2
881		AGGGGGAGGAGGGCGGGACTGAGCAGGCGGAGACGGACAA								h1
122		AGGGGGAGGAGGGCGGGACTGAGCAGGCGGAGACGGACAA								u2
5		-----								f1
3		-----								C13
3		-----								a1
3		-----								b1
3		-----								d2
3		-----								dd2
		930		940		950		960		
921		AGTCCGGGGACTATAAAGGCCGGTCCGGCAGCATCTGGGTC								b2
921		AGTCCGGGGACTATAAAGGCCGGTCCGGCAGCATCTGGGTC								h1
162		AGTCCGGGGACTATAAAGGCCGGTCCGGCAGCATCTGGGTC								u2
5		-----CGGGGACTATAAAGGCCGGTCCGGCAGCATCTGGGTC								f1
3		-----								C13
3		-----								a1
3		-----								b1
3		-----								d2
3		-----								dd2
		970		980		990		1000		
961		AGTCCCAGCTCAGAGGCCGCGCAACCTGACACAGCCATGCCCGG								b2
961		AGTCCCAGCTCAGAGGCCGCGCAACCTGACACAGCCATGCCCGG								h1
202		AGTCCCAGCTCAGAGGCCGCGCAACCTGACACAGCCATGCCCGG								u2
41		AGTCCCAGCTCAGAGGCCGCGCAACCTGACACAGCCATGCCCGG								f1
3		-----GGC-CG--CTGACACAGCCATGCCCGG								C13
3		-----GCG-CAACCTGACACAGCCATGCCCGG								a1
3		-----G--C--C--C--CACAGCCATGCCCGG								b1
3		-----GCCGCAACCTGACACAGCCATGCCCGG								d2
3		-----GCCGCAACCTGACACAGCCATGCCCGG								dd2

FIGURE 20A (Cont'd)

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	1010	1020	1030	1040	
1001	G C A A G A A C T C A G G A C G G	G T G A A T G G C T C T C A G A T G C T C C T G	b2		
1001	G C A A G A A C T C A G G A C G G	G T G A A T G G C T C T C A G A T G C T C C T G	h1		
242	G C A A G A A C T C A G G A C G G	G T G A A T G G C T C T C A G A T G C T C C T G	u2		
81	G C A A G A A C T C A G G A C G C	T G A A T G G C T C T C A G A T G C T C C T G	f1		
26	G C A A G A A C T C A G G A C G C	T G A A T G G C T C T C A G A T G C T C C T G	C13		
28	G C A A G A A C T C A G G A C G C	T G A A T G G C T C T C A G A T G C T C C T G	a1		
19	G C A A G A A C T C A G G A C G G	G T G A A T G G C T C T C A G A T G C T C C T G	b1		
29	G C A A G A A C T C A G G A C G G	G T G A A T G G C T C T C A G A T G C T C C T G	d2		
29	G C A A G A A C T C A G G A C G G	G T G A A T G G C T C T C A G A T G C T C C T G	dd2		
	1050	1060	1070	1080	
1041	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	b2		
1041	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	h1		
282	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	u2		
121	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	f1		
66	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	C13		
68	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	a1		
59	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	b1		
69	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	d2		
69	G T G T T G C T G G T G C T C T C G	T G G C T G C C G C A T G G G G G C G C C C	dd2		
	1090	1100	1110	1120	
1081	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	b2		
1081	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	h1		
322	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	u2		
161	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	f1		
106	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	C13		
108	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	a1		
99	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	b1		
109	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	d2		
109	T G T C T C T G G C C G A G G C G A G	C C G C G C A A G T T T C C C G G G A C C	dd2		
	1130	1140	1150	1160	
1121	C T C A G A G T T G C A C T	C C G A A G A C T C C A G A T T C C G A G A G T T G	b2		
1121	C T C A G A G T T G C A C T	C C G A A G A C T C C A G A T T C C G A G A G T T G	h1		
362	C T C A G A G T T G C A C T	C C G A A G A C T C C A G A T T C C G A G A G T T G	u2		
201	C T C A G A G T T G C A C T	C C G A A G A C T C C A G A T T C C G A G A G T T G	f1		
146	C T C A G A G T T G C A C A	C C G A A G A C T C C A G A T T C C G A G A G T T G	C13		
148	C T C A G A G T T G C A C T	C C G A A G A C T C C A G A T T C C G A G A G T T G	a1		
139	C T C A G A G T T G C A C T	C C G A A G A C T C C A G A T T C C G A G A G T T G	b1		
149	C T C A G A G T T G C A C T	C C G A A G A C T C C A G A T T C C G A G A G T T G	d2		
149	C T C A G A G T T G C A C T	C C G A A G A C T C C A G A T T C C G A G A G T T G	dd2		
	1170	1180	1190	1200	
1161	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	b2		
1161	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	h1		
402	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	u2		
241	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	f1		
186	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	C13		
188	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	a1		
179	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	b1		
189	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	d2		
189	C G G A A A C G C T A C G A G G A C C	T G C T A A C C A G G C T G C G G G C C A	dd2		

FIGURE 20A (Cont'd)

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	1210	1220	1230	1240	
1201	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				b2
1201	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				h1
442	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				u2
281	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				f1
226	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				C13
228	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				a1
219	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				b1
229	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				d2
229	A C C A G A G C T G G G A A G A T T C G A A C A C C G A C C T C G T C C C G G C				dd2
	1250	1260	1270	1280	
1241	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				b2
1241	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				h1
482	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				u2
321	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				f1
266	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				C13
268	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				a1
259	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				b1
269	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				d2
269	C C C T G C A G T C C G G A T A C T C A C G C C A G A A G T G C G G C T G G G A				dd2
	1290	1300	1310	1320	
1281	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				b2
1281	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				h1
522	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				u2
361	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				f1
306	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				C13
308	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				a1
299	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				b1
309	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				d2
309	T C C G G C G G C C A C C T G C A C C T G C G T A T C T C T C G G G C C G C C C				dd2
	1330	1340	1350	1360	
1321	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				b2
1321	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				h1
562	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				u2
401	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				f1
346	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				C13
348	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				a1
339	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				b1
349	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				d2
349	T T C C C G A G G G G C T C C C C G A G G C C T C C C G C C T T C A C C G G G C				dd2
	1370	1380	1390	1400	
1361	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				b2
1361	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				h1
602	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				u2
441	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				f1
386	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				C13
388	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				a1
379	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				b1
389	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				d2
389	T C T G T T C C G G C T G T C C C C G A C G G C G T C A A G G T C G T G G G A C				dd2

FIGURE 20A (Cont'd)

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	1410	1420	1430	1440	
1401	G T G A C A C G A C C G	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	b2		
1401	G T G A C A C G A C C G	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	h1		
642	G T G A C A C G A C C G	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	u2		
481	G T G A C A C G A C C G	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	f1		
426	G T G A C A C G A C C T	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	C13		
428	G T G A C A C G A C C G	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	a1		
419	G T G A C A C G A C C G	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	b1		
429	G T G A C A C G A C C G	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	d2		
429	G T G A C A C G A C C G	C T G C G G C G T C A G C T C A G C C T T G C A A G A C	dd2		
	1450	1460	1470	1480	
1441	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	b2			
1441	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	h1			
682	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	u2			
521	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	f1			
466	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	C13			
468	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	a1			
459	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	b1			
469	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	d2			
469	C C C A G G C G C C C G C G C T G C A C C T G C G A C T G T C G C C G C C G C C	dd2			
	1490	1500	1510	1520	
1481	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	b2			
1481	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	h1			
722	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	u2			
561	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	f1			
506	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	C13			
508	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	a1			
499	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	b1			
509	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	d2			
509	G T C G C A G T C G G A C C A A C T G C T G G C A G A A T C T T C G T C C G C A	dd2			
	1530	1540	1550	1560	
1521	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	b2			
1521	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	h1			
762	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	u2			
601	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	f1			
546	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	C13			
548	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	a1			
539	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	b1			
549	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	d2			
549	C G G C C C C A G C T G G A G T T G C A C T T G C G G C C G C A A G C C G C C A	dd2			
	1570	1580	1590	1600	
1561	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C C A C T G	b2			
1561	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C C A C T G	h1			
802	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C C A C T G	u2			
641	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C G A C T G	f1			
586	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C C A C T G	C13			
588	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C G A C T G	a1			
579	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C C A C T G	b1			
589	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C C A C T G	d2			
589	G G G G G C G C C G C A G A G C G C G T G C G C G C A A C G G G G A C C A C T G	dd2			

FIGURE 20A (Cont'd)

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28 / 34																																									
	1810										1820										1830										1840										
1801	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	b2
1801	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	h1
1042	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	u2
881	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	f1
826	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	C13
828	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	a1
819	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	b1
829	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	d2
829	C	A	G	C	G	C	C	C	T	G	C	T	G	C	G	T	G	C	C	C	G	C	C	A	G	C	T	A	C	A	A	T	C	C	C	A	T	G	G	T	dd2
	1850										1860										1870										1880										
1841	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	b2
1841	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	h1
1082	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	u2
921	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	f1
866	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	C13
868	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	a1
859	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	b1
869	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	d2
869	G	C	T	C	A	T	T	C	A	A	A	A	G	A	C	C	G	A	C	A	C	C	G	G	G	G	T	G	T	C	G	C	T	C	C	A	G	A	C	C	dd2
	1890										1900										1910										1920										
1881	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	b2
1881	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	h1
1122	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	u2
961	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	f1
906	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	C13
908	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	a1
899	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	b1
909	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	d2
909	T	A	T	G	A	T	G	A	C	T	T	G	T	T	A	G	C	C	A	A	A	G	A	C	T	G	C	C	A	C	T	G	C	A	T	A	T	G	A	G	dd2
	1930										1940										1950										1960										
1921	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	A	G	G	A	C	G	C	b2
1921	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	A	G	G	A	C	G	C	h1
1162	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	A	G	G	A	C	G	C	u2
1001	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	G	G	G	A	G	G	C	f1
946	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	G	G	G	A	G	G	C	C13
948	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	G	G	G	A	G	G	C	a1
939	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	A	G	G	A	C	G	C	b1
949	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	A	G	G	A	C	G	C	d2
949	C	A	G	T	C	C	T	G	G	T	C	C	T	T	C	C	A	C	T	G	T	G	C	A	C	C	T	G	C	G	C	G	G	A	G	G	A	C	G	C	dd2
	1970										1980										1990										2000										
1961	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	b2
1961	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	h1
1202	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	u2
1041	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	f1
986	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	C13
988	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	a1
979	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	b1
989	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	d2
989	G	A	C	C	T	C	A	G	T	T	G	T	C	C	T	G	C	C	C	T	G	T	G	G	A	A	T	G	G	G	C	T	C	A	A	G	G	T	T	C	dd2

FIGURE 20A (Cont'd)

FIGURE 20A (Cont'd)

2210 2220 2230

b2
h1
u2
f1
C13
a1
b1
d2
dd2

FIGURE 20A (Cont'd)

	170										180										190										200										
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	C13
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	a1
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	b1
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	b2
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	d2
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	dd2
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	f1
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	h1
161	P	P	P	S	Q	S	D	Q	L	L	A	E	S	S	S	A	R	P	Q	L	E	L	H	L	R	P	Q	A	A	R	G	R	R	R	A	R	A	R	N	G	u2

SUBSTITUTE SHEET (Rule 26)

WO 97/00958

PCT/AU96/00386

		32/34			
		210	220	230	240
201	DHCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				C13
201	D ¹ HCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				a1
201	DHCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				b1
201	DHCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				b2
201	DHCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				d2
201	DHCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				dd2
201	D ¹ HCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				f1
201	DHCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				h1
201	DHCPLGPGRCCRLHTVRLASLEDLGWADWVLSPREVQVTMC				u2
		250	260	270	280
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				C13
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				a1
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				b1
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				b2
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				d2
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				dd2
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				f1
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				h1
241	IGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASYN				u2
		290	300		
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				C13
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				a1
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				b1
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				b2
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				d2
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				dd2
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				f1
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				h1
281	PMVLIQKTD TGVS LQTYDDLLAKDCHCI.				u2

Decoration 'Decoration #1': Box residues that differ from C13protseq.def.

FIGURE 20B (Cont'd)

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C13SA/5HNUCLEOTIDE SEQUENCE

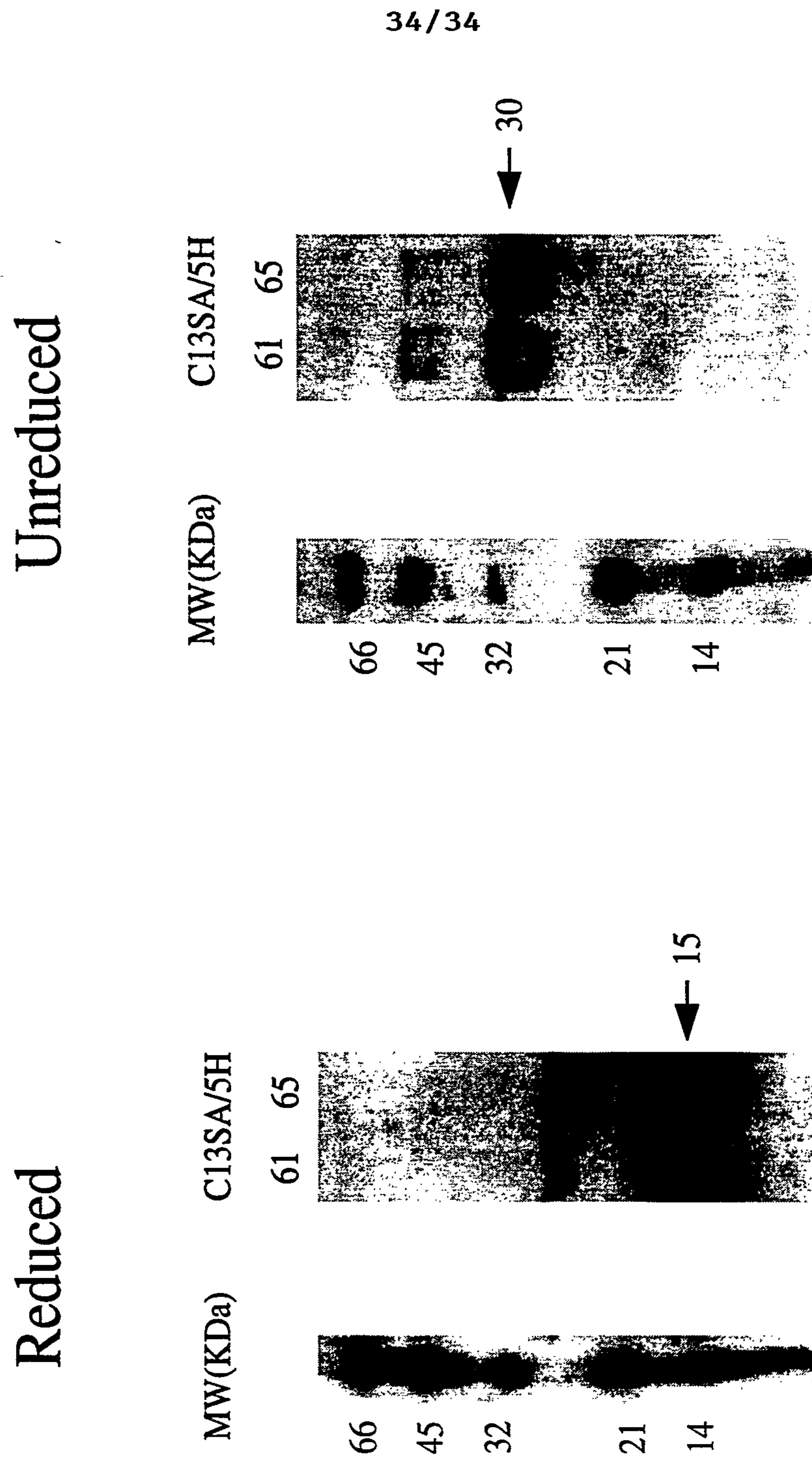
		10			20			30			40			50			60			70			80	
1		GCTAGCQCCA		TGGATTACTA		CAGAAAATAT		GCAGCTATCT		TTCTGGTCAC		ATTGTCGGTG		TTTCTGCATG		TTCTCCATTC		80						
81		CGCTCCTGAT		GAATTCCACC		ACCACCACCA		CCTGGTGCCC		CGCGGCTCCG		ACTACAAGGA		CGACGACGAC		AAGCTCCGCG		160						
161		CCTCCGTGGC		GCGCAACGGG		GACCACTGTC		CGCTCGGGCC		CGGGCGTTGC		TGCCGTCTGC		ACACGGTCCG		CGCGTCGCTG		240						
241		GAAGACCTGG		GCTGGGCCGA		TTGGGTGCTG		TCGCCACGGG		AGGTGCAAGT		GACCATGTGC		ATCGGCGCGT		GCCCGAGCCA		320						
321		GTTCCGGGCG		GCAAACATGC		ACGCGCAGAT		CAAGACGAGC		CTGCACCGCC		TGAAGCCCGA		CACGGTGCCA		GCGCCCTGCT		400						
401		GCGTGCCCGC		CAGCTACAAT		CCCATGGTGC		TCATTCAAAA		GACCGACACC		GGGGTGTCGC		TCCAGACCTA		TGATGACTTG		480						
481		TTAGCCAAAG		ACTGCCACTG		CATATGACTC		GAG									513							
		10			20			30			40			50			60			70			80	

TRANSLATED PROTEIN SEQUENCE

		10			20			30			40			50			60			70			80	
1		LEKREHHHHH		LVPRGSDYKD		<u>DDDK</u> LRASVA		RNGDHCPLGP		GRCCRLHTR		ASLEDLGWAD		WVLSPREVOV		TMCIGACPSQ		80						
81		FRAANMHAQI		KTSLHRLKPD		TVPAPCCVPA		SYNPMVLIQK		TDTGVSLQTY		DDLLAKDCHC		I •				142						
		10			20			30			40			50			60			70			80	

underline = FLAG epitope
 ===== = PKA site
 ↓ = yeast signal sequence cleavage site amino acid 5
 • = stop codon
 ————— = motif of 5 HIS residues for binding to metal chelate column
 coding region for bioactive part of clone 13 commences with amino acid 30
 LVPRGS = this sequence from amino acids 11-16 represents thrombin cleavage site

FIGURE 21

**FIGURE 22**