

Background

10

The present invention pertains in general to hematopoietic growth factors and to polynucleotides encoding such factors. The present application pertains in particular to mammalian pluripotent colony stimulating factors, specifically human pluripotent granulocyte colony-stimulating factor (hpG-CSF), to fragments and polypeptide analogs thereof and to polynucleotides encoding the same.

20 The human blood-forming (hematopoietic) system replaces a variety of white blood cells (including neutrophils, macrophages, and basophils/mast cells), red blood cells (erythrocytes) and clot-forming cells (megakaryocytes/platelets). The hematopoietic system of the average human male has been estimated to produce on the
25 order of 4.5×10^{11} granulocytes and erythrocytes every year, which is equivalent to an annual replacement of total body weight. Dexter et al., BioEssays, 2, 154-158 (1985).

30 It is believed that small amounts of certain hematopoietic growth factors account for the differentiation of a small number of progenitor "stem cells" into the variety of blood cell lines, for the tremendous proliferation of those lines, and for the ultimate differentiation of mature blood cells from those
35 lines. Because the hematopoietic growth factors are present in extremely small amounts, the detection and

5 result, a large number of names have been coined to
denote a much smaller number of factors. As an example
of the resultant confusion the terms, IL-3, BPA, multi-
CSF, HCGF, MCGF and PSF are all acronyms which are now
believed to apply to a single murine hematopoietic
10 growth factor. Metcalf, Science, 229, 16-22 (1985).
See also, Burgess, et al. J.Biol.Chem., 252, 1988
(1977), Das, et al. Blood, 58, 600 (1980), Ihle, et al.,
J.Immunol., 129, 2431 (1982), Nicola, et al.,
J.Biol.Chem., 258, 9017 (1983), Metcalf, et al.,
15 Int.J.Cancer, 30, 773 (1982), and Burgess, et al.
Int.J.Cancer, 26, 647 (1980), relating to various murine
growth regulatory glycoproteins.

The application of recombinant genetic tech-
niques has brought some order out of this chaos. For
20 example, the amino acid and DNA sequences for human
erythropoietin, which stimulates the production of
erythrocytes, have been obtained. (See, Lin, PCT
Published Application No. 85/02610, published June 20,
1985.) Recombinant methods have also been applied to
25 the isolation of cDNA for a human granulocyte-macrophage
colony-stimulating factor. See, Lee, et al., Proc.
Natl. Acad. Sci. (USA), 82, 4360-4364 (1985) and Wong,
et al., Science, 228, 810-814 (1985). See also Yokota,
et al. Proc. Natl. Acad. Sci. (USA), 81, 1070 (1984),
30 Fung, et al., Nature, 307, 233 (1984), and Gough, et
al., Nature, 309, 763 (1984) relating to cloning of
murine genes, as well as Kawasaki, et al., Science, 230,
291 (1985) relating to human M-CSF.

A human hematopoietic growth factor, called
35 human pluripotent colony-stimulating factor (hpCSF) or
pluripoietin, has been shown to be present in the cul-

5 hpCSF purified from this cell line has been reported to
stimulate proliferation and differentiation of pluri-
potent progenitor cells leading to the production of all
major blood cell types in assays using human bone marrow
progenitor cells. Welte et al., Proc. Natl. Acad. Sci.
10 (USA), 82, 1526-1530 (1985). Purification of hpCSF
employed: $(\text{NH}_4)_2\text{SO}_4$ precipitation; anion exchange
chromatography (DEAE cellulose, DE52); gel filtration
(AcA54 column); and C18 reverse phase high performance
liquid chromatography. A protein identified as hpCSF,
15 which is eluted in the second of two peaks of activity
in C18 reverse phase HPLC fractions, was reported to
have a molecular weight (MW) of 18,000 as determined by
sodium dodecyl sulphate (SDS)-polyacrylamide gel elec-
trophoresis (PAGE) employing silver staining. HpCSF was
20 earlier reported to have an isoelectric point of 5.5
[Welte, et al., J. Cell. Biochem., Supp 9A, 116 (1985)]
and a high differentiation activity for the mouse myelo-
monocytic leukemic cell line WEHI-3B D⁺ [Welte, et al.,
UCLA Symposia on Molecular and Cellular Biology, Gale,
25 et al., eds., New Series, 28 (1985)]. Preliminary
studies indicate that the factor identified as hpCSF has
predominately granulocyte colony-stimulating activity
during the first seven days in a human CFU-GM assay.

Another factor, designated human CSF-8, has
30 also been isolated from human bladder carcinoma cell
line 5637 and has been described as a competitor of
murine ¹²⁵I-labelled granulocyte colony-stimulating
factor (G-CSF) for binding to WEHI-3B D⁺ cells in a
dose-response relationship identical to that of
35 unlabelled murine G-CSF [Nicola, et al., Nature, 314,
625-628 (1985)]. This dose-response relationship had

5 also unique among CSF's in that they share a high degree
of ability to induce differentiation of WEHI-3B D⁺
cells. Nicola, et al., Immunology Today, 5, 76-80
(1984). At high concentrations, G-CSF stimulates mixed
granulocyte/macrophage colony-forming cells [Nicola, et
10 al., (1984) supra], which is consistent with preliminary
results indicating the appearance of granulocytic, mono-
cytic, mixed granulocytic/monocytic and eosinophilic
colonies (CFU-GEMM) after 14 days incubation of human
bone marrow cultures with hpCSF. CSF- β has also been
15 described as stimulating formation of neutrophilic
granulocytic colonies in assays which employed mouse
bone marrow cells, a property which has been a criterion
for identification of a factor as a G-CSF. On the basis
of these similarities, human CSF- β has been identified
20 with G-CSF (granulocytic colony stimulating factor).
Nicola et al., Nature, 314, 625-628 (1985).

Based upon their common properties, it appears
that human CSF- β of Nicola, et al., supra, and the hpCSF
of Welte, et al., supra, are the same factor which could
25 properly be referred to as a human pluripotent granulo-
cyte colony-stimulating factor (hpG-CSF). Characteriza-
tion and recombinant production of hpG-CSF would be
particularly desirable in view of the reported ability
of murine G-CSF to completely suppress an in vitro WEHI-
30 3B D⁺ leukemic cell population at "quite normal concen-
trations", and the reported ability of crude, injected
preparations of murine G-CSF to suppress established
transplanted myeloid leukemias in mice. Metcalf,
Science, 229, 16-22 (1985). See also, Sachs, Scientific
35 American, 284(1), 40-47 (1986).

5 material. It is noteworthy, for example, that restric-
tions appear to exist against commercial use of Human
Tumor Bank cells such as the human bladder carcinoma
cell line 5637 (A.T.C.C. HTB9) which have been reported
as sources of natural hpCSF isolates in Welte, et al.
10 (1985, supra).

Summary of the Invention

According to the present invention, DNA
15 sequences coding for all or part of hpG-CSF are pro-
vided. Such sequences may include: the incorporation
of codons "preferred" for expression by selected non-
mammalian hosts; the provision of sites for cleavage by
restriction endonuclease enzymes; and the provision of
20 additional initial, terminal or intermediate DNA
sequences which facilitate construction of readily
expressed vectors. The present invention also provides
DNA sequences coding for microbial expression of poly-
peptide analogs or derivatives of hpG-CSF which differ
25 from naturally-occurring forms in terms of the identity
or location of one or more amino acid residues (i.e.,
deletion analogs containing less than all of the resi-
dues specified for hpG-CSF; substitution analogs, such
as [Ser¹⁷]hpG-CSF, wherein one or more residues
30 specified are replaced by other residues; and addition
analogues wherein one or more amino acid residues is added
to a terminal or medial portion of the polypeptide) and
which share some or all the properties of naturally-
occurring forms.
35 Novel DNA sequences of the invention include
sequences useful in securing expression in procaryotic

stimulating factor. DNA sequences of the invention are specifically seen to comprise: (a) the DNA sequence set forth in Table VII and Table VIII or their complimentary strands; (b) a DNA sequence which hybridizes (under hybridization conditions such as illustrated herein or more stringent conditions) to the DNA sequences in Table VII or to fragments thereof; and (c) a DNA sequence which, but for the degeneracy of the genetic code, would hybridize to the DNA sequence in Table VII. Specifically comprehended in part (b) are genomic DNA sequences encoding allelic variant forms of hpG-CSF and/or encoding other mammalian species of pluripotent granulocyte colony-stimulating factor. Specifically comprehended by part (c) are manufactured DNA sequences encoding hpG-CSF, fragments of hpG-CSF and analogs of hpG-CSF which DNA sequences may incorporate codons facilitating translation of messenger RNA in microbial hosts. Such manufactured sequences may readily be constructed according to the methods of Alton, et al., PCT published application WO 83/04053.

Also comprehended by the present invention is that class of polypeptides coded for by portions of the DNA complement to the top strand human cDNA or genomic DNA sequences of Tables VII or VIII herein, i.e., "complementary inverted proteins" as described by Tramontano, et al., Nucleic Acids Res., 12, 5049-5059 (1984).

The present invention provides purified and isolated polypeptide products having part or all of the primary structural conformation (i.e., continuous sequence of amino acid residues) and one or more of the biological properties (e.g., immunological properties and

5 synthetic procedures or of procaryotic or eucaryotic
host expression (e.g., by bacterial, yeast, higher
plant, insect and mammalian cells in culture) of exog-
enous DNA sequences obtained by genomic or cDNA cloning
or by gene synthesis. The products of typical yeast
10 (e.g., Saccaromyces cerevisiae) or procaryote [e.g.,
Escherichia coli (E. coli)] host cells are free of asso-
ciation with any mammalian proteins. The products of
microbial expression in vertebrate (e.g., non-human
mammalian and avian) cells are free of association with
15 any human proteins. Depending upon the host employed,
polypeptides of the invention may be glycosylated with
mammalian or other eucaryotic carbohydrates or may be
non-glycosylated. Polypeptides of the invention may
also include an initial methionine amino acid residue
20 (at position -1).

Also comprehended by the invention are phar-
maceutical compositions comprising effective amounts of
polypeptide products of the invention together with
suitable diluents, adjuvants and/or carriers useful in
25 hpG-CSF therapy.

Polypeptide products of the invention may be
"labelled" by association with a detectable marker sub-
stance (e.g., radiolabelled with ^{125}I) to provide
reagents useful in detection and quantification of human
30 hpG-CSF in solid tissue and fluid samples such as blood
or urine. DNA products of the invention may also be
labelled with detectable markers (such as radiolabels
and non-isotopic labels such as biotin) and employed in
DNA hybridization processes to locate the human hpG-CSF
35 gene position and/or the position of any related gene
family in a chromosomal map. They may also be used for

5 may be useful, alone or in combination with other
hematopoietic factors or drugs in the treatment of
hematopoietic disorders, such as aplastic anemia. They
may also be useful in the treatment of hematopoietic
deficits arising from chemotherapy or from radiation
10 therapy. The success of bone marrow transplantation,
for example, may be enhanced by application of hpG-
CSF. Wound healing burn treatment and the treatment of
bacterial inflammation may also benefit from the appli-
cation of hpG-CSF. In addition, hpG-CSF may also be
15 useful in the treatment of leukemia based upon a
reported ability to differentiate leukemic cells.
Welte, et al., Proc. Natl. Acad. Sci. (USA), 82, 1526-
1530 (1985) and Sachs, supra.

Numerous aspects and advantages of the inven-
20 tion will be apparent to those skilled in the art upon
consideration of the following detailed description
which provides illustrations of the practice of the
invention in its presently preferred embodiments.

25 Brief Description of the Drawings

The Figure is a partial restriction endo-
nuclease map of the hpG-CSF gene accompanied by arrows
depicting the sequencing strategy used to obtain the
30 genomic sequence.

Detailed Description

According to the present invention, DNA
35 sequences encoding part or all of the polypeptide
sequence of hpG-CSF have been isolated and character-
ized.

5 resulting in such identification, and to the sequencing,
development of expression systems based on cDNA, genomic
and manufactured genes and verification of expression
hpG-CSF and analog products in such systems.

10 More particularly, Example 1 is directed to
amino acid sequencing of hpG-CSF. Example 2 is directed
to the preparation of a cDNA library for colony hybridi-
zation screening. Example 3 relates to construction of
hybridization probes. Example 4 relates to hybridiza-
15 tion screening, identification of positive clones, DNA
sequencing of a positive cDNA clone and the generation
of polypeptide primary structural conformation (amino
acid sequence) information. Example 5 is directed to
the identification and sequencing of a genomic clone
encoding hpG-CSF. Example 6 is directed to the con-
20 struction of a manufactured gene encoding hpG-CSF
wherein E.coli preference codons are employed.

Example 7 is directed to procedures for con-
struction of an E. coli transformation vector incor-
porating hpG-CSF-encoding DNA, the use of the vector in
25 procaryotic expression of hpG-CSF, and to analysis of
properties of recombinant products of the invention.
Example 8 is directed to procedures for generating
analogs of hpG-CSF wherein cysteine residues are
replaced by another suitable amino acid residue by means
30 of mutagenesis performed on DNA encoding hpG-CSF.
Example 9 is directed to procedures for the construction
of a vector incorporating hpG-CSF analog-encoding DNA
derived from a positive cDNA clone, the use of the
vector for transfection of COS-1 cells, and the cultured
35 growth of the transfected cells. Example 10 relates to
physical and biological properties or recombinant poly-
peptide products of the invention.

A sample (3-4 μ g, 85-90% pure of SDS, silver stain-PAGE) of hpG-CSF was obtained from Sloan Kettering Institute, New York, New York, as isolated and purified according to Welte, et al., Proc. Natl. Acad. Sci. (USA), 82, 1526-1530 (1985).

The N-terminal amino acid sequence of this sample of hpG-CSF was determined in a Run #1 by micro-sequence analysis using an AB407A gas phase sequencer (Applied Biosystems, Foster City, California) to provide the sequence information set out in Table I below. In Tables I-IV single letter codes are employed, "X" designates a residue which was not unambiguously determined and residues in parentheses were only alternatively or tentatively assigned.

TABLE I

1	5	10	15
K-P-L-G-P-A-S-K-L-K-Q-(G,V,S)-G-L-X-X-X			

A high background was present in every cycle of the run for which results are reported in Table I, indicating that the sample had many contaminating components, probably in the form of chemical residues from purification. The sequence was retained only for reference use.

In Run #2, a second sample (5-6 μ g, ~95% pure) was obtained from Sloan Kettering as for Run #1 and a sequencing procedure was performed as for Run #1. This sample was from the same lot of material employed to generate Fig. 4 of Welte, et al., Proc. Natl. Acad. Sci.

1 5 10 15 20
 T-P-L-G-P-A-S-(S)-L-P-Q-($\frac{S}{X}$)-M-($\frac{L}{M}$)-X-K-(R)-X-X-(R)-(L)-X-

10

Although more residues were identified, Run #2 did not provide a sufficiently long, unambiguous sequence from which a reasonable number of probes could be constructed to search for hpG-CSF DNA. It was calculated that at least 1536 probes would have been required to attempt isolation of cDNA based on the sequence of Table II. Again, contamination of the sample was believed to be the problem.

Accordingly, a third sample (3-5 μ g, ~40% pure) was obtained from Sloan Kettering as above. This preparation was electroblotted after separation by SDS-PAGE in an attempt at further purification. Sequence analysis of this sample yielded no data.

25

(B) Sequencing of Materials
Provided by Revised Methods

In order to obtain a sufficient amount of pure material to perform suitably definitive amino acid sequence analysis, cells of a bladder carcinoma cell line 5637 (subclone 1A6) as produced at Sloan-Kettering were obtained from Dr. E. Platzer. Cells were initially cultured in Iscove's medium (GIBCO, Grand Island, New York) in flasks to confluence. When confluent, the cultures were trypsinized and seeded into roller bottles (1-1/2 flasks/bottle) each containing 25 ml of preconditioned

dures. Eight grams of beads were introduced into a bottle and 400 ml of PBS was added. Beads were suspended by swirling gently for 3 hours. After allowing the beads to settle, the PBS was drawn off, the beads were rinsed in PBS and fresh PBS was added. The beads
10 were autoclaved for 15 minutes. Prior to use, the beads were washed in Iscove's medium plus 10% fetal calf serum (FCS) before adding fresh medium plus 10% FCS to obtain treated beads.

After removing all but 30 ml of the medium
15 from each roller bottle, 30 ml of fresh medium plus 10% FCS and 40 ml of treated beads were added to the bottles. The bottles were gassed with 5% CO₂ and all bubbles were removed by suction. The bottles were placed in roller racks at 3 rpm for 1/2 hour before
20 reducing the speed to 0.3 rpm. After 3 hours, an additional flask was trypsinized and added to each roller bottle containing beads.

At 40% to 50% of confluence the roller bottle cultures were washed with 50 ml PBS and rolled for 10
25 min. before removing the PBS. The cells were cultured for 48 hours in medium A [Iscove's medium containing 0.2% FCS, 10⁻⁸M hydrocortisone, 2mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin]. Next, the culture supernatant was harvested by centrifugation
30 at 3000 rpm for 15 min., and stored at -70°C. The cultures were refed with medium A containing 10% FCS and were cultured for 48 hours. After discarding the medium, the cells were washed with PBS as above and cultured for 48 hours in medium A. The supernatant was
35 again harvested and treated as previously described.

5 ml/min. and at a retentate rate of about 1000 ml/min. The concentrate was diafiltered with about 10 liters of 50mM Tris (pH 7.8) using the same apparatus and same flow rates. The diafiltered concentrate was loaded at 40 ml/min. onto a 1 liter DE cellulose column equilibrated in 50mM Tris (pH 7.8). After loading, the column was washed at the same rate with 1 liter of 50mM Tris (pH 7.8) and then with 2 liters of 50mM Tris (pH 7.8) with 50mM NaCl. The column was then sequentially eluted with six 1 liter solutions of 50mM Tris (pH 7.5) containing the following concentrations of NaCl: 75mM; 100mM; 125mM; 150mM; 200mM; and 300mM. Fractions (50 ml) were collected, and active fractions were pooled and concentrated to 65 ml on an Amicon[®] ultrafiltration stirred cell unit equipped with a YM5 membrane. This concentrate was loaded onto a 2 liter Aca54 gel filtration column equilibrated in PBS. The column was run at 80 ml/hr. and 10 ml fractions were collected. Active fractions were pooled and loaded directly onto a C4 high performance liquid chromatography (HPLC) column.

25 Samples, ranging in volume from 125 ml to 850 ml and containing 1-8 mg of protein, about 10% of which was hpG-CSF, were loaded onto the column at a flow rate ranging from 1 ml to 4 ml per minute. After loading and an initial washing with 0.1M ammonium acetate (pH 6.0-7.0) in 80% 2-propanol at a flow rate of 1/ml/min. One milliliter fractions were collected and monitored for proteins at 220nm, 260nm and 280nm.

As a result of purification, fractions containing hpG-CSF were clearly separated (as fractions 72 and 73 of 80) from other protein-containing fractions. HpG-CSF was isolated (150-300 µg) at a purity of about

5 Sequence analysis was carried out with an AB 470A
 sequencer according to the methods of Hewick, et al., J.
Biol. Chem., 256, 7990-7997 (1981) and Lai, Anal. Chim.
Acta, 163, 243-248 (1984). The results of Run #4 appear
 in Table III.

10

TABLE III

1	5	10
Thr - Pro - Leu - Gly - Pro - Ala - Ser - Ser - Leu - Pro-		
15	15	20
Gln - Ser - Phe - Leu - Leu - Lys -(Lys)- Leu -(Glu)- Glu-		
	25	30
20 Val - Arg - Lys - Ile -(Gln)- Gly - Val - Gly - Ala - Ala-		
Leu - X - X -		

In Run #4, beyond 31 cycles (corresponding to
 25 residue 31 in Table III) no further significant sequence
 information was obtained. In order to obtain a longer
 unambiguous sequence, in a Run #5, 14 µg of hpG-CSF
 purified from conditioned medium were reduced with 10 µl
 of β-mercaptoethanol for one hour at 45°C, then thor-
 30 oughly dried under a vacuum. The protein residue was
 then redissolved in 5% formic acid before being applied
 to a polybrenized glass fiber disc. Sequence analysis
 was carried out as for Run #4 above. The results of Run
 #5 are given in Table IV.

35

5 Thr - Pro - Leu - Gly - Pro - Ala - Ser - Ser - Leu - Pro -
 15 20
 Gln - Ser - Phe - Leu - Leu - Lys - Cys - Leu - Glu - Gln -
 25 30
 10 Val - Arg - Lys - Ile - Gln - Gly - Asp - Gly - Ala - Ala -
 35 40
 Leu - Gln - Phe - Lys - Leu - Gly - Ala - Thr - Tyr - Lys -
 45
 Val - Phe - Ser - Thr - (Arg) - (Phe) - (Met) -X-
 15

The amino acid sequence give in Table IV was
 sufficiently long (44 residues) and unambiguous to con-
 struct probes for obtaining hpG-CSF cDNA as described
infra.

20

Example 2

Among standard procedures for isolating cDNA
 sequences of interest is the preparation of plasmid-
 25 borne cDNA "libraries" derived from reverse transcrip-
 tion of mRNA abundant in donor cells selected on the
 basis of their expression of a target gene. Where sub-
 stantial portions of the amino acid sequence of a poly-
 peptide are known, labelled, single-stranded DNA probe
 30 sequences duplicating a sequence putatively present in
 the "target" cDNA may be employed in DNA/DNA hybridiza-
 tion procedures carried out on cloned copies of the cDNA
 which have been denatured to single stranded form.
 Weissman, et al., U.S. Patent No. 4,394,443; Wallace, et
 35 al., Nucleic Acids Res., 6, 3543-3557 (1979), and Reyes,
 et al., Proc. Natl. Acad. Sci. (USA), 79, 3270-3274

5 Manual for Genetic Engineering, Advanced Bacterial
Genetics", Cold Spring Harbor Laboratory, Cold Spring
Harbor, N.Y. (1980) at pp. 55-58 and 174-176, relating
to colony and plaque hybridization techniques.

Total RNA was extracted from approximately 1
10 gram of cells from a bladder carcinoma cell line 5637
(1A6) using a guanidinium thiocyanate procedure for quan-
titative isolation of intact RNA. [Chirgwin, et al.,
Biochemistry, 18, 5294-5299 (1979)].

The sterile aqueous RNA solution contained
15 total RNA from the 1A6 cells. To obtain only the
messenger RNA from the total RNA solution, the solution
was passed through a column containing oligodeoxy-
thymidylate [oligo(dT)] (Collaborative Research, Inc.,
Waltham, Massachusetts. Poly-Adenylated (poly-A⁺) tails
20 characteristic of messenger RNA adhere to the column
while ribosomal RNA is eluted. As a result of this
procedure, approximately 90 µg of poly-adenylated
messenger RNA (poly-A⁺ mRNA) were isolated. The iso-
lated poly-A⁺ messenger RNA was pre-treated with methyl-
25 mercury hydroxide (Alpha Ventron, Danvers,
Massachusetts) at a final concentration of 4 mM for 5
minutes at room temperature prior to use in a cDNA reac-
tion. The methylmercury hydroxide treatment denatured
interactions of messenger RNA, both with itself and with
30 contaminating molecules that inhibit translation.
Payvar, et al., J.Biol.Chem., 258, 7636-7642 (1979).

According to the Okayama procedure [Okayama,
et al., Molecular & Cellular Biology, 2, 161-170
(1982)], a cDNA bank was prepared using mRNA obtained
35 from 1A6 cells. The cDNAs were then transformed by
incubation into a host microorganism E.coli K-12 strain
HB101 for amplification.

5 the hpG-CSF amino terminal sequence of Table IV consisted of a set of 24 oligonucleotides each being 23 bases in length and containing three inosine residues. The probe oligonucleotides were manufactured according to the procedure of Caruthers, et al., Genetic
 10 Engineering, 4, 1-18 (1982) and labeled with γ -³²P ATP by kinasing with polynucleotide kinase. The probe oligonucleotides, corresponding to the messenger RNA for residues 23-30 of the sequence of Table IV, are illustrated in Table V.

15

TABLE V

hpG-CSF Probes

20 5' GC IGC ICC $\begin{smallmatrix} A \\ G \end{smallmatrix}$ TC ICC $\begin{smallmatrix} T \\ C \end{smallmatrix}$ TG $\begin{smallmatrix} G \\ A \\ T \end{smallmatrix}$ AT $\begin{smallmatrix} T \\ C \end{smallmatrix}$ TT3'

The assignment of neutrality to I's was based on the published work of Takahashi, et al., Proc. Natl. Acad. Sci. (USA), 82, 1931-1935 (1985) and Ohtsuka, et al., J. Biol. Chem., 260, 2605-2608 (1985). However, inosine may have a destabilizing effect if base paired with a G or T. In Takahashi, et al., inosines may appear to have a neutral effect because they average out as a group to near neutrality (e.g., three having paired favorably with C and two not favorable to pairing with T).

To test the effect of having I's base pair with G's, control experiments were designed using an N-myc gene sequence and clone. The sequences picked from the N-myc gene had the same overall G and C content at

35

included) as the hpG-CSF probes.

Two sets of N-myc test probes were constructed according to the procedure of Caruthers, et al., supra. Set I, as illustrated in Table VI included: 1, a 23 mer with perfect match; 2, in which three third position C's were replaced with I's generating the worst possible case for adding I's; and 3, in which four third position C's were replaced with I's. The second set of test probes was designed to represent a more random distribution of inosine base pairs, that might give an overall neutral base pairing effect. Set II, as illustrated in Table VI, included: 4, containing two I's that will base pair with C's and one with a G; and 5, identical to 4 with the addition of one more I:G base pair.

TABLE VI

N-myc Test Probes

25

1. 5' CAC AAC TAT GCC GCC CCC TCC CC^{3'}

2. 5' CAC AAC TAT GCI GCC CCI TCI CC^{3'}

30

3. 5' CAI AAC TAT GCI GCC CCI TCI CC^{3'}

4. 5' AAC GAG CTG TGI GGC AGI CCI GC^{3'}

5. 5' AAI GAG CTG TGI GGC AGI CCI GC^{3'}

35

5 hybridized as described in Example 4 for the hpG-CSF probes except the period of hybridization was only 6 hours. Filters were washed three times at room temperature then once at 45°C., 10 minutes each. The filters were monitored with a Geiger counter.

10 The filter representing N-myc probe 3 gave a very weak signal relative to the other four probed filters and was not washed any further. After a 10 minute 50°C. wash, the Geiger counter gave the following percent signal with probe one being normalized to 100%:

15 Probe 2, 20%; Probe 3 (45°C.), 2%; Probe 4, 92%; and Probe 5, 75%. After a 55°C. wash, the percentages were: Probe 2, 16%; Probe 4, 100%; and Probe 5, 80%. A final wash at 60°C. yielded the following percentages: Probe 2, 1.6%; Probe 4, 90%; and Probe 5, 70%.

20 Thus, in the presence of three I's, as in probes 2 and 4, up to a 60-fold difference in signal is observed as the theoretical T_m (I's not included in the calculation) is approached [based upon a worst case I base pairing (Probe 2) and a relatively neutral I base

25 pairing case (Probe 4)].

The standardization information gained by the N-myc test hybridizations was utilized in washing and monitoring of the hpG-CSF hybridization as indicated below, to gauge the degree of confidence with which the

30 results of less than stringent washing might be accepted.

Example 4

35 According to the procedure of Hanahan, et al., J. Mol. Biol., 166, 557-580 (1983), bacteria containing

5 colonies which were replica plated to 24 other nitro-
cellulose filters. The replicas were incubated until
distinct colonies appeared. The bacteria on the filters
were lysed on sheets of Whatman 3 MM paper barely satu-
rated with sodium hydroxide (0.5M) for 10 minutes, then
10 blotted with Tris (1M) for 2 minutes, followed by
blotting with Tris (0.5M) containing NaCl (1.5M) for 10
minutes. When the filters were nearly dry, they were
baked for 2 hours at 80°C. in a vacuum oven prior to
nucleic acid hybridization. [Wahl, et al., Proc. Natl.
15 Acad. Sci. (USA), 76, 3683-3687 (1979)]; and Maniatis,
et al., Cell, 81, 163-182 (1976).

The filters were prehybridized for 2 hours at
65°C. in 750 ml of 10X Denhardt's, 0.2% SDS and 6X
SSC. The filters were rinsed in 6X SSC, then placed
20 four in a bag and hybridized for 14 hours in 6X SSC and
10X Denhardt's. There was approximately 15 ml of solu-
tion per bag containing 50×10^6 cpm of ^{32}P -labeled
probe (oligonucleotides).

After hybridization, the filters were washed
25 three times in 6X SSC (1 liter/wash) at room temperature
for 10 minutes each. The filters were then washed two
times at 45°C. for 15 minutes each, once at 50° for 15
minutes and once at 55°C. for 15 minutes using 1 liter
volumes of 6X SSC. The filters were autoradiographed
30 for 2 hours at -70°C. using an intensifying screen and
Kodak XAR-2 film. On this autoradiograph, there were
40-50 positive signals detected including 5 very intense
signals.

The areas containing the strongest five
35 signals and an additional five positives were scraped
from the master plates and replated for a secondary

5 Based on the N-myc probe study of Example 3, the final
wash temperature in the second screening was raised
because the aggregate melting temperature for the 24 23-
mers was 60-68°C., similar to that of the N-myc
probes. Just after the second 55°C. wash, the filters
10 were left damp and an autoradiograph was made. Compari-
son of this autoradiograph with a second autoradiograph
taken for a similar period of time after a final wash at
60°C. showed that only two of the 10 clones being tested
did not suffer a substantial loss in signal in rising
15 from 55-60°C. These two clones were later shown to be
of nearly identical lengths and restriction endoclease
patterns. One clone designated Ppo2, was selected for
sequencing.

Sequencing of the recombinant hpG-CSF cDNA
20 clone, Ppo2, obtained by the above procedure was accom-
plished by the dideoxy method of Sanger, et al., Proc.
Natl. Acad. Sci. (USA) 74, 5463-5467 (1977). The
single-stranded DNA phage M-13 was used as a cloning
vector for supplying single-stranded DNA templates from
25 the double-stranded cDNA clones. The Sanger, et al.,
method revealed the sequence as set forth in Table VII
accompanied by its amino acid translation and a comple-
mentary strand in the polypeptide coding region.

30

35

HindIII

[illegible]

<u>HgiAI</u>										<u>ApaI</u>									
10										20									
Ala	Ser	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu	Lys	Cys	Leu	Glu	Gln	Val	Arg	Lys	Met
GCC	AGC	TCC	CTG	CCC	CAG	CAG	AGC	TTC	CTG	CTC	AAG	TGC	TAA	GAG	CAA	GTG	AGG	AAG	ATG
CGG	TCG	AGG	GAC	GGG	GTC	GTC	TCG	AAG	GAC	GAG	TTC	ACG	AAT	CTC	GTT	CAC	TCC	TTC	TAC

30										40									
Gly	Asp	Gly	Ala	Ala	Leu	Gln	Glu	Lys	Leu	Cys	Ala	Thr	Tyr	Lys	Leu	Cys	His		
GGC	GAT	GGC	GCA	GCG	CTC	CAG	GAG	AAG	CTG	TGT	GCC	ACC	TAC	AAG	CTG	TGC	CAC		
CCG	CTA	CCG	CGT	CGC	GAG	GTC	CTC	TTC	GAC	ACA	CGG	TGG	ATG	TTC	GAC	ACG	GTG		

Glu	Leu	Val	Leu	Leu	Gly	His	Ser	Leu	Gly	Ile	Pro	Trp	Ala	Pro	Leu	Ser
GAG	CTG	GTG	CTG	CTC	GGA	CAC	TCT	CTG	GGC	ATC	CCC	TGG	GCT	CCC	CTG	AGC
CTC	GAC	CAC	GAC	GAG	CCT	GTG	AGA	GAC	CCG	TAG	GGG	ACC	CGA	GGG	GAC	TCC

Ser	Gln	Ala	Leu	Gln	Leu	Ala	Gly	Cys	Leu	Ser	Gln	Leu	His	Ser	Gly	Leu	Phe
AGC	CAG	CCC	CTG	CAG	CTG	GCA	GGC	TGC	TTG	AGC	CAA	CTC	CAT	AGC	GGC	CTT	TTC
TCC	GTC	CGG	GAC	GTC	GAC	CGT	CCG	ACG	AAC	TCG	GTT	GAG	GTA	TCG	CCG	GAA	AAG

90										100										
Gln	Gly	Leu	Leu	Leu	Ala	Gln	Gln	Ala	Leu	Glu	Gly	Ile	Ser	Pro	Glu	Leu	Gly	Pro	Thr	Leu
CAG	GGG	CTC	CTG	CTG	GCC	CAG	CAG	GCC	CTG	GAA	GGG	ATC	TCC	CCC	GAG	TTG	GGT	CCC	ACC	TTG
GTC	CCC	GAG	GAC	GAC	CGG	GTC	GTC	CGG	GAC	CTT	CCC	TAG	AGG	GGG	CTC	AAC	CCA	GGG	TGG	AAC

TABLE VII (cont'd.)

110	Leu Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Thr Ile Trp Gln Gln Met Glu Glu	120	CTG CAG CTG GAC GTC GAC GGC CTG AAA CGG TGG TGG TAG ACC GTC CAG CAG ATG GAA GAA	130	Met Ala Pro Ala Leu Gln Pro Thr Thr Gln Gln Gly Ala Met Pro Ala Phe Phe	140	ATG GCC CCT GCC GCG GAC GTC GGC TGG TGG TGG TGG TGG TGG TGG TGG TGG TGG TGG	150	Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Phe	160	GCG GCC CGT CCT CCC CAG GGC GTC GTC GTC GTC GTC GTC GTC GTC GTC GTC GTC	170	Arg Val Leu Arg His Leu Ala Gln Pro OP	174	CGC GTT CTA CGC CAC CAC CTT GCC CAG CCC TGA GCC AAG CCC TCC CCA TCC CAT GTA		CGC CAA GAT GCG GTG GAA CCG GTC GGC GGC GGC GGC GGC GGC GGC GGC GGC GGC		TCT ATT TAA TAT TTA TGT CTA TTT AAG CCT CAT ATT TAA AGA CAG GGA AGA GCA		AGC CCC AGG CCT CTG TGT CCT TCC CTG CAT TTC TGA GTT TCA TTC TCC TCC TGC CTG		<u>StuI</u>		TGA GAA AAA GCT CCT CTC CTC CCA TCC CCT GGA CTG GGA GGT AGA TAG GTA AAT		TAT TTA TTA CTA TGA CTG CTC CCC AGC CCT GGC TCT GCA ATG GGC ACT GGC ATG		TGT GAG CCC CTG GTC CTG AGG GTC CCC ACC TGG GAC CCT TGA GAG TAT CAG GTC
-----	---	-----	---	-----	---	-----	---	-----	---	-----	---	-----	--	-----	---	--	---	--	---	--	---	--	-------------	--	---	--	---	--	---

TABLE VII (cont'd.)

GTG GGA GAC AAG AAA TCC CTG TTT AAT ATT TAA ACA GCA GTG TTC CCC ATC TGG (

GCA CCC CTC ACT CTG GCC TCA GCC GAC TGC ACA GCG GCC CCT GCA TCC CCT TGG (

GGC CCC TGG ACA AGC AGA GGT GGC CAG AGC TGG GAG GCA TGG CCC TGG GGT CCC /

TTG CTG GGG AAT CTC GTT TTT CTT CTT AAG ACT TTT GGG ACA TGG TTT GAC TCC (

TCA CCG ACG TGT CTC CTG TTT TTC TGG GTG GCC TCG GGA CAC CTG CCC TGC CCC (

GGT CAG GAC TGT GAC TCT TTT TAG GGC CAG GCA GGT GCC TGG ACA TTT GCC TTG (

GGG GAC TGG GGA TGT GGG AGG GAG CAG ACA GGA GGA ATC ATG TCA GGC CTG TGT (

GGA AGC TCC ACT GTC ACC CTC CAC CTC TTC ACC CCC CAC TCA CCA GTG TCC CCT (

TCA CAT TGT AAC TGA ACT TCA GGA TAA TAA AGT GTT TGC CTC CA

StuI

[f150-200 base poly A plus 25-30 bases plasmid DNA preceding a PvuII res

5 determined unambiguously by the Sanger, et al. method
due to the preceding multiple G's. The sequence there-
after reveals a series of 12 codons encoding a portion
of a putative leader sequence for the polypeptide.
Based on correspondence to the amino terminal sequence
10 of natural isolates of hpCSF described in Example 1, the
initial threonine residue of the putative "mature" form
of hpG-CSF is indicated by +1. Mature hpG-CSF is
thereafter revealed to include 174 residues as
indicated. Following the "stop" codon (the OP codon,
15 TGA) are approximately 856 bases of an untranslated 3'
sequence and multiple A's of the poly A "tail". Unique
HgiAi, and ApaI restriction endonuclease recognition
sites, as well as two StuI sites (discussed infra with
respect to construction of procaryotic and eucaryotic
20 expression systems) are also designated in Table VII.
Owing to the lack of asparagine residues in the
polypeptide, there are no apparent sites for N-
glycosylation. The underscored 6 bases near the end of
the 3' untranslated sequence represent a potential
25 polyadenylation site.

It is noteworthy that each of two additional
cDNA clones identified by the hybridization procedures
described above from among a total of 450,000 clones
failed to include DNA encoding the entire leader
30 sequence from the transcription initiation site
onward. Indeed, all three hpG-CSF clones terminated in
the 5' region at exactly the same site, indicating that
secondary structure of the mRNA transcribed severely
hinders cDNA formation beyond this site. As a practical
35

5 not have readily applied to isolation of hpCSF DNA
because such isolation systems ordinarily rely upon the
presence of a full length cDNA transcript in the clones
assayed.

The above sequence is not readily susceptible
10 for securing direct expression of hpG-CSF in a microbial
host. To secure such expression, the hpG-CSF coding
region should be provided with an initial ATG codon and
the sequence should be inserted in a transformation
vector at a site under control of a suitable promoter/-
15 regulator DNA sequence.

Example 5

In this example, cDNA encoding hpG-CSF as
20 isolated in the previous example was used to screen a
genomic clone. A phage lambda human fetal liver genomic
library [prepared according to the procedure of Lawn, et
al. Cell, 15, 1157-1174 (1978) and obtained from T.
Maniatis] was screened using a nick translated probe
25 consisting of two hpG-CSF cDNA fragments isolated by
digestion with HgiAI and StuI (HgiAI to StuI, 649 b.p.;
StuI to StuI, 639 b.p.). A total of approximately
500,000 phage were plated on 12 (15 cm) petri dishes and
plaque lifted and hybridized to probe using the
30 Benton/Davison procedure [Benton, et al., Science, 196,
180 (1977)]. A total of 12 positive clones were
observed. Three clones (1-3) yielding the strongest
signals upon autoradiography in a secondary screening
were grown in 1 liter cultures and mapped by restriction
35 enzyme digestion and Southern blotting using a radio-
labeled 24-mer oligonucleotide (kinased with γ -³²P ATP)

tion. DNA from clone 2 was digested with XI to release
an 8500 bp hpG-CSF containing fragment which was subse-
quently subcloned into pBR322 and further mapped by
restriction endonuclease digests, Southern Blotting, M13
subcloning and sequencing. The sequence obtained is as
10 set out in Table VIII.

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TABLE VIII

GGGACAGGCTTGAGAAATCCCAAAGGAGAGGGGCAAGGACACTGCCCCCGCAAGTCTGCCAGAGCAGAGAGGGAGACCCCGACTCAGCTGG

CACAGGCTCGTGCCCGCTTCCAGGCGTCTATCAGCGGCTCAGCCCTTGTTCAGCTGTTCTGTTCAAACACTCTGGGGCCATTTCAGGCCCTGGG

GGGAGGAAGGAGTTTGAGGGGGGCAAGGCGACGTCAAAGGAGGATCCACAAATTTTCACAAAACCTTTGCGAAACAGCTTTTGTGT

CCTGCATTGTCTTGGACACCAAAATTTGCATAAATCCTGGGAAGTTATTACTAAGCCCTTAGTCGTGGCCCCCAGGTAATTTCCTCCAGGCCCT

-30

MetAlaGlyProAlaThrG

TATGTATAAAGGGCCCCCTAGAGCTGGGGCCCCAAACAGCCCCGGAGCCCTGCAGCCCCCAGCCCCCATGGCTGGACCTGCCACCCC

-20 -18

etLysLeuMetA

TGAAGCTGATGGGTGAGTGTCTTGGCCCCAGGATGGGAGAGCCCCCTGCCATGGGAGGGAGGCTGGTGTACAGAGGGGCTGGGGAT

-

1aL

GGGAATGGGGATTAAAGGCACCCAGTGTCCCCGAGAGGGCCCTCAGGTGGTAGGGAACAGCATGTCTCCTGAGCCCCGCTCTGTCCCCCAGCCCC

TABLE VIII (cont'd.)

-10	-1 +1	10
euLeuTrpHisSerAlaLeuTrpThrValGlnGluAlaThrProLeuGlyProAlaSerSerLeuProGlnSerPheLeuLeuLysCysLeu		
TGCTGTGGCACAGTGCACACTCTGGACAGTGCAGGAAGCCACCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCTGCTCAAGTGTCTTAA		
1ArgLysIleGlnGlyAspGlyAlaAlaLeuGlnGluLysLeu	30	35
GAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGGTGAGTGAGGTGGGTGAGAGGGCTGTGTGGAGGGAAGCCCCGGTGGGGAGAG		
GATGGAAC TGCAGGGCCAAACATCCCTCTGGAAGGGACATGGGAGAATATTAGGAGCAGTGGAGCTGGGGAAGGCTGGGAAGGGACTTGGGGAA		
TGGTGGGGACAGTGTCTCGGGAGGGCTGGGATGGGAGTGGAGGCATCACATTTCAGGAGAAAGGGCAAGGGCCCCCTGTGAGATCAGAGAA		
CAGGGCAGAGAGGAACTGAACAGCCTGGCAGGACATGGAGGGAGGGGAAGACCAGAGAGTCTGGGGAGGACCCGGGAAGGAGCGGCGACCC		
	36	40
		50
	CysAlaThrTyrLysLeuCysHisProGluGluLeuValLeuLeuGlyHisSerLeuGlyI	
GAGTCTCACTCAGCATCCTTCCATCCCCAGTGTGCCACCTACAAAGCTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCA		

TABLE VIII (cont'd.)

60 70 71

laProLeuSerSerCysProSerGlnAlaLeuGlnLeu
CTCCCCTGAGCAGCTGCCCCCAGCCAGGCCCTGCAGCTGGTGAGTGTCAAGAAAGGCTAATGAGGAGGGGGAAGGAGAGGAGGAACA

72

AlaGlyCysI

CTCCCCCATGTCTCCAGGTTCCAAGCTGGGGGCCCTGACGTATCTCAGGCAGCACCCCTTAACCTCTCCGCTCTGTCTCACAGGCAGGCTGCT

80

90

100

LeuHisSerGlyLeuPheLeuTyrGlnGlyLeuLeuGlnAlaLeuGluGlyIleSerProGluLeuGlyProThrLeuAspThrLeuGlnLeu
CTCCATAGCGGGCCTTTTCCTCTACCAAGGGGCTCCTGCAGGCCCTGGAAGGGATCTCCCCCGAGTTGGGTCCCACCTTGGACACACTGCAGCT

120

laAspPheAlaThrThrIleTrpGlnGln

CCGACTTTGCCACCACCATCTGGCAGCAGGTGAGCCTTGTGGGCAGGGTGGCCAAAGGTCGTGTGGCATTTCTGGGCACCACAGCCGGGGCC

GCCCCTGTCCATGTGTCAAGCCCCCAGCATTTCCCTCATTTGTATAAAGCCCCACTCAGAAGGGCCCCAACCACTGATCACAGCTTTCCCCCAC

TABLE VIII (cont'd.)

	130	140	150
luLeuGlyMetAlaProAlaLeuGlnProThrGlnGlyAlaMetProAlaPheAlaSerAlaPheGlnArgAlaGlyValLeuVal			
AACTGGGAATGGCCCCCTGCCCTGCAGCCCCACCCAGGGTGCCATGCCGGCCCTTCGCCTCTGCTTCCAGCGCCGGGCAGGAGGGTCCCTGGT			
	160	170	174
sLeuGlnSerPheLeuGluValSerTyrArgValLeuArgHisLeuAlaGlnProOP			
TCTGCAGAGCTTCCTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCCCAGCCCTTGAGCCCAAGCCCCCTCCCATGTATTATCTCTA			
TTATGTCTATTTAAGCCTCATATTTAAAGACAGGGAAGAGCAGAACGGAGCCCCAGGCCCTCTGTGTCTTCCCTGCATTTCTGAGTTTCAT			
TGTAGCAGTGAGAAAAGCTCCTGTCTCTCCCATCCCCCTGGACTGGGAGGTAGATAGGTAAATACCAAGTATTATTACTATGACTGCTCCCC			
CTCTGCAATGGGCACCTGGGATGAGCCGCTGTGAGCCCCCTGGTCTCTGAGGGTCCCCCACCTGGGACCCCTTGAGAGTATCAGGTCTCCACACGTGG			
AAATCCCTGTTTAATATTAAACAGCAGTGTTCCTCCCATCTGTGGTCTTGCACCCCTCACTCTGGCCTCAGCCGACTGCACAGCGGCCCTGG			
GGCTGTGAGGCCCTGGACAAGCAGAGGTGGGCCAGAGCTGGGAGGCATGGCCCTGGGGTCCCCACGAATTGTCTGGGGAATCTCTCGTTTTTCT			
TTTTGGGACATGGTTTGACTCCCGAACATCACCGACGTGTCTCTGTTTTCTGTGGTGGCCTCGGGACACCTGCCCTGCCCCACGAGGGT			

TABLE VIII (cont'd.)

GACTCTTTTAGGGCCAGGCAGGTGCCCTGGACATTTGCCCTTGCTGGATGGGCACTGGGGATGTGGGAGGGAGCAGACAGGAGGAATCATGT
GTGTGAAAGGAAGCTCCACTGTCAACCTCCACCTCTTCACCCCCCACTCACCAAGTGTCCCCCTCCACTGTCACATTTGTAACTGAACCTTCAGG
TGTTTGCCCTCCAGTCAAGTCCTTCTCCTTCTTGAGTCCAGCTGGTGCTGGGCCAGGGGCTGGGGAGGTGAGGGTGGGAGAGGCCCA
CGGGAGGAGGTCTGGGAGGAGGTCCAGGGAGGAGGAAAGTTCTCAAGTTCGTCTGACATTCATTCGGTTAGCACATATTTATCTG
CTGTGCAGACGCTGGGCTAAGTGCTGGGGACACAGCAGGGAACAAGGCAGACATGGAATCTGCACTCGAG

depict the sequencing strategy used to obtain the genomic sequence. The boxed regions are those found in the cDNA clone with the dashed open ended box representing sequence not present in the cDNA clone, but identified by probing mRNA blots. The identification of coding sequences proposed for exon one was carried out by Northern blot analysis. A 24 mer oligonucleotide probe, 5'CAGCAGCTGCAGGGCCATCAGCTT³', spanning the predicted splice junctures for exons 1 and 2 was hybridized to hpG-CSF mRNA in a Northern blot format. The resulting blot shows an mRNA the same size (~1650 bp) as that seen with an exon 2 oligonucleotide probe. This data combined with the ability to direct expression of hpG-CSF from the pSVGM-Ppol vector (Example 9) using the Met initiation codon depicted in Table VIII, defines the coding sequences contained in exon 1. Exons 2-5 are defined by the coding sequences obtained in the cDNA clone (Ppo2) of the hpG-CSF gene (Table VII).

25

Example 6

This example relates to preparation of a manufactured gene encoding hpG-CSF and including E.coli preference codons.

30

Briefly stated, the protocol employed was generally as set out in the disclosure of co-owned Alton, et al., PCT Publication No. WO83/04053, which is incorporated by reference herein. The genes were designed for initial assembly of component oligonucleotides into multiple duplexes which, in turn, were assembled into three discrete sections. These sections

35

the construction of sections I, II and III is illustrated in Table IX though XIV. In the construction of Section I, as illustrated in Tables IX and X, oligonucleotides 1-14 were assembled into 7 duplexes (1 and 8); 2 and 9; 3 and 10; 4 and 11; 5 and 12; 6 and 13; and 10 7 and 14). The 7 duplexes were then ligated to form Section I as shown in Table X. It may also be noted in Table X that Section I includes an upstream XbaI sticky end and a downstream BamHI sticky end useful for ligation to amplification and expression vectors and for 15 ligation to Section II.

20

25

30

35

5	CTAGAAAAAACCAAGGAGGTAATAAA	1
	TAATGACTCCATTAGGTCCTGCTTCTTCT	2
	CTGCCGCAAAGCTTTCTGCTGAAATGTCTGG	3
	AACAGGTTTCGTAAAATCCAGGGTGACGGT	4
	GCTGCACTGCAAGAAAACTGTGCGCTA	5
10	CTTACAAACTGTGCCATCCGGAAGAGC	6
	TGGTACTGCTGGGTCATTCTCTTGG	7
	CATTATTTATTACCTCCTTGGTTTTTT	8
	GCAGAGAAGAAGCAGGACCTAATGGAGT	9
	TGTTCCAGACATTTTCAGCAGAAAGCTTTGCG	10
15	CAGCACCGTCACCCTGGATTTTACGAACC	11
	TAAGTAGCGCACAGTTTTTCTTGCAGTG	12
	ACCAGCTCTTCCGGATGGCACAGTTTG	13
	GATCCCAAGAGAATGACCCAGCAGT	14

20

25

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TABLE X

EChpG-CSFDNA SECTION I

10	1	20	30	40	2	50	60
CTAGAAAA	ACCAAGGAGG	TAAATAATAA	TGACTCCATT	AGGTCCTGCT			TCTTCTCTGC
TTTTT	TGGTTCCTCC	ATTATTTATT	ACTGAGGTAA	TCCAGGACGA			AGAAAGAGACG

8

XbaI

70	3	80	90	100	4	110	120
CGCAAAGCTT	TCTGCTGAAA	TGTCTGCAAC	AGGTCGTAA	AATCAGGGT			GACGGTGCTG
GCGTTTCGAA	AGACGACTTT	ACAGACCTTG	TCCAAGCATT	TTAGGTCCCA			CTGCCACGAC

10

11

130	5	140	150	160	6	170	180
CACTGCAAGA	AAAACGTGC	GCTACTTACA	AACTGTGCCA	TCCGGAAGAG			CTGGTACTGC
GTGACGTTCT	TTTGTGACACG	CGATGAATGT	TTGACACGGT	AGGCCTTCTG			GACCATGACG

12

13

7	190	100
TGGGTCATT	CTTTGG	
ACCCAGTAAG	AGAACCCCTAG	

14

BamHI

5 and 30). These 8 duplexes were then ligated to form
 Section II, as shown in Table XII. As further shown in
 Table XII, Section II has an upstream BamHI sticky end
 and a downstream EcoRI sticky end useful for ligation to
 an amplification vector and for ligation to Section I.
 10 Near its downstream end, Section II also includes a
 downstream SstI site useful in the eventual ligation
 Sections II and III.

TABLE XI

15

EChpG-CSFDNA SECTION II

	GATCCCGTGGGCTCCGCTGTCTTCT	15
20	TGTCCATCTCAAGCTCTTCAGCTGGC	16
	TGGTTGTCTGTCTCAACTGCATTCTGGT	17
	CTGTTCTGTATCAGGGTCTTCTG	18
	CAAGCTCTGGAAGGTATCTCTCCGGA	19
	ACTGGGTCCGACTCTGGACACTCTGCA	20
25	GCTAGATGTAGCTGACTTTGCTACTACT	21
	ATTTGGCAACAGATGGAAGAGCTCAAAG	22
	GACAAGAAGACAGCGGAGCCCACGG	23
	ACCAGCCAGCTGAAGAGCTTGAGATG	24
	ACAGACCAGAATGCAGTTGAGACAGACA	25
30	CTTGCAGAAGACCCTGATACAGGA	26
	CAGTTCCGGAGAGATACCTTCCAGAG	27
	TAGCTGCAGAGTGTCCAGAGTCGGACC	28
	AAATAGTAGTAGCAAAGTCAGCTACATC	29
	AATTCTTTGAGCTCTTCCATCTGTTGCC	30
35		

TABLE XII

EChpG-CSFDNA SECTION II

10	15	20	30	40	50	60
GATCCCGTG	GGCTCCGCTG	TCTTCTTGTC	CATCTCAAGC	TCTTCAGCTG	GCTGGTTGTC	
GGCAC	CCGAGGCGAC	AGAAGAACAG	GTAGAGTTTCG	AGAAGTCGAC	CGACCAACAG	
	<u>23</u>			<u>24</u>		
<u>BamHI</u>						
70	17	80	90	100	110	120
TGTCTCAACT	GCAATCTGGT	CTGTTCCTGT	ATCAGGGTCT	TCTGCAAGCT	CTGGAAGGTA	
ACAGAGTTGA	CGTAAGACCA	GACAAGGACA	TAGTCCCAGA	AGACGTTCTGA	GACCTTCCAT	
	<u>25</u>		<u>26</u>		<u>27</u>	
130	140	20	150	160	170	180
TCTCTCCGGA	ACTGGGTCCG	ACTCTGGACA	CTCTGCAGCT	AGATGTAGCT	GACTTTGCTA	
AGAGAGGCCT	TGACCCAGGC	TGAGACCTGT	GAGACGTCTGA	TCTACATCGA	CTGAAACGAT	
		<u>28</u>			<u>29</u>	
190	200	22	210			
CTACTATTG	GCAACAGATG	GAGAGCTCA	AAG			
GATGATAAAC	CGTTGTCTAC	CTTCTCGAGT	TTCTTAA			
		<u>30</u>				
		<u>SstI</u>		<u>ECORI</u>		

5 and 42). The 6 duplexes were then ligated to form Section III as depicted in Table XIV. As also shown in Table XIV, Section III includes an upstream BamHI sticky end and a downstream EcoRI sticky end useful for ligating into an amplification vector, and at least in the
 10 case of the EcoRI end, into an expression vector. In addition, Section II has an upstream SstI site useful in the eventual ligation of Sections II and III.

15

TABLE XIII

EChpG-CSFDNA SECTION III

20	GATCCAAAGAGCTCGGTATGGCACCAG	31
	CTCTGCAACCGACTCAAGGTGCTATGCCG	32
	GCATTCGCTTCTGCATTCCAGCGTCGTGC	33
	AGGAGGTGTACTGGTTGCTTCTCATCTG	34
	CAATCTTTCCTGGAAGTATCTTACCGTGT	35
25	TCTGCGTCATCTGGCTCAGCCGTAATAG	36
	AGAGCTGGTGCCATACCGAGCTCTTTG	37
	ATGCCGGCATAGCACCTTGAGTCGGTTGC	38
	TCCTGCACGACGCTGGAATGCAGAAGCGA	39
	ATTGCAGATGAGAAGCAACCAGTACACC	40
30	CAGAACACGGTAAGATACTTCCAGGAAAG	41
	AATTCTATTACGGCTGAGCCAGATGACG	42

35

TABLE XIV

EChpG-CSFDNA SECTION III

10	31	20	30	40	32	50
GATCCAAAG	AGCTCGGTAT	GGCACCAGCT	CTGCAACCGA	CTCAAGGTGC	TATGCCG	
GTTTC	TCGAGCCATA	CCGTGGTCGA	GACGTTGGCT	GAGTCCACG	ATACGGC	
	<u>37</u>				<u>38</u>	
<u>BamHI</u>	<u>SstI</u>					
70	33	80	90	100	34	110
TTGGCTTCTG	CATTCCAGCG	TCGTGCAGGA	GGTGACTGG	TTGCTTCTCA	TCTGCAA	
AAGCGAAGAC	GTAAGGTCGC	AGCACGTCCT	CCACATGACC	AACGAAGAGT	AGACGTT	
	<u>39</u>				<u>40</u>	
35	130	140	150	36	160	170
TTCCCTGGAAG	TATCTTACCG	TGTTCTGCGT	CATCTGGCTC	AGCCGTAATA	G	
AAGGACCTTC	ATAGAATGGC	ACAAGACGCA	GTAGACCGAG	TCGGCATTTAT	CTTAA	
	<u>41</u>			<u>42</u>		

ECOR

stage, Sections I and II have been joined in proper orientation. Next, another M13mp11 vector is opened by BamHI to EcoRI digestion and then ligated with the BamHI to EcoRI fragment formed by Section III.

10 The vector containing Sections I and II is digested with XbaI and SstI. Likewise, the vector containing Section III is digested with SstI and EcoRI. Both of the smaller of the two fragments resulting from each digestion are ligated into a a plasmid pCFM1156
15 which is previously opened with XbaI and EcoRI. The product of this reaction is an expression plasmid containing a continuous DNA sequence, as shown in Table XV, encoding the entire hpG-CSF polypeptide with an amino terminal methionine codon (ATG) for E.coli translation
20 initiation.

25

30

35

TABLE XV

[illegible]

TABLE XV (cont'd.)

150	160
Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg	
GGT GTA CTG GTT GCT TCT TCT CAT CTG CAA TCT TTC CTG GAA GTA TCT TAC CG	
170	174
His Leu Ala Gln Pro	
CAT CTG GCT CAG CCG TAA TAG AAT T	

5 European Patent Application NO. 138,490. pCFM836 is
first cut with NdeI and then blunt-ended with Poll such
that both existing NdeI sites are destroyed. Next, the
vector is digested with ClaI and SacII to remove an
existing polylinker before ligation to a substitute
10 polylinker as illustrated in Table XVI. This substitute
polylinker may be constructed according to the procedure
of Alton, et al., supra. Control of expression in the
expression pCFM1156 plasmid is by means of a lambda P_L
promoter, which itself may be under the control of a
15 C_I857 repressor gene (such as is provided in E.coli
strain K12ΔHtrp).

20

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30

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TABLE XVI

1 ATCGATTTCATTCTAGAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC
 TAGCTAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGCCATG

1 Clal, 12 XbaI, 29 NdeI, 35 HincII, HpaI, 39 MluI, 47 E
 53 HgiCI KpnI, 57 NcoI StyI,

61 GGAAGCTTACTCGAGGATCCGGGATAAATAAGTAACGATCC
 CCTTCGAATGAGCTCCTAGGGCCCTATTATTTCATTGCTAGG

63 HindIII, 70 AvaI XhoI, 75 BamHI Xho2, 79 Sac2,

5 ing [Met ⁻] hpCSF. The sequence employed was partially
 synthetic and partially cDNA-derived. The synthetic
 sequence employed E. coli preference codons.
 Plasmid Ppo2, containing the hpG-CSF gene
 shown in Table VII, was digested with HgiAI and StuI
 10 providing an approximately 645 base pair fragment
 including the gene for mature hpCSF (as shown in Table
 VII) with seven of the leader sequence residue codons at
 the 5' end and about 100 base pairs of the 3' non-coding
 region. HgiAI digestion leaves a 5', 4-base sticky end
 15 identical to that of PstI, and StuI leaves a blunt
 end. This allows for ready insertion of the fragment
 into M13 mp8 (Rf) cut with PstI and with the blunt-end-
 forming restriction enzyme, HincII. Upon amplification
 in M13, the hpG-CSF DNA was excised by digestion with
 20 ApaI and BamHI which cut, respectively, at the ApaI site
 spanning the codons for residues +3 to +5 of hpCSF and
 at a BamHI site "downstream" of the HincII site in the
 M13 mp8 restriction polylinker. In order to allow for
E. coli expression of the hpG-CSF polypeptide, a
 25 synthetic fragment was prepared as set out in Table XVII
 below.

TABLE XVII

30 5' - C TAG AAA AAA CCA AGG AGG TAA TAA ATA
 3' - TTT TTT GGT TCC TCC ATT ATT TAT
XbaI

 -1 +1
 Met Thr Pro Leu
 ATG ACA CCT CTG GGC C - 5'
 35 TAC TGT GGA GAC -3'
ApaI

5 specifying codons deleted upon ApaI digestion of the M13 DNA described above and employing codons preferentially expressed in E. coli), a translation initiating ATG, a sequence of 24 base pairs providing a ribosome binding site, and an XbaI sticky end.

10 The expression vector employed for E. coli expression was that described as pCFM536 in European Patent Application No. 136,490, by Morris, published April 10, 1985. (See also, A.T.C.C. 39934, E. coli JM103 harboring pCFM536). Briefly, plasmid pCFM536 was
15 digested with XbaI and BamHI. The hpG-CSF fragment (ApaI/BamHI) and linker (XbaI/ApaI) described above were then ligated thereinto to form a plasmid designated p536Ppo2.

Plasmid p536Ppo2 was transformed into a phage
20 resistant variant of the E. coli AM7 strain which has previously been transformed with plasmid pMW1 (A.T.C.C. No. 39933) harboring a CI^{857} gene. Transformation was verified on the basis of the antibiotic (amp) resistance marker gene carried on the pCFM536 progenitor plasmid.
25 Cultures of cells in LB broth (ampicillin 50 μ g 1ml) were maintained at 28°C. and upon growth of cells in culture to A600 = 0.5, hpCSF expression was induced by raising the culture temperature to 42°C. for 3 hours. The final O.D. of the culture was A600 = 1.2.

30 The level of expression of hpG-CSF by the transformed cells was estimated on a SDS-poly acrylamide gel stained with coomassie blue dye to be 3-5% of total cellular protein.

Cells were harvested by centrifugation at
35 3500 g for 10 minutes in a JS-4.2 rotor. Cells at 25% (w/v) in water were broken by passing 3 times through a

5 acid, 50 mM Tris, pH 8.7. The solubilized pellet
material was centrifuged at 15,000 g for 10 minutes and
to the supernatant CuSO_4 was added to 20 mM. After 1
hour, this sample was loaded onto a C4 HPLC column for
purification according to the procedures of example
10 1 (B) with adjustments made for volume and concentra-
tion.

A second purification procedure was developed
to yield larger quantities of hpG-CSF formulated in a
nonorganic-containing buffer. This material is suitable
15 for in vivo studies. One hundred and fifty grams of
cell paste was resuspended in about 600 ml of 1 mM DTT
and passed 4 times through a Manton Gualin Homogenizer
at about 7000 PSI. The broken cell suspension was
centrifuged at 10,000 g for 30 minutes and the pellet
20 was resuspended in 400 ml of 1% deoxycholate (DOC), 5 mM
EDTA, 5 mM DTT, and 50 mM Tris, pH 9. This suspension
was mixed at room temperature for 30 minutes and
centrifuged at 10,000 g for 30 minutes. The pellet was
resuspended in about 400 ml of water and centrifuged at
25 10,000 g for 30 minutes. The pellet was solubilized in
100 ml of 2% Sarkosyl and 50 mM at pH 8. CuSO_4 was
added to 20 μM and the mixture was stirred 16 hours at
room temperature, and then centrifuged at 20,000 g for
30 minutes. To the supernatant was added 300 ml
30 acetone. This mixture was put on ice for 20 minutes and
then centrifuged at 5000 g for 30 minutes. The pellet
was dissolved in 250 ml of 6 M guanidine and 40 mM
sodium acetate at pH 4, and put over a 1,200 ml G-25
column equilibrated and run in 20 mM sodium acetate at
35 pH 5.4. The hpG-CSF peak (about 400 ml) was pooled and
put on a 15 ml CM-cellulose column equilibrated in 20 mM

5 with 37 mM sodium chloride. 150 ml of this eluent was
concentrated to 10 ml and applied to a 300 ml G-75
column equilibrated and run in 20 mM sodium acetate and
100 mM sodium chloride at pH 5.4. The peak fractions
10 comprising 35 ml were pooled and filter sterilized. The
final concentration of hpG-CSF was 1.5 mg/ml, is greater
than 95% pure as determined by analysis on a gel, and
contained less than 0.5 ng of pyrogen per 0.5 mg of
hpG-CSF. The pyrogen level was determined using a
Limulus Amebocyte Lysate (LAL) test kit (M. A.
15 Bioproducts, Walkersville, Maryland).

Example 8

This example relates to the use of recombinant
20 methods to generate analogs of hpG-CSF wherein cysteine
residues present at positions 17, 36, 42, 64 and 74 were
individually replaced by a suitable amino acid resi-
due.

Site directed mutagenesis procedures according
25 to Souza, et al., published PCT Application No.
WO85/00817, published February 28, 1985, were carried
out on [Met⁻¹] encoding DNA of plasmid p536Ppo2, des-
cribed infra, using synthetic oligonucleotides ranging
in size from 20 to 23 bases as set out in Table XVIII
30 below. Oligonucleotide No. 1 allowed for formation of a
gene encoding [Ser¹⁷]hpG-CSF; oligonucleotide No. 2
allowed for formation of [Ser³⁶]hpG-CSF, and so on.

1. 5'-CTG CTC AAG TCC TTA GAG CAA GT-3'
 2. 5'-GAG AAG CTG TCT GCC ACC TACA-3'
 3. 5'-TAC AAG CTG TCC CAC CCC GAG-3'
 4. 5'-TGA GCA GCT CCC CCA GCC AG-3'
 5. 5'-CTG GCA GGC TCC TTG AGC CAA-3'

10

The Cys to Ser site directed mutagenesis restrictions were carried out using M13 mp10 containing an XbaI-BamHI hpG-CSF fragment isolated from p536Ppo2 as a template. DNA from each M13mp10 clone containing a Cys-Ser substitution was treated with XbaI and BamHI. The resulting fragment was cloned into expression vector pCFM746 and expression products were isolated as in Example 7.

20 The plasmid pCFM746 may be constructed by cleaving a plasmid pCFM736 (the construction of which from deposited and publically available materials is described in Morris, published PCT Application No. WO85/00829, published February 28, 1985) with ClaI and BamHI to remove an existing polylinker and by substituting the following polylinker.

TABLE XIX

30 ClaI
 5' CGATTTGATTCTAGAATTCGTTAACGGTACCATGGAA
 3' TAAACTAAGATCTTAAGCAATTGCCATGGTACCTT

 GCTTACTCGAGGATCCGCGGATAAATAAGTAAC^{3'}
 35 CGAATGAGCTCCTAGGCGCCTATTTATTCATTGCTAG^{5'}
Sau3a

5 DTT and passed 3 times through a French Pressure Cell at
10,000 psi. The broken cell suspension was centrifuged
at 1,000 g for 30 minutes. The pellet was resuspended
in 1% DOC, 5 mM EDTA, 5 mM DTT, 50 mM Tris, pH 9 and
allowed to mix 30 minutes at room temperature. The
10 mixture was centrifuged at 10,000 g for 30 minutes,
resuspended in 40 ml H₂O, and recentrifuged as 10,000 g
for 30 minutes. The pellet was dissolved in 10 ml of 2%
Sarkosyl, 50 mM DTT, 50 mM Tris, pH 8. After mixing for
1 hour, the mixture was clarified by centrifugation at
15 20,000 g for 30 minutes, and then applied to a 300 ml
G-75 column equilibrated and run in 1% Sarkosyl, 50 mM
Tris, pH 8. Fractions containing the analog were pooled
and allowed to air oxidize by standing with exposure to
air for at least one day. Final concentrations ranged
20 from 0.5 - 5 mg/ml.

Example 9

In this example, a mammalian cell expression
25 system was devised to ascertain whether an active poly-
peptide product of hpG-CSF DNA could be expressed in and
secreted by mammalian cells (COS-1, A.T.C.C. CRL-
1650). This system was designed to provide for secre-
tion of a polypeptide analog of hpGCSF via expression
30 and secretory processing of a partially synthetic, par-
tially cDNA-derived construction encoding [Ala¹] hpG-CSF
preceded by a leader polypeptide having the sequence of
residues attributed to human GM-CSF in Wong, et al.,
Science, 228, 810-815 (1985) and Lee, et al., Proc.
35 Natl. Acad. Sci. (USA), 82, 4360-4364 (1985).

5 human GM-CSF. There follow codons specifying an alanine
residue, a proline residue and a leucine residue. The
proline and leucine residues duplicate the amino acids
present at positions +2 and +3 of hpG-CSF, while the
alanine residue is duplicative of the initial amino
10 terminal (+1) residue of GM-CSF rather than hpG-CSF.
Replacement of threonine by alanine was designed to be
facilitative of proper host cell "processing off" of the
GM-CSF leader by cellular mechanisms ordinarily involved
in GM-CSF secretory processing.

15 Plasmid pSVDM-19 was digested with KpnI and
the site was blunt ended with Klenow enzyme. Thereafter
the DNA was cut with HindIII. The resulting large frag-
ment was combined and ligated with the HindIII/PvuII
fragment shown in Table VII (isolated from plasmid Ppo2
20 as the second largest fragment resulting from HindIII
digestion and partial digestion with PvuII) to form
plasmid pSV-Ppol. The manufactured GM-CSF leader
sequence fragment of Table VIII was then ligated into
pSV-Ppol (following its cleavage with HindIII and ApaI)
25 to yield plasmid pSVGM-Ppol.

Calcium phosphate precipitates (1-5 μ g) of
plasmid pSVGM-Ppol DNA was transformed into duplicate 60
mm plates of COS-1 cells essentially as described in
Wigler, et al., Cell, 14, 725-731 (1978). As a control,
30 plasmid pSVDM-19 was also transformed into COS-1
cells. Tissue culture supernatants were harvested 5
days post-transfection and assayed for hpG-CSF acti-
vity. Yields of [Ala¹]hpG-CSF from the culture super-
natant were on the order of 1 to 2.5 μ g/ml.

35 Following successful expression of the
[Ala¹]hpG-CSF product encoded plasmid pSVGM-Ppol in

5 at that position. Briefly, an oligonucleotide was synthesized (^{5'}CAGCATCTCTACACCTCTGGG) for site-directed mutagenesis (SDM). The HindIII to BamHI hpG-CSF fragment in pSVGM-Ppol was ligated into M13mp10 for the SDM. The newly synthesized hpG-CSF gene containing a
 10 Thr codon in position one was isolated by cleavage with HindIII and EcoRI. The fragment was then cloned into pSVDM-19 prepared by cleavage with the same two restriction endonucleases. The resulting vector pSVGM-Ppo(Thr) was transformed into COS cells and the yields of hpG-CSF
 15 measured in the culture supernates ranged from 1 to 5 µg/ml.

Finally, the genomic sequence whose isolation is described in Example 5 was employed to form an expression vector for mammalian cell expression of
 20 hpG-CSF. More specifically, pSVDM-19 was digested with KpnI and HindIII and the large fragment used in a four-way ligation with a synthetic linker with HindIII and NcoI sticky ends, as shown in Table XXI. An NcoI-BamHI fragment containing exon 1 isolated from pBR322 (8500
 25 hpG-CSF), a genomic subclone, and a BamHI-KpnI fragment containing exons 2-5 isolated from the plasmid pBR322 (8500 hpG-CSF genomic subclone). The resulting mammalian expression vector, pSV/ghG-CSF produced 1 to 2.5 µg/ml of hpG-CSF from transformed COS cells.

30

TABLE XXI

HindIII
 5' AGCTTCCAACAC
 35 AGGTTGTGGTAC^{5'}
NcoI

1. Molecular Weight

Recombinant hpG-CSF products of E. coli expression as in Example 7 had an apparent molecular weight of 18.8 kD when determined in reducing SDS-PAGE (as would be predicted from the deduced amino acid analysis of Table VII., whereas natural isolates purified as described in Example 1 had an apparent molecular weight of 19.6 kD. The presence of N-glycans associated with the natural isolates could effectively be ruled out on the basis of the lack of asparagine residues in the primary sequence of hpG-CSF in Table VII and therefore a procedure was devised to determine if O-glycans were responsible for molecular weight differences between natural isolates and the non-glycosylated recombinant products. Approximately 5 µg of the natural isolate material was treated with neuraminidase (Calbiochem, LaJolla, California), a 0.5 µg sample was removed, and the remaining material was incubated with 4 mU O-Glycanase (endo-x-n-acetylgalactoseaminidase, Genzyme, Boston, Massachusetts) at 37°C. Aliquots were removed after 1/2, 2 and 4 hours of incubation. These samples were subjected to SDS-PAGE side by side with the E. coli derived recombinant material. After neuraminidase treatment, the apparent molecular weight of the isolate shifted from 19.6 kD to 19.2 kD, suggestive of removal of a sialic acid residue. After 2 hours of treatment with O-glycanase, the molecular weight shifted to 18.8 kD -- identical to the apparent molecular weight of the E. coli derived material. The sensitivity of the carbohydrate structure to neuraminidase and O-glycanase

2. ³H-Thymidine Uptake

Proliferation induction of human bone marrow cells was assayed on the basis of increased incorporation of ³H-thymidine. Human bone marrow from healthy donors was subjected to a density cut with Ficoll-Hypaque (1.077g/ml, Pharmacia) and low density cells were suspended in Iscove's medium (GIBCO) containing 10% fetal bovine serum and glutamine pen-strep. Subsequently, 2x10⁴ human bone marrow cells were incubated with either control medium or the recombinant E. coli material of Example 7 in 96 flat bottom well plates at 37°C. in 5% CO₂ in air for 2 days. The samples were assayed in duplicate and the concentration varied over a 10,000 fold range. Cultures were then pulsed for 4 hours with 0.5 μ Ci/well of ³H-Thymidine (New England Nuclear, Boston, Massachusetts). ³H-Thymidine uptake was measured as described in Ventua, et al., Blood, 61, 781 (1983). In this assay human hpG-CSF isolates can induce ³H-Thymidine incorporation into human bone marrow cells at levels approximately 4-10 times higher than control supernatants. The E. coli-derived hpG-CSF material of Example 6 had similar properties.

A second human bone marrow cell proliferation study was carried out using culture medium of transfected COS-1 cells as prepared in Example 9 and yielded similar results, indicating that encoded polypeptide products were indeed secreted into culture medium as active materials.

5 monocytic leukemic cell line WEHI-3B D⁺ was assayed in
semi-solid agar medium as described in Metcalf, Int. J.
Cancer, 25, 225 (1980). The recombinant hpG-CSF product
and media controls were incubated with -60 WEHI-3B D⁺
cells/well at 37°C. in 5% CO₂ in air for 7 days. The
10 samples were incubated in 24 flat bottom well plates and
the concentration varied over a 2000-fold range.
Colonies were classified as undifferentiated, partially
differentiated or wholly differentiated and colony cell
counts were counted microscopically. The E. coli recom-
15 binant material was found to induce differentiation.

4. CFU-GM, BFU-E and CFU-GEMM Assays

Natural isolates of pluripotent human G-CSF
(hpG-CSF) and the recombinant pluripotent human G-CSF
20 (rhpG-CSF) were found to cause human bone marrow cells
to proliferate and differentiate. These activities were
measured in CFU-GM [Broxmeyer, et al., Exp.Hematol., 5,
87, (1971)] BFU-E and CFU-GEMM assays [Lu, et al.,
Blood, 61, 250 (1983)] using low density, non-adherent
25 bone marrow cells from healthy human volunteers. A
comparison of CFU-GM, BFU-E and CFU-GEMM biological
activities using either 500 units of hpG-CSF or rhpG-CSF
are shown in Table XXII below.

All the colony assays were performed with low
30 density non-adherent bone marrow cells. Human bone
marrow cells were subject to a density cut with Ficoll-
Hypaque (density, 1.077 g/cm³; Pharmacia). The low
density cells were then resuspended in Iscove's modified
Dulbecco's medium containing fetal calf serum and placed
35 for adherence on Falcon tissue culture dishes (No. 3003,
Becton Dickenson, Cockeysville, MD.) for 1-1/2 hours at
37°C.

Medium	0±0	26±1	0±0
natural			
hpG-CSF	83±5.4	83±6.7	4±0
rhG-CSF	87±5	81±0.1	6±2

10

Medium control consisted of Iscove's modified Dulbecco medium plus 10% FCS, 0.2 mM hemin and 1 unit of recombinant erythropoietin.

15

For the CFU-GM assay target cells were plated at 1×10^5 in 1 ml of 0.3% agar culture medium that included supplemented McCoy's 5A medium and 10% heat inactivated fetal calf serum. Cultures were scored for colonies (greater than 40 cells per aggregate) and morphology assessed on day 7 of culture. The number of colonies is shown as the mean $\pm$ SEM as determined from quadruplicate plates.

20

For the BFU-E and CFU-GEMM assays, cells (1×10^5) were added to a 1 ml mixture of Iscove's modified Dulbecco medium (Gibco), 0.8% methylcellulose, 30% fetal calf serum 0.05 mM 2-mercaptoethanol, 0.2 mM hemin and 1 unit of recombinant erythropoietin. Dishes were incubated in a humidified atmosphere of 5% CO₂ and 5% O₂. Low oxygen tension was obtained using an oxyreducer from Reming Bioinstruments (Syracuse, N.Y.). Colonies were scored after 14 days of incubation. The number of colonies is shown as the mean $\pm$ SEM, as determined from duplicate plates.

25

Colonies formed in the CFU-GM assay were all found to be chloracetate esterase positive and non-specific esterase (alpha-naphthyl acetate esterase)

30

35

9 CFU-GM assay. The BFU-E and CFU-GEMM data in Table XXII
are representative of three separate experiments and
similar to the data reported previously for natural
hpG-CSF. It is important to note that the rhpG-CSF is
extremely pure and free of other potential mammalian
10 growth factors by virtue of its production in E.coli.
Thus rhpG-CSF is capable of supporting mixed colony
formation (CFU-GEMM) and BFU-E when added in the
presence of recombinant erythropoietin.

15 5. Cell Binding Assays

It was previously reported that WEHI-3B(D⁺)
cells and human leukemic cells from newly diagnosed
leukemias will bind ¹²⁵I-labeled murine G-CSF and that
this binding can be complete for by addition of
20 unlabeled G-CSF or human CSF-8. The ability of natural
hpG-CSF and rhpG-CSF to compete for binding of
¹²⁵I-hpG-CSF to human and murine leukemic cells was
tested. Highly purified natural hpG-CSF (>95% pure;
1µg) was iodinated [Tejedor, et al., Anal.Biochem., 127,
25 143 (1982)] was separated from reactants by gel filtra-
tion and ion exchange chromatography. The specific
activity of the natural ¹²⁵I-hpG-CSF was approximately
µCi/µg protein. Murine WEHI-3B(D⁺) and two human peri-
pheral blood myeloid leukemic cell preparations (ANLL,
30 one classified as M4, the other as M5B) were tested for
their ability to bind ¹²⁵I-hpG-CSF.

The murine and freshly obtained human peri-
pheral blood myeloid leukemic cells were washed three
times with PBS/1% BSA. WEHI-3B(D⁺) cells (5 x 10⁶) or
35 fresh leukemic cells (3 x 10⁶) were incubated in dupli-
cate in PBS/1% BSA (100 µl) in the absence or presence

5 resuspended and layered over 200 μ l ice cold FCS in a
350 μ l plastic centrifuge tube and centrifuged (1000 g;
1 min.). The pellet was collected by cutting off the
end of the tube and pellet and supernatant counted
separately in a gamma counter (Packard).

10 Specific binding (cpm) was determined as total
binding in the absence of a competitor (mean of dupli-
cates) minus binding (cpm) in the presence of 100-fold
excess of unlabeled hpG-CSF (non-specific binding). The
non-specific binding was maximally 2503 cpm for
15 WEHI-3B(D⁺) cells, 1072 cpm for ANLL (M4) cells and 1125
cpm for ANLL (M5B) cells. Experiments one and two were
run on separate days using the same preparation of
125I-hpG-CSF and display internal consistency in the
percent inhibition noted for 2000 units of hpG-CSF.

20 Data obtained are reported in Table XXIII below.

25

30

35

TABLE XXIII

<u>Competitor</u>	<u>(U/ml)</u>	<u>WEHI-3B(D⁺)</u> <u>cpm</u>	<u>% Inhib.</u>	<u>ANLL (M4)</u> <u>cpm</u>	<u>% Inhib.</u>	<u>ANLL</u> <u>cpm</u>
<u>Exp. 1</u>						
none	0	6,608	-	1,218	-	122
natural						
hpG-CSF:	10,000	685	90			
	2,000	1,692	74	34	97	-376
	200	2,031	69			
rhpg-CSF:	10,000	0	100			
	2,000	1,185	82	202	83	0
	200	2,330	65			
<u>Exp. 2</u>						
none	0	2,910	0			
natural						
hpG-CSF:	2,000	628	78			
GM-CSF:	2,000	3,311	0			

5 GM-CSF. In addition, binding of natural hpG-CSF to
human myelomonocytic leukemic cells (ANLL, M4) was
observed. The binding to these cells is paralleled in
response to natural hpG-CSF in liquid cultures by
differentiation into mature macrophages as judged by
10 morphology. The absence of binding of natural
 ^{125}I -hpG-CSF to monocytic leukemic cells from another
patient (ANLL, M5B) suggests that certain leukemias may
differentially express or lack receptors for hpG-CSF.
The ability of rhpG-CSF to compete for the binding of
15 natural ^{125}I -hpG-CSF, similar to natural hpG-CSF,
suggests that the receptors recognize both forms equally
well.

These studies demonstrating the binding of
natural ^{125}I -labeled hpG-CSF to leukemic cells are
20 paralleled in culture by the ability of natural hpG-CSF
to induce granulocytic and monocytic differentiation of
light density bone marrow cells obtained from one
patient with an acute promyelocytic leukemia (M3) and a
second patient with an acute myeloblastic leukemia
25 (M2). Cells from each patient were cultured for four
days in medium alone or in the presence of 1×10^5 units
of rhpG-CSF. Cells from the M3 control cultures incu-
bated in medium alone were still promyelocyte in type;
while cells cultured in the presence of rhpG-CSF showed
30 mature cells of the myeloid type including a metamyelo-
cyte, giant band form and segmented neutrophils and
monocyte. The actual differentials for this patient, on
100 cells evaluated for the control, 100% promyelocytes,
and for the rhpG-CSF treated cells, 22% blasts plus
35 promyelocytes, 7% myelocytes, 35% metamyelocytes, 20%
band forms plus segmented neutrophils, 14% monocytes and

5 clone. Cells from the second patient with a myelo-
blastic leukemia (M2) were also cultured for four days
in the presence of absence of rhpG-CSF. Visual analysis
of M2 cells cultured in medium alone revealed large
"blast-like" cells, some of which had nucleoli. Some of
10 the M2 cells, when treated with rhpG-CSF, differentiated
to mature segmented neutrophils displaying residual auer
rods in the center neutrophil suggesting differentiation
occurring in a cell belonging to the leukemic clone.
The actual differentiation of 100 cells evaluated mor-
15 phologically revealed that control cells consisted of
100% blasts. The rhpG-CSF treated cells consisted of
43% blasts, 1% myelocytes, 15% metamyelocytes, 28% band
forms plus segmented neutrophils, 2% promonocytes and
11% monocytes. The leukemic cells were also examined
20 for differentiation at four other concentrations of
rhpG-CSF (5×10^3 , 1×10^4 , 2.5×10^4 and 5×10^4 U/ml,
data not shown). Even at the lowest concentration of
rhpG-CSF tested (5×10^3 U/ml), there was significant
differentiation (cells differentiated beyond myelocytes)
25 of the M3 (50%) and M2 (37%) leukemic cells.

6. Immunoassay

To prepare polyclonal antibodies for immuno-
assay use the antigen employed was pluripotent G-CSF
30 purified from the human bladder carcinoma cell line 5637
(1A6) as prepared in Example 1 (B). This material was
judged to be 85% pure based on silver nitrate staining
of polyacrylamide gels. Six week-old Balb/C mice were
immunized with multiple-site subcutaneous injections of
35 antigen. The antigen was resuspended in PBS and emulsi-
fied with equal volumes of Freund's complete adjuvant.

5 later mouse serum was taken to test for the antibody specific to human pluripotent G-CSF.

Dynatech Immulon II Removawell strips in holders (Dynateck Lab., Inc., Alexandria, Virginia) were coated with hpG-CSF 5 µg/ml in 50mM carbonate-bicarbonate buffer, pH 9.2. Wells were coated with 0.25 µg in a volume of 50 µl. Antigen coated plates were incubated 2 hours at room temperature and overnight at 4°C. The solution was decanted and the plates were incubated 30 minutes with PBS containing 5% BSA to block the reactive surface. This solution was decanted and the diluted preimmune or test sera were added to the wells and incubated for 2 hours at room temperature. Sera were diluted with PBS, pH 7.0 containing 1% BSA. The serum solution was decanted and plates were washed three times with Wash Solution (KPL, Gaithersburg, Maryland). Approximately 200,000 cpm of iodinated rabbit anti-mouse IgG (NEN, Boston, Massachusetts) in 50 µl PBS, pH 7.0 containing 1% BSA was added to each well. After incubating 1-1/2 hours at room temperature, the solution was decanted and plates were washed 5 times with Wash Solution. Wells were removed from holder and counted in a Beckman 5500 gamma counter. High-titered mouse sera showed greater than 12-fold higher reactivity than the corresponding preimmune sera at a dilution of 1:100.

The immunological properties of E. coli-derived hpG-CSF were determined by reactivity to high-titered mouse serum specific to mammalian-cell derived hpG-CSF. 0.25 µg of 90% pure E. coli-derived protein was coated to Immulon II Removawells in a volume of 50 µl and mouse serum was assayed as described above.

7. Serine Analog Bioassays

[Ser¹⁷]hpG-CSF, [Ser³⁶]hpG-CSF, [Ser⁴²]hpG-CSF, [Ser⁶⁴]hpG-CSF, and [Ser⁷⁴]hpG-CSF products prepared according to Example 9 were assay for hpG-CSF activity in the ³H-thymidine uptake, CFU-GM, and WEHI3B D⁺ assays. In each assay, the [Ser¹⁷] analog had activity comparable to that of recombinant molecules having the native structure. The remaining analogs had on the order of 100-fold lesser activity in the ³H-thymidine uptake assay, 250-fold lesser activity in the CFU-GM assay, and 500-fold lesser activity in the WEHI-3B D⁺ assay. This data is supportive of the proposition that cysteines at positions 36, 42, 64 and 74 may be needed for full biological activity.

20

8. In vivo Bioassay

Alzet[®] osmotic pumps (Alzet Corp., Palo Alto, CA; Model 2001) were connected to indwelling right jugular vein catheters and implanted subcutaneously in seven male Syrian golden hamster. Four of the pumps contained a buffer [20 mM sodium acetate (pH 5.4) and 37 mM sodium chloride] and 1.5 mg/ml E.coli-derived hpG-CSF while 3 contained buffer alone. The claimed pumping rate for the osmotic pumps was 1 microliter/hr. for up to seven days. At the third day after implantation of the pumps, the mean granulocyte count of the four treated hamsters was six-fold higher than that of the three (buffer) controls and the increased granulocyte count was reflected in a four-fold increase in total lymphocytes. Erythrocyte count was unchanged by treatment. These results indicate that the recombinant

5 other hpG-CSF products such as polypeptide analogs of
hpG-CSF and fragments of hpG-CSF. Following the proce-
dures of the above-noted published application by Alton,
et al. (WO/83/04053) one may readily design and manufac-
10 tures having primary conformations which differ from
that herein specified for in terms of the identity or
location of one or more residues (e.g., substitutions,
terminal and intermediate additions and deletions).
Alternately, modifications of cDNA and genomic genes may
15 be readily accomplished by well-known site-directed
mutagenesis techniques and employed to generate analogs
and derivatives of. Such products would share at least
one of the biological properties of hpG-CSF but may
differ in others. As examples, projected products of
20 the invention include those which are foreshortened by
e.g., deletions; or those which are more stable to
hydrolysis (and, therefore, may have more pronounced or
longer lasting effects than naturally-occurring); or
which have been altered to delete one or more a poten-
25 tial sites for o-glycosylation (which may result in
higher activities for yeast-produced products); or which
have one or more cysteine residues deleted or replaced
by, e.g., alanine or serine residues and are potentially
more easily isolated in active form from micrbial
30 systems; or which have one or more tyrosine residues
replaced by phenylalanine and may bind more or less
readily to hpG-CSF receptors on target cells. Also
comprehended are polypeptide fragments duplicating only
a part of the continuous amino acid sequence or
35 secondary conformations within hpG-CSF, which fragments
may possess one activity of (e.g., receptor binding) and

5 173-175 (1982)] or utility in other contexts, such as in
assays of hpG-CSF antagonism. Competitive antagonists
may be quite useful in, for example, cases of
overproduction of hpG-CSF.

According to another aspect of the present
10 invention, the DNA sequence described herein which
encodes hpG-CSF polypeptides is valuable for the infor-
mation which it provides concerning the amino acid
sequence of the mammalian protein which has heretofore
been unavailable despite analytical processing of
15 isolates of naturally-occurring products. The DNA
sequences are also conspicuously valuable as products
useful in effecting the large scale microbial synthesis
of hpG-CSF by a variety of recombinant techniques. Put
another way, DNA sequences provided by the invention are
20 useful in generating new and useful viral and circular
plasmid DNA vectors, new and useful transformed and
transfected microbial procaryotic and eucaryotic host
cells (including bacterial and yeast cells and mammalian
cells grown in culture), and new and useful methods for
25 cultured growth of such microbial host cells capable of
expression of hpG-CSF and its related products. DNA
sequences of the invention are also conspicuously suit-
able materials for use as labelled probes in isolating
hpG-CSF and related protein encoding human genomic DNA
30 as well as cDNA and genomic DNA sequences of other
mammalian species. DNA sequences may also be useful in
various alternative methods of protein synthesis (e.g.,
in insect cells) or in genetic therapy in humans and
other mammals. DNA sequences of the invention are
35 expected to be useful in developing transgenic mammalian
species which may serve as eucaryotic "hosts" for pro-

5 polypeptide analogs of the invention are reports of the
immunological activity of synthetic peptides which sub-
stantially duplicate the amino acid sequence extant in
naturally-occurring proteins, glycoproteins and nucleo-
10 weight polypeptides have been shown to participate in
immune reactions which are similar in duration and
extent to the immune reactions of physiologically signi-
ficant proteins such as viral antigens, polypeptide
hormones, and the like. Included among the immune reac-
15 tions of such polypeptides is the provocation of the
formation of specific antibodies in immunologically
active animals. See, e.g., Lerner, et al., Cell, 23,
309-310 (1981); Ross, et al., Nature, 294, 654-656
(1981); Walter, et al., Proc. Natl. Acad. Sci. (USA),
20 77, 5197-5200 (1980); Lerner, et al., Proc. Natl. Acad.
Sci. (USA), 78, 3403-3407 (1981); Walter, et al., Proc.
Natl. Acad. Sci. (USA), 78, 4882-4886 (1981); Wong, et
al., Proc. Natl. Acad. Sci. (USA), 78, 7412-7416 (1981);
Green, et al., Cell, 28, 477-487 (1982); Nigg, et al.,
25 Proc. Natl. Acad. Sci. (USA), 79, 5322-5326 (1982);
Baron, et al., Cell, 28, 395-404 (1982); Dreesman, et
al., Nature, 295, 185-160 (1982); and Lerner,
Scientific American, 248, No. 2, 66-74 (1983). See,
also, Kaiser, et al., Science, 223, 249-255 (1984)
30 relating to biological and immunological activities of
synthetic peptides which approximately share secondary
structures of peptide hormones but may not share their
primary structural conformation.

While the present invention has been described
35 in terms of preferred embodiments, it is understood that
variations and modifications will occur to those skilled

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pluripotent granulocyte colony-stimulating factor (hpG-CSF) of the sequence set forth in Table VIII, b) is a non-naturally occurring polypeptide; and c) is the product of procaryotic or eucaryotic expression of an exogenous DNA sequence.

2. A polypeptide according to Claim 1 wherein the exogenous DNA sequence is a cDNA sequence.

3. A polypeptide according to Claim 1 wherein the exogenous DNA sequence is a genomic DNA sequence.

4. A polypeptide according to Claim 1 wherein the exogenous DNA sequence is carried on an autonomously replicating DNA plasmid or viral vector.

5. A polypeptide according to Claim 1 further characterized by being covalently associated with a detectable label substance.

6. A polypeptide according to Claim 5 wherein said detectable label is a radiolabel.

7. A DNA sequence which codes upon expression in a procaryotic or eucaryotic host cell for a polypeptide product having at least a part of the primary structure and one or more of the biological properties of naturally-occurring pluripotent granulocyte colony-stimulating factor, said DNA sequence being selected

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sequences defined in (a) or fragments thereof; and

(c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) or (b) and which sequences code for a polypeptide having the same amino acid sequence.

8. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to Claim 7 in a manner allowing the host cell to express said polypeptide product.

9. A host cell according to Claim 8 wherein the host is E. coli.

10. A host cell according to Claim 8 wherein the host cell is a mammalian cell.

11. A non-naturally occurring polypeptide product of the expression of a DNA sequence of Claim 7 in a procaryotic or eucaryotic host.

12. A cDNA sequence according to Claim 7.

13. A genomic DNA sequence according to Claim 7.

14. A DNA sequence according to Claim 7 and including one or more codons preferred for expression in E. coli cells.

15. A DNA sequence according to Claim 7 and coding for

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17. A DNA sequence according to Claim 12 or 13 coding for expression of human pluripotent granulocyte colony-stimulating factor.

18. A DNA sequence according to Claim 7 covalently associated with a detectable label substance.

19. A DNA sequence according to Claim 18 wherein the detectable label is a radiolabel.

20. A single-stranded DNA sequence according to Claim 18.

21. A biologically functional plasmid or viral DNA vector including a DNA sequence according to Claim 7.

22. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to Claim 21.

23. A process for the production of a polypeptide having part or all of the primary structure and one or more of the biological properties of naturally occurring pluripotent granulocyte colony-stimulating factor, which process is characterized by culturing under suitable nutrient conditions, procaryotic or eucaryotic host cells transformed or transfected with a DNA sequence according to Claim 7 in a manner allowing the



granulocyte colony-stimulating factor, which process is characterized by culturing under suitable nutrient conditions, procaryotic or eucaryotic host cells transformed or transfected with a DNA sequence set forth in Table VIII in a manner allowing the host cells to express said polypeptide, and isolating desired polypeptide products of the expression of the DNA sequence.

25. A pharmaceutical composition comprising an effective amount of a polypeptide according to Claim 1 and/or produced by the process of Claim 23 or 24 and a pharmaceutically acceptable diluent, adjuvant or carrier.

26. A pharmaceutical composition according to Claim 25 further characterized by being free of association with any human protein.

27. Use of a polypeptide according to Claim 1 for the manufacture of a medicament for providing hematopoietic therapy to a mammal.

28. Use of a polypeptide according to Claim 1 for the manufacture of a medicament for arresting proliferation of leukemic cells.

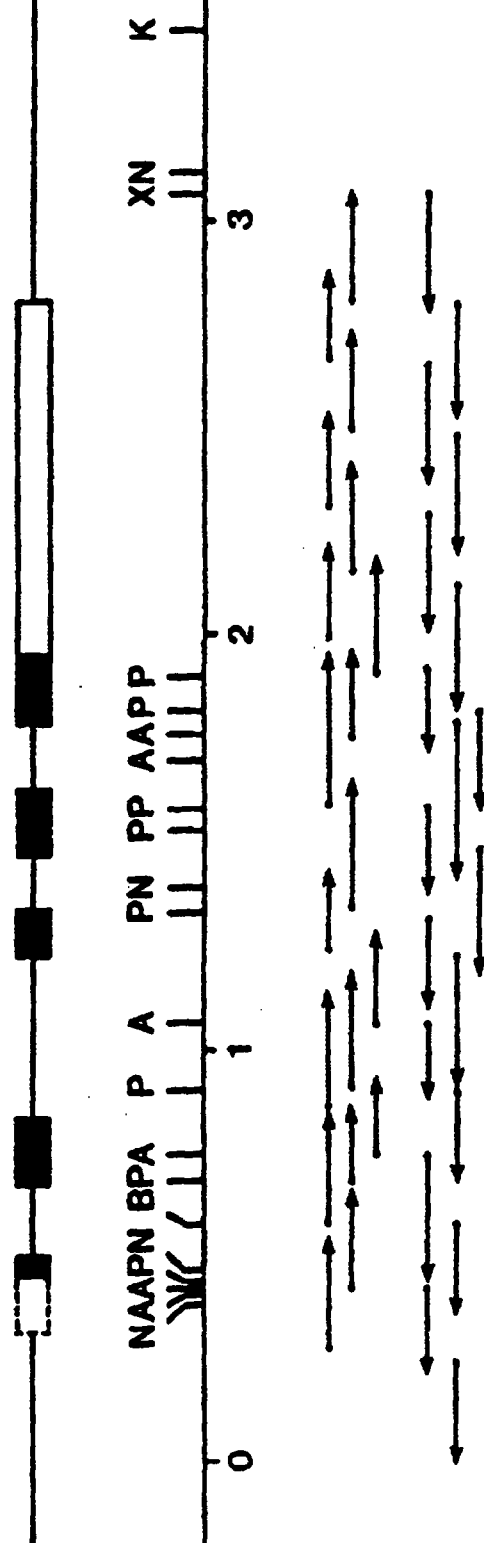


29. A polypeptide according to Claim 1 preceded by a methionine residue.

30. A polypeptide having the hematopoietic biological properties of naturally occurring pluripotent granulocyte colony-stimulating factor, said polypeptide having an amino acid sequence selected from the polypeptide sequence set forth in Table VIII or any allelic variants, derivatives, deletion analogs, substitution analogs, or addition analogs thereof, and characterized by being non-naturally occurring and by being the product of procaryotic or eucaryotic expression of an exogenous DNA sequence.

31. A pharmaceutical composition comprising an effective amount of the polypeptide according to Claim 29 and a pharmaceutically acceptable diluent, adjuvant or carrier.

FIG.1



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Disclosed are novel polypeptides possessing part or all of the primary structural conformation and one or more of the biological properties of a mammalian (e.g., human) pluripotent granulocyte colony-stimulating factor ("hpG-CSF") which are characterized in preferred forms by being the product of procaryotic or eucaryotic host expression of an exogenous DNA sequence. Sequences coding for part or all of the sequence of amino acid residues of hpG-CSF or for analogs thereof may be incorporated into autonomously replicating plasmid or viral vectors employed to transform or transfect suitable procaryotic or eucaryotic host cells such as bacteria, yeast or vertebrate cells in culture. Products of expression of the DNA sequences display, e.g., the physical and immunological properties and in vitro biological activities of isolates of hpG-CSF derived from natural sources. Disclosed also are chemically synthesized polypeptides sharing the biochemical and immunological properties of hpG-CSF.

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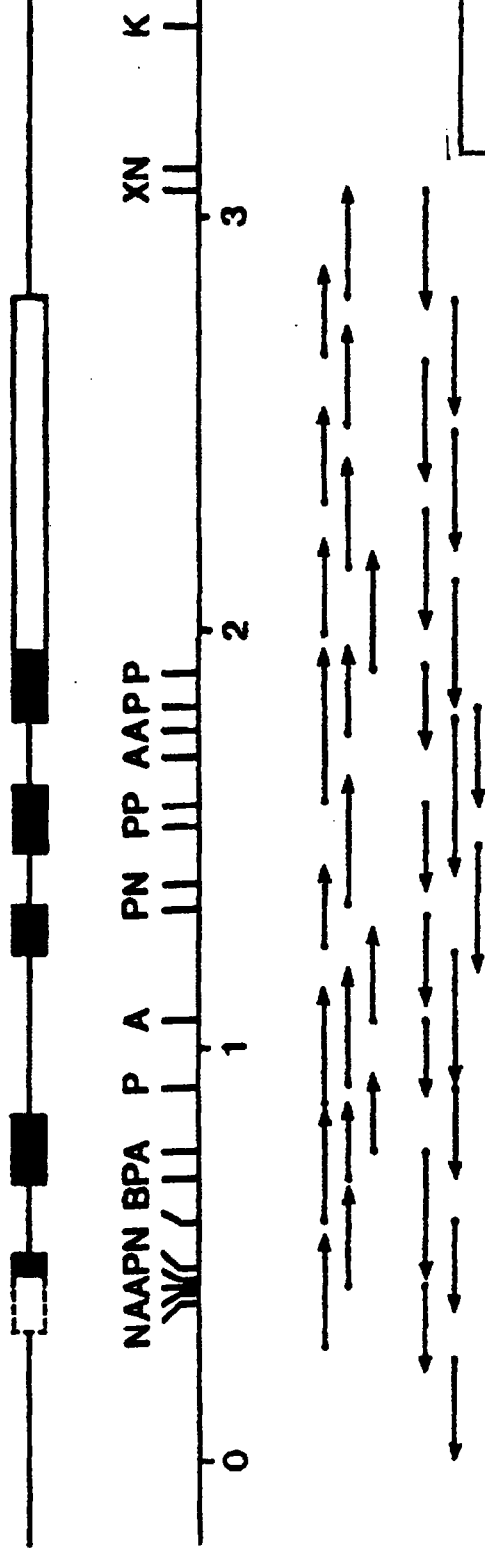
Disclosed are novel polypeptides possessing part or all of the primary structural conformation and one or more of the biological properties of a mammalian (e.g., human) pluripotent granulocyte colony-stimulating factor ("hpG-CSF") which are characterized in preferred forms by being the product of procaryotic or eucaryotic host expression of an exogenous DNA sequence. Sequences coding for part or all of the sequence of amino acid residues of hpG-CSF or for analogs thereof may be incorporated into autonomously replicating plasmid or viral vectors employed to transform or transfect suitable procaryotic or eucaryotic host cells such as bacteria, yeast or vertebrate cells in culture. Products of expression of the DNA sequences display, e.g., the physical and immunological properties and in vitro biological activities of isolates of hpG-CSF derived from natural sources. Disclosed also are chemically synthesized polypeptides sharing the biochemical and immunological properties of hpG-CSF.

(Figure 1)

REPLACEMENT SHEET

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FIG. 1



1. A polypeptide which:

(a) consists only of the 1-174 amino acid primary structure set forth in Table VIII, and

(b) is the product of procaryotic or eucaryotic host cell expression of an exogenous DNA sequence encoding the same.

2. A polypeptide according to Claim 1 preceded by a methionyl residue.

3. A glycosylated polypeptide according to Claim 1 which is a Chinese Hamster Ovary host cell expression product of an exogenous DNA sequence.

4. A DNA sequence which codes upon expression in a procaryotic or eucaryotic host cell for a polypeptide according to Claim 1 or 2.

5. A biologically functional plasmid or viral DNA vector including a DNA sequence according to Claim 4.

6. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA according to Claim 4.

7. A process for the production of a polypeptide according to Claim 1, 2 or 3 which process is characterized by culturing under suitable nutrient conditions, procaryotic or eucaryotic host cells transformed or transfected with DNA sequence according to Claim 4 in a manner allowing the host cell to express said polypeptide,

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8. A pharmaceutical composition comprising an effective amount of a polypeptide according to Claim 1, 2 or 3 and/or produced by the process according to Claim 7 and a pharmaceutically acceptable diluent, adjuvant or carrier.

9. Use of a polypeptide according to Claim 1, 2 or 3 and/or produced by the process according to Claim 7 as a culture medium component for the growth of hematopoietic cells in vitro, said use optionally including the use of at least one other hematopoietic factor.

10. Use of a polypeptide according to Claim 1, 2 or 3 and/or produced by the process according to Claim 7 for the manufacture of a medicament for enhancing production and/or release of granulocytes in a mammal.

REPLACEMENT SHEET

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1. A process for the production of a human pG-CSF product having the *in vivo* granulocytopoietic biological property of naturally occurring human pG-CSF comprising the steps of:

(a) culturing under suitable nutrient conditions, mammalian cells, comprising promoter DNA, other than human pG-CSF promoter DNA, operatively linked to DNA encoding the hpG-CSF amino acid sequence of Figure 7; and

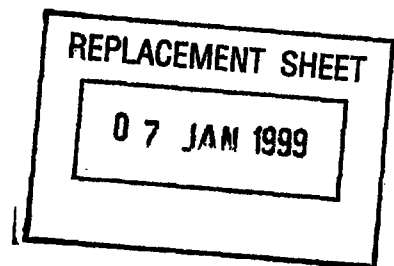
(b) isolating said human pG-CSF expressed by said cells.

2. A process according to Claim 1, wherein the promoter DNA is viral promoter DNA.

3. A process according to Claim 1, wherein said cells are primate cells.

4. A process according to Claim 3, wherein said primate cells are COS cells.

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1. A process for the production of a human pG-CSF product having the *in vivo* granulocytopoietic biological property of naturally occurring human pG-CSF comprising the steps of:

(a) culturing under suitable nutrient conditions, mammalian cells, comprising promoter DNA, other than human pG-CSF promoter DNA, operatively linked to DNA encoding the hpG-CSF amino acid sequence of Table VII
~~Figure 7~~; and

(b) isolating said human pG-CSF expressed by said cells.

2. A process according to Claim 1, wherein the promoter DNA is viral promoter DNA.

3. A process according to Claim 1, wherein said cells are primate cells.

4. A process according to Claim 3, wherein said primate cells are COS cells.

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