United States Patent

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[56] PRODUCTION OF ERYTHROPOIETIN


[22] Filed: Jun. 7, 1995


[57] ABSTRACT

Disclosed are novel polypeptides possessing or all of the primary structural conformation characteristic or some of the biological properties of a human erythropoietin ("EPO") which are characterized in preferred forms by being the product of recombinant or non-recombinant host expression of an estron DNA sequence. Illustratively, genomic DNA, cDNA and non-manufactured DNA sequences encoding for part or all of the sequence of amino acid residues of EPO or analogs thereof are incorporated into autonomously replicating plasmid or viral vector employed in transfected or transformed suitable prokaryotic or eukaryotic host cells such as bacteria, yeast or vertebrate cells in culture. Upon induction from culture media or cellular lysates or fragments, product expression of the DNA sequences may, e.g., be the immunological properties and in vivo and in vivo biological activities of EPO or analogs thereof. Disclosed also are chemically synthesized polypeptides serving the biochemical and immunological properties of EPO. Also disclosed are improved methods for the detection of specific epitope-specific polypeptides in a heterogeneous cellular or viral sample derived from, e.g., DNA present in a plasmid or virus-forming DNA or genomic DNA library.

14 Claims, 27 Drawing Sheets
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**FIG. 3**

Diagram showing a circular DNA structure labeled as **pSVgHuEPO**. The diagram includes various restriction enzyme sites such as **PstI**, **ApR**, **Ri**, **SV40ori**, and **BamHI**. The structure also indicates the presence of **HuEPO**.
FIG. 4

pDSVL-γHuEPO

γHuEPO
FIG. 5A

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PAM3A
GATCCGCCGCCGCCCTGGACAGCCGCCCTCTCTCTGAGCCGCGCTGCGCGCC

CGCTGAACCTCCCCGGAGATGAGACCTCCCCCGTGGGGTCCCGGGCTGAG

-27
Mat Gly Val His Glu Cys Pro Ala Trp

GGACCCCAGCCAGGCGCGAGATG GGG GTG CAC GAA TGT CCT GCC TGG

-10
Leu Trp Leu Leu Leu Ser Leu Val Ser Leu Pro Leu Gly Leu Pro
CTG TGG CTT CTC CTG TCT CTC GTG TCG CTC CCT CTG GGC CTC CCA

-1  +1  10
Val Pro Gly Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu
GTC CCG GCC GCC CCA CCA CGC CTC ATC TGT GAC AGC CGA GTC CTG

20  *
Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Val Thr Met
GAG AGG TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT GTC ACG ATG

30  *
Gly Cys Ser Glu Ser Cys Ser Leu Asn Glu Asn Ile Thr Val Pro
GCC TGT TCC GAA AGC TGC AGC TTG AAT GAG AAT ATC ACC GTC CCA
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FIG. 5B

50
Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly
GAC ACC AAA GTT AAC TTC TAT GCC TGG AAG AGG ATG GAG GTC GCG

60
Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu
CAG CAG GCT GTA GAA GTC TGG CAG GCC CTG GCC CTG CTC TCA GAA

70
Ala Val Leu Arg Gly Gln Ala Val Leu Ala Asn Ser Ser Gln Pro
GCT GTC CTG CGG GCC CAG GCC GTC TGG GCC AAC TCT TCC CAG CCT

80
Pro Leu Gln Leu His Met Asp Lys Ala Ile Ser Gly Leu
TTC GAG CCC CTG CAG CTG CAC ATG GAT AAA GCC ATC AGT GCC CCT

90
Arg Ser Ile Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Glu Ala
GCC AGC ATC ACC ACT CTG CTT CGG GCG CTG GGA GCC CAG GAA GCC

100
Ala Ser Leu Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile
ATC TCC CTC CCA GAT GCG GCC TCG GCT CCA CTC GGA ACC ATC

110
Thr Ala Asp Thr Phe Cys Lys Leu Phe Arg Val Tyr Ser Asn Phe
ACT GCT GAC ACT TTC TGC AAA CTC TTC CGA GTC TAG TCC AAT TTC
FIG. 5C

150
Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Arg
CTC CGG GGA AAG CTC AAG CTC TAC ACG GAA GAG GCC TGC AGG AGA

160
Gly Asp Arg DP
GCG GAC AGA TGA CCA TGG GTC CAT CAC CAC CAG GAC

CTG CCT GGC CAC ACC CTC CTC GCC
CGG GAG AAG GGC AAT GAC GCT CAC GAG

165
tat
CAG TGC CAG TAC ACC TCA CAG GAG

CTG TGC CAG TAC ACC TCA CAG GAG

AGA AGC ATT CAG AAG GCA CGT

GCC AAG CAC TGG CTA AGG

AGA AGC ATT CAG AAG GCA CGT

TTT TGC CAT ACC CTC CAG GAG

CTG AAG GGT TCG CAG

TTT TGC CAT ACC CTC CAG GAG

GAG TAC TGG TGG GAG GAA GAT CAC GAG

AGA AGC ATT CAG AAG GCA CGT

AGA AGC ATT CAG AAG GCA CGT

AGA AGC ATT CAG AAG GCA CGT

Hind III
FIG. 8

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-1 +1

MgII

ACCTGGATA AAGAGGCTCC ACCAGGATGG ACTGCGACT CAGCAGTTTT
ACCTAT TTTCGCAAG TGCTCTTAC TACGACTGA GCTTGCAAAA

GGAAAGAATG TTGTGGGACG CTAGAGGACG TCAAAACATC ACCACTGGTT
GCTTCTTAGC AACAACCTTC GATTTCTTG ACTTTTTTAC TGTCGACCAA

GTGCTGAAAAC CTGTTCTTTTG AGCAGAACAC TAGCGCTACC AGACACCCAG
CAGCGCTTGT GACGACAAAC TTGCTTTTTG AATGGCAATGC TCTGTGCTTC

GTTAAGCTTTG ACCCTGGAA AAGTATGCAA GTTGGCTCAAC AGCTGTATTG
GCTTGGAAAG TGCCGACCTT TGCTGACCTT CAAACGTTG TGCGACCAAC

AGTTGCGGCA GTTTGGGCTT TGTATCGGA AGCTGGTGGC AGGGCTCGAC
TCCAACCGTT CCAGACCCGA ACAATAGACT TGCGACCAAC TGCCACGTTT

CCCTTCTGGT TAACTCTTTT CAACCAAGGG ACCCTTGGCA ATTCGACCTC
GGAAACACCA ATTCAGGAAG GTTGGTACCC TTGGTAACCT TACGCTGGAC

GAAGGAGCCG TCTCTGGTTG GAGATCTTGG ACTACCTTGF TGGGACTATT
CTATCCTGCC AGAGACCCAA CTCTAGAAAC TGGGAGACCA ACTCTGAGAA

GGTGTCTCAA AGCAAGGGCA TTTCCCCACC AGGCGCTGCT TCTGCGCTTC
CAGCAGGATT TTCTCTGGTT AAAGGCTGAC TGCACGCGAG AGACGCGGAG

CATCGACGAC CATCAGCTG TACACCTTCA GAGATTATT CAGATTTAC
CTACTCTTTG TCTAAGACGA CTAGGAAAT CTGTCAATG TCTCAATATG

TGCAACCTCTT TGCAAGGCTAA ATTCAGTTTG TACACCGGTT AGCCCTGTGG
AGGTTGAGA ACTCTCCATT TACCTCCAC ATGCGCCCAA TTGGGACACCT

AAGCTGGGAC AGATAAGGCC GACGTGATAA AAGAAGTATT TGAGGCACCTG
TTGACCACTG TCTATTGGGA CTAAGTTATG TTGTCACATC

SacII

ATGTAACAAA GC
PACATTGTTT CGGCT
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FIG. 10

1. AATTCTAGAAACCATGAGGGTTAATAAATA
2. CCATTATTTATTACCCCTATGGTTCCTAG
3. ATGGCTCCGCCGCGTGCTGATCTGGCGAC
4. CTCGAGTGCGACAGCACGCCGGCGGAG
5. TCGAGAGTTCTGGAAACGTTACCTGCTG
6. CTTCCAGCGGTAACGTTCCAGAACT
7. GAAGCTAAAGAAGCTGAAAAACATC
8. GTGGTGATGTTTTCAGCTTTTTTAG
9. ACCACTGTTTGCCTGAAACACTGCC
10. CAAAAGAAGCTGTTCCAGCAAAAC
11. TTTGAACGAAAAACATTACGGTACC
12. GATCCGGTACCGTAATGTTTCGPT
FIG. 11

XbaI
BglII

AATTCTAG AAACCATGAC GCTAAATAAAA TAATGCTGCC GCGCGCTCTG
GATC TTGGTACTC CCATTATTTT ATTACCGAGG CCGCGCCAGAC

ATCTGCGCTT CTGGAGTCTT CTGGAGTTAC CTCTGAGAG CTAAAGAAGC
TAGACCTGTA GCTCTCAGAA CCTTCGACAG GACGACCTTC GATTCTTCCG

TGAAAGAACAC ACCATGCTTT CTGCTGAACA CTGCTTTTCG AACGAAACA
ACTTTTGTAC TGGTGAACAA CAGGACCTGT AACGAAAGAAG TGGCTTTTTG

TTGCCTGACC G
AAATCCATGC CCTAG

FIG. 12

1. AATTCGCTACCAGACACCAAGGT
2. GTTAACCTTGTTGTCTGGAACCG
3. TAACTTCTACGCTTTGGAACGTAT
4. TTCCATACGTCTTCCAAAGCGTAGA
5. GGAAGTTTGGTCAACAACGAGTTGAGT
6. CCAAACCTTCACCTGGTTGGACCAC
7. TGGCGCAGGGTCTGGCACTGCTGAGCG
8. GCCCTGCTCAGCAGTGCCAGACCCCTG
9. AGGCTGTACTGGCTGGCCAGGCA
10. GCAGTGCCTGGGCCAGCAGTACA
11. CTGCTGGTAAACTCTCTCAGCGT
12. TTCCCACGGGCTGAGAGGAGTTTACCA
13. GGGAAACCGCTCGACGCTGATGTTGAC
14. GCTTTGTCACATGCAGCTGACACGG
15. AAAGCAGTATCTGGCCTGAGATCTG
16. GATCCAGATCTCAGGCCAGATACT
FIG. 14

1. GATCCAGATCTCTGACTACTCTGC
2. ACGCAGCAGAGTAGTCAGAGATCTG
3. TGCCGTGCTCTGGGTGCACAGAAAGAGG
4. GATAGCCTTTPCTGTGCACCCAGAGC
5. CTATCTCTGCCCGGAATGCTGATCTCT
6. CAGCAGATGCAGCATCCGGGCGGAGA
7. GCTGCACCGCTGCCTACCATCCACTG
8. ATCAAGCAGTGTGATGGTACGCAGCGGTG
9. CAGATACCTTCCGCAAACGTGTTCG
10. ATACACGAAACAGTTTGGCGGAAGGTT
11. TGATATCTCTAACTTCTGCTGTTGGTA
12. CAGTTTACCACGCAGGAAGTTAGAGT
13. AACTGAAACTGTATACTGCGGAAGC
14. GGCAATGCTTCGCCAGTATACAGTTT
15. ATGCCGTACTGGTGACCGCTAATAG
16. TCGACTTTTAGCGGTCACCAGTAC
FIG. 16

1. AATTCAGCTTGGGATAAAAGAGCT
2. GTGGAGCTCTTTTATCCAGCTTTG
3. CCACCAAGATTTGATCTGACTTC
4. TCTCGAGTCACAGATCAATCTTG
5. GAGAGTTTTGGGAAGATACCTTTTG
6. CTTCCCAACAAGTATCTTTGCCAAAAC
7. GAAGCTAAAGAAGCTGAAAAACATC
8. GTGATTGTCTTTTACAGCTTTCTTTAG
9. ACCACTGGTTGTGCTGAACACTGTTTC
10. CAAAGAAGAGTGGTACAGCACAACCA
11. TTTGAGCGAAAAACATTACCGTACC
12. GATCCGGGTACCTGAATTTTACGTT
FIG. 17

EcoRI  HindIII
AATTCA AGCTTGCTAG

G TCGAAGCTAT

3

AAAGACCTCC ACCAGATTTG ATCTGACT CGAGATTTT
TTGTGCAGG TGGTTCTAAG TAGACACTGA GCTTCGAAAA

4

5

GGAARGATAC TTGTGCAAG CTAARGAGAC TGAAAACATC ACCACTGCTT
CTTTCTAG AACCATCTTC GATTTCCTCC ACTTTTCTAG TGGTGACCAA

6

2

GTTCTGACAC CTGTTCTTG AAGGAAACA TTACGTTACC G
CAGGAACTTG TACAGAGAC TGGCTTTTCT TAGCCCATGG CCTAG

12
1. AATTCGCTACCAGACCACCAAGGT
2. GTTAACCTTGTTGCTGTGCTACCG
3. TAACTTCTACGCTTGGAAACGCGATAT
4. TTCCATACGTTTCCCCAAGCGTGGAA
5. GGAAGTTGGTCAAACAAGCAGTTGAAGT
6. CCAAACCTCTCAACTGCTTGTTTGACCAAC
7. TTGGCAAGGTTTGGGTGTTGTTATCTG
8. GCTTCAGATAAACAAGGCCAAACCTTGT
9. AAGCTGTTTTGAGAGGTGAAGCCT
10. ACAAGGCTTGGACCTCTCTACAAC
11. TGTTGGTTAATCTTTCTCACCATGGG
12. TGGTTCCCATGGTTGAGAAAGAGTTAACCC
13. AACCATTGCAAATGGCAGTGAT
14. CTTTATCGACGTGCAATTGCAA
15. AAAGCCGTCTCTGTTTGGAGATCTG
16. GATCCAGATCTCAACCACAGAGACGG
FIG. 19

EndI

EndII

A ATTCGGTACC AGACACCAAG GCCATGG TCTGCGTTTC

GTTATCTCT AGGCGTGGAA AGCTATGGAA GTTGGCTAAC AAGCTTTTGA
CACAGAAC TGCAGACCT TGCAGACCT CACCCGTTT TGGCACACCT

GTTATGGCAA GTTGGCGCT TGTATCTGA AGCTGTTTGG AAGGCTCAG
TCCGAGCTT CCGACGGA ACATAGACT TGGGAAACAC TCCGAGCTTC

CCTGGTTCT TACCTCTCT CAACCATGCC AACCATGCC ATTCGACCTC
GGACACCC AGTACAGAG CTTGGTACCC TTGTAAAGCT TACGCTCACG

GATGAAAGCC TCTCGGTTT GAGATCG
CTATTTGCGC AGGACACAA CTCTGACCTA G

BglII BamHI
FIG. 20

1. GATCCAGATCTTTGGACTACTTTTGT
2. TCTCAACAAAGTAGTCAAAGATCTG
3. GAGAGCTTTGGGCTGCTCRAAAGGAA
4. ATGCGTCTCCTTTTGGCAACCACAAGAC
5. CCATTCCCCACCCAGACGGCTGCTT
6. GCAGAAGCAGCGCTCTGGGTGGGAA
7. CTGCCGCTCCATTGGAAACCATC
8. CAGTGATGGTTCTCAATGGAGCCG
9. ACTGCTGATACCCCTGACAAAGTT
10. GAATAACTTTCTGAAGGTATCAG
11. ATTCAGAGGTATTACTCCAAACTCT
12. CTCAAGAAAGTTGGAGTAAACTCT
13. TGAGAGGTAARITGAGTTGTAACAC
14. ACCGGGTGTAACAACCTTTCAATTTACCT
15. CGGTGAAGGCTGTAAGACTGGT
16. CTGTCACCAGTTCTAGAGGCTTTC
17. GACAGATAAGCGCCGACTGATAA
18. GTTGTATACGTCGGGCTTTAT
19. CAAACAGTGTAGTGAACAAAAG
20. TCGACTTTTTGTTACATCTGACT
FIG. 21

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GATC CAGATCTTTG ACTACTTTGT TTHAGCTTT
GACTAGACG AGATGCAACA ACTCTCCAAA

GCACTCTCA AAGGAGCCCA TTTCCCCACC AAGGCTGCT TCTGCCCCTC
CAGCAGCTT TCTCTGCGT AAGGGCGTGC TCTGCCAGCA AGACGCAGG

CATTGACAC CAGTCGCTT GATACTTTCA GACACTTAYT CAGCAAGTAC
GACTCTTTG GATGCAACG CAGTCCAAGT CTTTCAATA GCTCBAAC

TCCAAGCTCT TTHAGGCTTG AATGAGCTTG TACACGCGTG AAGGCTGTAG
AGGTTGACG ACTCTCCATT TTAGGCAAC ATGCGGCAAC TCTCCAGATC

AGTCTGTCG AGATAGCCCC GACGTATAAC AGACGGTTAG
TGACTCTGTG TCTATGCAGG CTTACTATTG TTTCAGATG

ATCTAAAAGA G
TACATCTT CAGCT
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1 PRODUCTION OF ERYTHROPOIETIN

This is a continuation of application Ser. No. 08/202,874, filed Feb. 28, 1994, and now abandoned, which was a continuation-in-part of U.S. application Ser. No. 07/131,178, filed Oct. 23, 1992, now abandoned, which was a continuation-in-part of U.S. application Ser. No. 06/678,288, filed Nov. 30, 1990, and issued Oct. 27, 1992 as U.S. Pat. No. 5,070,008 which was a continuation-in-part of U.S. Ser. No. 06/655,841, filed Sept. 28, 1990, and now abandoned, which was a continuation-in-part of U.S. application Ser. No. 06/582,155, filed Feb. 21, 1990, and now abandoned, which was a continuation-in-part of U.S. application Ser. No. 06/561,024, filed Dec. 13, 1990, and now abandoned.

BACKGROUND

The present invention relates generally to the manipulation of genetic material and, more particularly, to recombinant procedures making possible the production of polypeptides possessing part or all of the primary structural conformation and/or one or more of the biological properties of naturally-occurring erythropoietin.

A. Manipulation of Genetic Material

Genetic material may be broadly defined as those chemical substances which program for and guide the manufacture of constituents of cells and viruses and direct the responses of cells and viruses. A long chain polymeric substance known as deoxyribonucleic acid (DNA) comprises the genetic material of the cell itself, and viruses except for certain viruses which are programmed by ribonucleic acids (RNA). The repeating units in DNA polymers are four different nucleotides, each of which consists of either a pyrimidine (thymine) or a purine (adenine or guanine) base joined to a deoxyribose sugar to which a phosphate group is attached. Concentration of nucleotides in linear polymeric form is by means of a chain of the 3' phosphate of one nucleotide to the 5' hydroxyl group of another. Functional DNA occurs in the form of stable double stranded associations of single strands of nucleotides (known as deoxyribonucleosides), which associations occur by means of hydrogen bonding between guanine and cytosine bases (i.e., "complementary" associations existing either between adenine (A) and thymine (T) or guanine (G) and cytosine (C)). By convention, nucleotides are referred to by the names of their constituent purine or pyrimidine base, and the complementary association of nucleotides in double stranded DNA (i.e., A T and G C) are referred to as "base pairs". Ribonucleic acid is a polynucleotide comprising adenine, guanine, cytosine and uracil rather than thymine, bound to ribose and a phosphate group.

Most briefly put, the programming function of DNA is generally achieved through a process wherein specific DNA nucleotide sequences (known as "promoters") interact with specific RNA polymerases to form a complex known as RNA polymerase-DNA. The DNA serves as a template for the formation of functional RNA molecules (RNA polymerase) which are transported and align individual amino acids along the RNA strand to allow for formation of polypeptides in proper amino acid sequence. The mRNA "message" derived from DNA and the proteins provided by the RNA supply and constitute all of the atomic units in the two amino acid polypeptide "carrier". The sequence is the deoxyribonucleic acid (DNA) in base pairs in the form of a "hybrid"-vectors which are formed which include the selected exogenous DNA segment "sliced into" the viral or circular DNA plasmid.

Transformation of compatible unicellular host organisms with the hybrid vectors results in the formation of multiple copies of the exogenous DNA in the host cell population. In some instances, the desired result is the expression of the foreign DNA and the "product" harvested from the cells. More frequently, the goal of transformation is the expression of the desired product in cells of the desired DNA in the form of large scale synthesis of recombinant products that have significant biological activity. The use of cells vectors—sequential growth of three nucleotide bases. In one sense, the formation of a protein is the ultimate form of expression of the programmed genetic message provided by the nucleotide sequence of a gene.

"Promoter" DNA sequences usually "precede" a gene in a DNA polymer and provide a site for initiation of the transcription into mRNA. "Regulatory" DNA sequences also usually "underlie" of (i.e., preceding) a gene in a given DNA polymer, and provide the sites that determine the frequency (or rate) of transcriptional initiation. Generally, these sequences which precede a selected gene (or series of genes) in a functional DNA polymer cooperate to determine whether the transcription (and eventual expression) of a gene will occur. DNA sequences which "follow" a gene in a DNA polymer and provide a site for termination of transcription into mRNA are referred to as transcriptional "terminator" sequences.

A future of microbiological processing for the near decade has been the attempt to manufacture industrially and pharmaceutically significant substances using organisms which either do not naturally have genetically coded information concerning the desired product included in their DNA, or (in the case of mammalian cells in culture) do not ordinarily express a commercial gene at appreciable levels. Simply put, a gene that specifies the structure of a desired polypeptide product is either isolated from a "donor" organism or chemically synthesized and then newly introduced into another organism which is preferably a self-replicating unicellular organism such as bacteria, yeast or mammalian cells in culture. Once this is done, the existing machinery for gene expression in the "transformed" or "transfected" microbial host cells operates to construct the desired product using the exogenous DNA as a template for transcription of mRNA which is then translated into a continuous sequence of amino acid residues.

The art is rich in patent and literature publications relating to "recombinant DNA" methodologies for the isolation, synthesis, purification and amplification of genetic materials for use in the transformation of selected host organisms. U.S. Pat. No. 4,273,553 to Cohen et al., for example, relates to transformation of unicellular host organisms with "hybrid" viral or circular plasmid DNA which includes exogenous expression DNA sequences. The procedures of the Cohen et al. patent first involve manufacture of a transformation vector by enzymatically cleaving viral or circular plasmid DNA to linear form. DNA strands selected ("exogenous" or "heterologous") DNA strands usually including sequences coding for desired product are prepared in linear form through use of similar enzymes. The linear viral or plasmid DNA is isolated with the foreign DNA in the presence of ligating enzymes capable of effecting a reassociation process and "hybrid" vectors are formed which include the selected exogenous DNA segment "sliced into" the viral or circular DNA plasmid.

The development of specific DNA sequences for splicing into DNA vectors is accomplished by a variety of tech-
3

5,547,933

4

5

6
for human HLA-DR using a 177 base pair synthetic oligonucleotide. Finally, Anderson, et al., *H.A.S. (U.S.A.)*, 80, pp. 6335-6342 (1983) report the isolation of genomic clone for bovine pancreatic trypsin inhibitor (BPTI) using a single probe 98 base pairs in length and constructed according to the known amino acid and nucleotide sequence of 304 bp. The authors note a determination of poor prospects for isolating mRNA suitable for synthesis of a cDNA library due to apparent low levels of mRNA in initially targeted porcine gland and lung tissue sources and then address the prospects of success in pooling a genomic library using a mixture of labeled probes, stating: "More generally, hybridization of oligo-DNA probes have been used to induce protein genes of known sequence from cDNA Libraries. Such probes are typically mixtures of 8-12 oligonucleotides, 14-17 nucleotides in length, representing every possible codon combination for a small stretch (5-8 residues) of amino acid sequence. Under stringent hybridization conditions that discriminate against incorrectly base-paired probes, these mixtures are capable of identifying specific gene sequences in clone libraries of low to moderate complexity. Nevertheless, because of their short length and heterogeneity, such probes often lack the specificity required for providing sequences as complex as a mammalian genome. This makes such a method impractical for the isolation of mammalian protein genes when the corresponding mRNAs are unavailable." (Citation continued.)

There thus continues to exist a need in the art for improved methods for effecting the rapid and efficient isolation of cDNA clones in instances where it is known of the amino acid and nucleotide sequences of the polypeptide coded for and where "unlabeled" tissue sources of mRNA are not readily available for use in constructing cDNA libraries. Such improved methods would be especially useful if they were applicable to isolating mammalian genome clones where sparse information is available concerning amino acid sequences of the polypeptide coded for by the gene sought.

B. Erythropoietin As A Polypeptide Of Interest

Erythropoiesis, the production of red blood cells, occurs continuously throughout the human life span to effect cell destruction. Erythropoiesis is a very precisely controlled physiological mechanism in which millions of red blood cells are produced to replace the millions of red blood cells that are lost in the normal daily turnover of the blood. In the bone marrow and in the spleen the production and destruction of red blood cells is carefully monitored to maintain a constant level of circulating red blood cells.

Erythropoietin, an acidic glycoprotein of approximately 34,000 dalton molecular weight, may occur in three forms: a, b, and c. The a and b forms differ slightly in carbohydrate components, but have the same polypeptide, biological activity and molecular weight. The c form is an αβ form with an additional carbohydrate (starch acid) removed. Erythropoietin is present in very low concentrations in plasma when the body is in a healthy state, whereas tissues receive sufficient oxygenation from the existing number of erythrocytes. This normal low concentration is enough to stimulate replacement of red blood cells which are lost normally through aging.

The amount of erythropoietin in the circulation is increased under conditions of hypoxia when oxygen transport by blood cells in the circulation is reduced. Hypoxia may be caused by loss of large amounts of blood through hemorrhage; destruction of red blood cells by over-exposure to radiation; reduction in oxygen delivery due to high altitude; or prolonged unconsciousness, or various forms of anemia. In response to tissues undergoing hypoxic stress, erythropoietin will increase the production of red blood cells. By stimulating the conversion of primitive precursor cells in the bone marrow into precursors which subsequently mature, the erythrocytes and are released into the circulation as red blood cells. When the number of red blood cells in circulation is greater than needed for normal tissue oxygen requirements, erythropoietin in circulation is decreased.


provides low yields of a crude solid extract containing erythropoietin.

Initial attempts to isolate erythropoietin from urine yielded unstable, biologically inactive preparations of the hormone. U.S. Pat. No. 5,385,801 describes a method for stabilizing the biological activity of a crude substance containing erythropoietin recovered from urine. The resulting crude preparation containing erythropoietin purportedly retains 90% of erythropoietin activity, and is stable.

Another method of purifying human erythropoietin from urine of patients with aplastic anemia is described in Minakawa et al., *J. Biol. Chem.*, Vol. 252, No. 15 (Aug. 10, 1977), pp. 5559-5564. The proposed procedure includes ion exchange chromatography, gel filtration, and adsorption chromatography, and yields a pure erythropoietin preparation with a purity of 70-80% of total protein in 21% yield.

U.S. Pat. No. 4,367,840 to Takagawa et al. describes methods for preparing “purified erythropoietin” from healthy human urine specimens with very high base 100 exchangers and processes that the low molecular weight products contained “no inhibitory effect” against erythropoietin.

E.P. Patent Application No. 2,083,897 by Sugarman et al., filed May 6, 1982, describes a process for the production of hybrid human-kidney cells, secreting rapid levels ranging from 5 to 40 Units of erythropoietin per ml of suspension of cells (distributed into the culture dishes in a murine hemopoietic test) containing up to 10^8 cells per ml. At the highest production levels obtained, to 20 units have been obtained, the rate of erythropoietin production could be calculated to be from 30 to approximately 1,000 Units/ml of cells/48 hours in vitro cultures following transfer of cells from in vivo propagation systems. (See also the equivalent: U.S. Pat. No. 4,377,517.) Numerous patents have been made for isolation of erythropoietin from these cell lines, including nucleotide sequences, but the yields have been quite low. See, e.g., Itohara et al., *Exp. Hematol.*, 7(1), 281-288 (1983); Takahashi et al., *J. Nippon Med. Sch.*, 51:380-384 (1983); Kuroki et al., *J. Nippon Med. Sch.*, 51:380-384 (1983); Ogasawara et al., *J. Nippon Med. Sch.*, 51:380-384 (1983); and Kaibara et al., *J. Nippon Med. Sch.*, 51:380-384 (1983).

Other isolation techniques utilized to obtain purified erythropoietin involve immunological procedures. A polyclonal, serum-derived antibody directed against erythropoietin is developed by injecting an animal, preferably a rabbit, with human erythropoietin. The injected erythropoietin is recognized as a foreign antigenic substance by the immune system of the animal and elicits production of antibodies against the antigen. Eroting cells responding to the immune system's response produce and release into circulation antibodies substantially different from those produced by other responding cells. The antibody activity remains in the serum of the animal when its blood is collected. While unpurified or partially purified preparations purified as a serum immunoglobulin G fraction may then be used in arrays to detect and complex with human erythropoietin, the materials suffer from a major disadvantage. Two serum monobody produced at all the different antibodies produced by individual cells, are polyclonal in nature and will complex with components in crude extracts other than erythropoietin alone.

Of interest to the background of the present invention are recent advances in the art of developing continuous cell cultures capable of producing a single species of antibody which is specifically immunologically reactive with a single antigenic determinant of a selected antigen. See, generally


While polyclonal and monoclonal antibodies as described above provide highly useful materials for use in immunore-
BRIEF SUMMARY

The present invention provides, for the first time, novel 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 in vivo and in vitro biological activity) of naturally occurring erythropoietin, including allelic variants thereof. These polypeptides are also uniquely characterized by being the product of proerythropoietin or erythropoietin cDNA cloning or by gene synthesis. Fraud of minimal expression in vertebrates (e.g., mammalian and avian) cells may be further characterized by freedom from association with human proteins or other contaminants which may be associated with erythropoietin in its natural mammalian cellular environment or in ex vivo cell culture systems, or in plasma, or urine. The products of polypeptide transcription (e.g., baculovirus) or proerythropoietin (e.g., E. coli) host cells are free of association with any mammalian proteins. Depending upon the host employed, polypeptides of the invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine unit and residue (position 1).

Nucleic acid products of the invention include those having a primary structural conformation sufficiently duplicative of that of a naturally-occurring (e.g., human) erythropoietin to allow possession of one or more of the biological properties thereof and having an average carbohydrate composition which differs from that of naturally-occurring (e.g., human) erythropoietin.

Vertebrates (e.g., COS-1 and CHO) cells provided by the present invention comprise the first cells ever available which can be propagated in vitro continuously and which upon growth in culture are capable of producing, in the medium of their growth in excess of 100 (preferably in excess of 500) 10 units of erythropoietin per 107 cells in 48 hours as determined by kidney assay.

Also provided by the present invention are synthetic polypeptides wholly or partially duplicative of continuous sequences of erythropoietin amino acid residues which are herein for the first time sterilized. These sequences, by virtue of sharing primary, secondary or tertiary structural and conformational characteristics with naturally-occurring erythropoietin may possess biological activity and/or immunological properties in concert with the naturally-occurring product such that they may be employed as biologically active or immunologically active substances for erythropoietic in therapeutic and immunological processes. Correspondingly provided are monoclonal and polyclonal antibodies generated by established means which are immunoreactive with such polypeptides and, preferably, also immunoreactive with naturally-occurring erythropoietin.

Illustrating the present invention are cloned DNA sequences of glycosylated human polypeptides of polypeptide sequences suitably deduced therefrom which
represent, respectively, the primary structural and functional elements of monkey and human species origins.

Also provided by the present invention are novel biologically functional viral and circular plasmid DNA vectors comprising DNA sequences of the invention together with suitable dienes, histones, and/or histone variants which allow for provision of the vectors in viable host cells in vivo. These vectors can also be used in gene therapy for the treatment of human diseases. The advantage of these vectors is that they are capable of delivering genetic material to the desired cells in vivo.

Polypeptide products of the invention may be labeled by covalent association with a detectable marker substance (e.g., metabolically labeled with 3H) or provide reagents useful in detection and quantification of polynucleotides in solid tissue and tissue samples such as blood or urine. DNA products of the invention may also be labeled with detectable markers such as radioisotopes and non-isotopic labels such as biotin and employed in DNA hybridization processes to locate the polynucleotides in a test sample. 

Isolation and purification of recombinantly expressed polypeptides provided by the invention may be by conventional means including, e.g., preparative chromatographic separations and immunological separations involving monoclonal and/or polyclonal antibody preparations.

Having herein described the sequence of amino acid residues of recombinant polypeptides, the present invention provides for the production and use of recombinant polypeptides in the form of DNA sequences coding for polypeptides and includes several advantages characterized as incorporation of codons "preferred" for expression by selected non-mammalian hosts, provision of sites for cleavage by restriction endonuclease enzymes in the molecular and synthesis of recombinant polypeptides. Additionally, the present invention provides for the manufacture and use of recombinant polypeptides. The DNA sequences coding for polypeptides are recombined in a vector that is compatible with the expression system used to produce the recombinant polypeptide. The vector can be introduced into a host cell by transformation or transfection. 

Novel DNA sequences of the invention include all sequences useful in preparing in a sequence 13 parallel or eastermost 14JC of polypeptides produced having at least a portion of the primary structure thereof, and at least one or more of the biological properties of the polypeptides which are comprised by the DNA sequences set out in FIGS. 5 and 6 herein or any additional sequences encoding therefrom. 

DNA sequences which hybridize under hybridization conditions which are identical as stringent as the conditions to DNA sequences described in (a) of FIG. 6 are DNA sequences which hybridize under less stringent conditions to DNA sequences described in (a). Other sequences described in (a) are DNA sequences which encode DNA sequences which hybridize to DNA sequences defined in (a) and (b) above. Specifically, recombinant DNA sequences encoding the DNA variant forms of monkey and human sequences encoding the DNA sequences described herein are DNA sequences which encode polypeptides which are essentially identical to the polypeptides encoded by the DNA sequence described herein, i.e., "complementary inverted proteins" as described by Tannenbaum et al., Nucleic Acids Research, 2: pp. 3009-3019 (1984).
allowed for the rapid isolation of three positive clones from 
within a screening of 1,500,000 plaque-purified plaques constituting 
a human genomic library. This was accomplished through 
use of the above-noted mixture of 128 20-mer probes together with 
a second set of 128 17-mer probes based on 
amino acid analysis of 2 different continuous sequences of 
a different human epihepatic enzyme.

The above-noted illustrative procedures constitute the 
first known instance of the use of multiple mixed oligo-
nucleotide probes in DNA/DNA hybridization processes 
corrected toward isolation of mammalian genomic clones and the 
first known instance of the use of a mixture of more than 
250 oligonucleotide probes in the isolation of EPO clones.

Numerous aspects and advantages of the invention will be 
aparent not only to those skilled in the art upon consideration of the 
foresaid detailed description which provides illustrations of the 
practices of the invention in their presently preferred embodiment.

Reference is made to FIGS. 1 through 21, wherein:

FIG. 1 is a graphic representation of a radiomunoassay analysis of products of the invention;
FIG. 2 shows vector pdSVL-MbE.
FIG. 3 shows vector pSV3HPdrE.
FIG. 4 shows vector pdSVL-gHdE.
FIGS. 6A, 6B and 6C (collectively referred to as FIG. 6) show the sequence of monkey EPO cDNA and the encoded EPO.
FIGS. 6A, 6B, 6C, 6D and 6E (collectively referred to as FIG. 6) show the sequence of human EPO cDNA and the encoded EPO.
FIG. 7 shows the sequence of the hEPO gene.
FIG. 8 shows the sequence of the MV-2EPO gene.
FIG. 9 shows a comparison of the human and monkey EPO polypeptides.
FIG. 10 shows the EPO gene section 1 of oligonucleotides.
FIG. 11 shows section 1 of the EPO gene.
FIG. 12 shows the EPO gene section 2 of oligonucleotides.
FIG. 13 shows section 2 of the EPO gene.
FIG. 14 shows the EPO gene section 3 of oligonucleotides.
FIG. 15 shows section 3 of the EPO gene.
FIG. 16 shows the EPO gene section 1 of oligonucleotides.
FIG. 17 shows section 1 of the VEGF gene.
FIG. 18 shows the EPO gene section 2 of oligonucleotides.
FIG. 19 shows section 2 of the EPO gene.
FIG. 20 shows the EPO gene section 3 of oligonucleotides.
FIG. 21 shows the section 3 of the EPO gene.

DETAILED DESCRIPTION

According to the present invention, DNA sequences 
encoding part or all of the polypeptide sequence of human and 
monkeys species epihepatic enzymes, at least, “EPO,” have been isolated and characterized. Further, the 
monkeys and human species DNA has been made the subject of enzymatic and non-enzymatic expression, providing isolable 
fraction of the polypeptides having biological (e.g., 
immunological) properties of naturally occurring EPO as 
well as both in vivo and in vitro biological activities of EPO.

The DNA of monkey species origin was obtained from 
a plasmid library constructed with DNA derived from 
the kidney tissue of a monkey in a chemically induced anemic state 
and whose serum was immunologically determined to include 
high levels of EPO compared to normal monkey serum. 
The isolation of the desired cDNA clones containing EPO encoding DNA was accomplished through use of DNA/DNA 
hybridization employing a pool of 238 mixed, radiolabeled, 20-mer oligonucleotide probes and the 
rapid screening of 25,000 colonies. Design of the oligo-
nucleotide probes was based on amino acid sequence information provided by enzymatic fragmentation and sequencing of a small sample of human EPO.

The DNA of human species origin was isolated from a 
human genomic DNA library. The isolation of clones contain-
ing EPO encoding DNA was accomplished through 
DNA/DNA hybridization employing the above noted pool of 238 mixed, 20-mer oligonucleotide probes and a 
second pool of 128 radiolabeled 17-mer probes whose 
sequences were based on amino acid sequence information obtained from a different epihepatic human EPO fragment.

Positive clones and plaques were verified by means of 
direct sequencing of cloned DNA using a subset of 16 
sequences from the pool of 20-mer selected and positive clones were subjected to nucleotide sequence analysis 
resulting in elucidation of primary structural configuration of the 
EPO polypeptides encoded therein. The deduced 
polypeptide sequences displayed high degree of homology 
to each other and to a partial sequence generated by amino 
acid analysis of human EPO fragments.

A selected positive monkey cDNA clone and a selected 
positive human genomic clone were each inserted into a “shuttle” DNA vector which was amplified in E. coli and 
employed to transfected mammalian cells in culture. Cultured 
growth of transfected host cells resulted in culture medium 
supernatant preparations containing as much as 
3000 mU of EPO per ml of culture fluid.

The following examples are presented by way of illus-
tration of the invention and are specifically directed to 
procedures carried out prior to identification of EPO encoding 
monkey cDNA clones and human genomic clones, to 
procedures resulting in such identification, and to the 
sequencing, development of expression systems and immuno-
ological verification of EPO expression in such systems.

More particularly, Example 1 is directed to amino acid 
sequencing of human EPO fragments and comparison of 
mixtures of mammalian plaques based on the results of this 
sequencing.

Example 2 is generally directed to procedures 
involved in the identification of positive monkey cDNA 
clones and thus provides information concerning animal 
medium, and pre-existing mammograms (MFA) analy-
sis of animal sera. Example 3 is directed to the preparation 
of the cDNA library, cloning, hybridization screening and 
verification of positive clones. DNA sequencing of a posi-
tive cDNA clone and the generation of monkey EPO 
polypeptide primary structure configuration, amino acid 
and sequence information. Example 4 is directed to procedures 
involved in the identification of positive human genomic 
clones and thus provides information concerning the source 
of the genomic library, plaque hybridization procedures, 
and verification of positive clones. Example 5 is directed to 
DNA sequencing of a positive genomic clone and the generation 
of human EPO polypeptide amino acid sequence information 
including a comparison thereof in the monkey EPO 
sequence information.

Example 6 is directed to procedures for construction 
of a vector incorporating EPO encoding DNA derived 
from a positive monkey cDNA clone, the use 
of the vector for transformation of COS-1 cells and culti-

vated growth of the transfected cells. Example 7 is directed to 
procedures for construction of a vector incorporating 
EPO encoding DNA derived from a positive human genomic 
clone, the use of the vector for transformation of COS-1 cells and 
cultivated growth of the transfected cells.

Example 8 is directed to procedures performed on the aggregate of transformed cells obtained from the cultivated growth of 
transfected cells.
species EPO and EPO analogs, which genes include a number of preference codons for expression in E. coli and yeast host cells, and to expression systems based thereon. In Example 12, relates to the immunological and biological activity profiles of expression products of the systems of Example 1).

**EXAMPLE 2**

A. Monkey EPO Fragment Amino Acid Sequencing

Human EPO was isolated from urine and subjected to trypsin digestion resulting in the development and isolation of 2 distinct fragments in quantities approximating 160-180 picomoles.

Further analysis revealed that within Fragment No. T35 there existed a series of 7 unique acid reactions (Val, Pro, Phe, Tyr, Ala, Trp, Lys, Tyr) which could be uniquely characterized as unmodified for one of 128 possible DNA sequences spanning 20 base pairs. A first set of 128-mer oligonucleotides was therefore synthesized by standard phosphoramidite methods (see, e.g., Beaucage et al., Tetrahedron Letters, 22, pp. 1859-1862 (1981)) in solid support according to the sequence set out in Table II, below.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Val</th>
<th>Asp</th>
<th>Phe</th>
<th>Tyr</th>
<th>Ala</th>
<th>Thr</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>CAA</td>
<td>T7G</td>
<td>AAG</td>
<td>AAG</td>
<td>CCA</td>
<td>ACC</td>
<td>TT</td>
</tr>
<tr>
<td>T</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>O</td>
</tr>
<tr>
<td>C</td>
<td>O</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

Oligonucleotide probes were labelled at the 5' end with gamma-32P-ATP, 7000-8000 Counts/min (C/M) using T4 polynucleotide kinase (NEB).

B. Purification Procedures

Forma Cynomolgus monkeys were sacrificed (2-3 kg, 1.5-2 years old) without the use of anesthetic and a pool of plasma containing 320-350 microliters of plasma were collected into a 25 ml polyethylene amber stoppered bottle. The plasma was centrifuged for 1 hr at 20,000 x g to remove the red blood cells. The supernatant was filtered through a 0.45 micron membrane and stored at -70°C.

B. RIA for EPO

Radionuclide procedures applied for quantitative detection of EPO in samples were conducted according to the following procedures:

1. An anticlotting standard or unknown sample was incubated together with antiserum for two hours at 37°C. After the two hour incubation, the sample tubes were cooled on ice, and 1.5 ml of a 0.2% RISA solution was added. The tubes were incubated at 0°C for at least 15 more hours. Each assay tube contained 500 ul of solution and an antibody mixture consisting of 50 ul of diluted immune sera, 10,000 cpm of [32P]EPO, 50 ul of trypsin and 0.25 ul of other EPO standard or unknown sample, with BSA containing 0.1% RISA resulting in the remaining volume. The serum protein was the second test bleed of a rabbit immunized with 1% pure preparation of human urinary erythropoietin. The total antisera dilution on the assay was adjusted so that the...
antibody-bound. Each Epo did not exceed 10–20% of the
input total counts. In general, this corresponded to a final
elution volume of 100,000 to 1,103,000.

The antibody-bound Epo-erythropoietin was precipitated
by the addition of 150 ml of 0.5 M NaCl. After 40 min. incubation,
the samples were centrifuged and the pellets were washed
twice with 0.75 ml of 10 mM Tris- HCl pH 8.2 containing
0.15 M NaCl, 2 mM EDTA, and 0.05% Triton X-100. The
washed pellets were counted in a gamma counter to deter-
mine the percent of antibody-bound Epo-erythropoietin.

The counts bound by pre-immune sera were subtracted from all final values to
correct for nonspecific precipitation. The erythropoietin con-
tent of the unknown samples was determined by comparison to
the standard curve.

The above procedure was applied to monkey serum
obtained in Part A, above, as well as to the untreated monkey
serum. Normal serum levels were assayed to contain approxi-
mately 35 units/ml while treated monkey serum con-
tained from 1000 to 10,000 mIU/ml.

EXAMPLE 3

A. Monkey cDNA Library Construction

Messenger RNA was isolated from normal and anemic
monkey kidneys by the guanidinium thiocyanate procedure
of Chirgwin, et al., Biochemistry, 18, p. 5294 (1979) and
puriﬁed by two runs of oligo(dT)-cellulose chromatography as described in pp.
197–198 in Maniatis, et al., "Molecular Cloning, A Laboratory Manual" (Cold Spring Harbor Laboratory, Cold
Spring Harbor, N.Y., 1982). The cDNA library was con-
structed according to a modiﬁcation of the general proce-

The key features of the presently preferment proce-
dures were as follows: (1) pUCS was used as the sole vector;
(2) blunt ends were ligated to the oligo dT tail of 60–80 bases
in length; (3) HindIII digestion was used to remove the oligo
dT tail from one end of the vector; (4) PCR methodology
and oligo dG tailing was carried out according to the
published procedure. BamHI digestion was employed to
remove the oligo dG tail from one end of (1) vector, and (5)
replacement of the RNA strand by DNA was in the presence
of two linkers (GATCTAAAGAAGCTTCCCCCCCCT and
AAGCTTTACG in a three-base overhang) over the oligo
dG tail existed. The replacement of the RNA strand by DNA was
in the presence of two linkers

B. Colony Hybridization Procedures For Screening Monkey
cDNA Library

Transformed E. coli were spread out at a density of 3000
colony-forming units per 10 cm plate on nutrient plates containing
30 micrograms/ml ampicillin. GeneScreen filters (New
England Nuclear Catalog No. NIP-027) were prepared on a
BioRad plate (800 mm X 800 mm) at 37 gL.

Asparagine acids 2.5 ml (150 mg of solution), containing 500
micrograms/ml chloramphenicol) and were used to instruct
the colonies on the plate. The colonies were grown in the
incubator at 37°C for 12 hours, or longer to amplify the plasmid copy
number. The amplified colonies (colony colonies) were treated
by serially placing the plasmid over 2 pieces of
Whatman 3 MM paper saturated with each of the following
solutions:

1. 30 ml RNAase—25 ml 3 M NaCl (pH 8.0)—10 mM

EDTA (pH 8.0) for five minutes,

2. 0.5 M NaOH for ten minutes, and

3. 1.0 M Tris-HCl (pH 7.5) for three minutes.

The filters were then air dried in a vacuum at 30°C for

The filters were then subjected to Proteinase K digestion
through treatment with a solution containing 50 micrograms/ml
of the protease enzyme in buffer K (0.1 M Tris-HCl (pH
8.0)—0.15 M NaCl—10 mM EDTA (pH 8.0)—0.05 M Tris- HCl, pH 6.8, containing

1.0 M Tris-HCl (pH 8.0) for five minutes,

2. 0.5 M NaOH for ten minutes, and

3. 1.0 M Tris-HCl (pH 7.5) for three minutes.

The filters were then air dried in a vacuum at 30°C for
two hours.
TABLE IV—continued

<table>
<thead>
<tr>
<th>Location</th>
<th>Recognition Motif</th>
<th>Apparent Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From: 5,547,933

8. Plaque Hybridization Procedures For Screening Human Genomic Library

Phage particles were added to the DNA's were fixed to filters (53,000 plaques per filter) according to the procedures of \textit{Nature}, \textit{Nature}, \textit{Journal of Virology}, 58, pp. 355-362 (1979) except for the use of Glass.QLabel Plus filters (New Zealand Nuclear Catalog No. NEP-172) and NEP-AM (NNE, 7 g, MgCl₂ 4H₂O, 2 g, Na₂HPO₄, 10 g, yeast extract, 5 g, casamino acids, 2 g, maltose, 2 g; and agar, 15 g per liter). The pre-washed filters were washed at 80°C for 1 hour and then digested with Proteinase K as described in Example 3, Para. B. Hybridization was carried out with 41% formamide - 1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Para. D. Hybridization was carried out with 1.0 M NaCl - 0.1% SDS buffer at 35°C for 1 hour or more after which the buffer was removed. Hydro
of the human EPO gene, two base pairs of which ("X") have not yet been unambiguously sequenced.

FIG. 6 thus serves to identify the primary structural conformation (amino acid sequence) of mature EPO, as depicted in Table 16, and the amino acid residues estimated, with M.W. = 18,000. Also included in the Table is the DNA sequence coding for a 22 residue leader sequence along with 5' and 3' DNA sequences which may be significant to promoter/operator functions of the human gene. Sites for potential glycosylation of the mature human EPO polypeptide are designated in the Figure by asterisks. It is worthy of note that the specific amino acid sequence of FIG. 6 likely consists of a naturally occurring site form of human erythropoietin. Support for this position is found in the results of continued efforts to sequence of urinary isolates of human erythropoietin in which the finding that a significant number of erythropoietin molecules therein have a methionine at residue 128 as opposed to a serine, as shown in the Figure.

FIG. 9 illustrates the extent of polypeptide sequence homology between human and monkey EPO. In the upper continuous line of the Figure, single letter designations are employed to represent the deduced translated polypeptide sequences of human EPO commencing with residue 27 and the lower continuous line shows the deduced polypeptide sequence of monkey EPO commencing at assigned residue number 27. Asterisks are employed to highlight the sequence homology. It should be noted that the deduced human and monkey EPO sequences reveal a "potential" lysine (K); residue at position 116. Cross-reference in FIG. 6 indicates that this residue is at the margin of a putative mRNA splice junction in the genomic sequence. Presence of the lysine residue in the human polypeptide sequence was further verified by sequencing of a cDNA human sequence clone isolated from mRNA isolated from COS-7 cells transformed with the human genomic DNA in Example 7, infra.

EXAMPLE 6

The expression system selected for initial attempts at microbial synthesis of isolable quantities of EPO polypeptide material code for by the monkey EPO cDNA provided by the procedures of Example 3 was one involving mammalian host cells, i.e., COS-7 cells, ATCC No. CRL-1650. The cells were transfected with a "shuttle" vector capable of autonomous replication in E. coli host (by virtue of the presence of pBR322-derived DNA) and the mammalian host (by virtue of the presence of SV40 virus-derived DNA).

More specifically, an expression vector was constructed according to the following procedures. The plasmid clone 83 provided in Example 3 was excised in E. coli and the approximately 1.4 kb monkey EPO-expressing DNA was isolated by EcoRI and HindEII digestion. Separately isolated was an approximately 4.0 kb, LinIII/Sall fragment from pBR322. An approximately 30 bp, EcoRI/Sall "linker" fragment was obtained from M13mp18(1) DNA (Ph. 1, Laboratories). This linker included, in series, an EcoRI sticky end, followed by Sall, Smal, and HindEII recognition sites and a Sall sticky end. The above three fragments were ligated to generate an approximately 5.4 kb intermediate plasmid ("pERS") wherein the EPO DNA was flanked on one side by a "basic" useful restriction endonuclease recognition sites and theSall sequence was a "stitched" sticky end. The two fragments were ligated to generate an approximately 3.0 kb intermediate plasmid ("pELS") wherein the EPO DNA was flanked on one side by a "basic" useful restriction endonuclease recognition site and theSall site was a "stitched" sticky end. The EPO DNA was inserted into the BamHI site of pBR322 and another M13mp18(1) HindIII/BamHI RF fragment linker also having approximately 50 bp. The M13 flanking fragment was characterized by a Sall sticky end, followed by PstI, SalI, XbaI recognition sites and a BamHI sticky end. The ligated product was, again, a useful intermediate plasmid ("pBR-EPO") including the EPO DNA flanked on both sides by BamHI recognition site.

The vector chosen for expression of the EPO DNA in COS-1 cells ("pSVL1") had previously been constructed to allow for selection and autonomous replication in E. coli. These characteristics are provided by the origin of replication and ampicillin resistance gene DNA sequences present in the region spanning nucleotides 2448 through 4362 of pBR322. This sequence was modified by the addition of a linker providing a BamHI recognition immediately adjacent nucleotide 2448 prior to incorporation in the vector. Among the selected vectors, a useful property was the capacity to autonomously replicate in COS-1 cells and the presence of a viral promoter sequence functional in mammalian cells. These characteristics are provided by the origin of replication DNA sequences and "site gene" viral vector DNA sequence present in 3'-2 bp sequence spanning nucleotide numbers 2448 through 270 of the SV40 genome. A unique restriction site (BamHI) was provided in the vector and immediately adjacent the viral promoter sequence through use of a commercially available linker sequence (Collaborative Research). Also incorporated in the vector was a 241 base pair sequence derived from nucleotide numbers 2353 through 2470 of SV40 containing the "site gene" viral EPO polymerase signal (commonly referred to as a transcription terminator). This fragment was positioned in the vector in the proper orientation via a via the "site gene" viral promoter via the unique BamHI site. Also present in the vector was another unique genetic at the position to allow potential transcription of a gene inserted at unique BamHI site, between the viral promoter and terminator sequences. The orientation gene comprised an approximately 2500 bp mouse hypoxia-induced transducer (OHTR) substituting into an internal pBS/LV as in Gasser et al., PA.N.S. (U.S.A.), 79, pp. 6522-6525, (1982). Again, the major operational component of pBS/LV comprises nucleotides 2448 through 4362 of pBR322 along with nucleotides 2448 through 270 (242 bp) and 2353 through 2470 (257 bp) of SV40 DNA.

Following procedures described, e.g., in Maeda et al., supra, the EPO-expressing DNA was isolated from plasmid pBR-EPO as a BamHI fragment and digested into BstEII and BamHI. Reduction unique analysis was employed to confirm insertion of the EPO gene in the correct orientation in two of the resulting cloned vectors (BstEII and BstEII). As shown in FIG. 2, illustrating plasmid pBS/LV, vectors with EPO genes in the correct orientation vectors F-X and Q) were saved for use as positive controls in transfection experiments designed to determine EPO expression levels in host transformed with vectors having EPO DNA in the correct orientation.

Vectors H, E, X and Q were combined with carrier DNA (mouse liver and spleen DNA) were employed to transduce duplicate 50 mm plates by calcium phosphate transfection methods. Duplicate 50 mm plates were also transfected with carrier DNA as a "mock" transfection negative control. After five days all culture media were tested for the presence of polypeptide possessing the immunological properties of naturally occurring EPO.
EXAMPLE 7

A. Initial EPO Expression System Involving COS-1 Cells

The system selected for initial attempts at microbial synthesis of immobilized proteins of the human EPO polypeptide material coded for by the human genomic DNA EPO clone, also involved expression in mammalian COS-1 cells (COS-1 cells, ATCC No. CCL-104). The human EPO gene was first subcloned into an "expression" vector which is capable of autonomous replication in both E. coli hosts (by virtue of the presence of pBR322 derived DNA) and in the mammalian cells COS-1. By virtue of the presence of SV40 virus derived DNA. The shuttle vector, containing the EPO gene, was then transfected into COS-1 cells. EPO polypeptide material was produced in the transfected cells and secreted into the cell culture media.

More specifically, an expression vector was constructed according to the following procedures. DNA isolated from lambda clone K11, containing the human genomic EPO gene, was digested with BamHI and HindIII restriction endonucleases, and a 5.6 kb DNA fragment known to contain the entire EPO gene was isolated. This fragment was mixed with the bacterial plasmid pUC18 (Bethesda Research Laboratories, Inc.) which had been similarly digested, creating the intermediate plasmid "pUC8-HEX", providing a convenient source of the restriction fragment.

The vector chosen for the expression of the EPO DNA in COS-1 cells (pSV865) had previously been constructed. Plasmid (SV865) consisted of DNA sequences allowing selection and autonomous replication in E. coli. These characteristics are provided by the origin of replication and Ampicillin resistance gene DNA sequences present in the originally spanning nucleotides 2148 through 4362 of the E. coli plasmid pUC19. The origin of autonomous replication (nucleotide numbers 3171 through 270) in this fragment had been modified by the addition of a linker providing a HindIII recognition site immediately adjacent to nucleotide 2448. Plasmid (pSV865) was also capable of autonomous replication in COS-1 cells. This characteristic was provided by a 342 bp fragment containing the SV40 virus origin of replication (nucleotide numbers 3171 through 270). This fragment had been modified by the addition of a linker providing an EcoRI recognition site adjacent to nucleotide 270 and a linker providing a SalI recognition site adjacent to nucleotide 3171. A 165 bp fragment of SV40 was also present in this vector between nucleotides 1715 through 2252, again providing a SalI recognition site next to nucleotide 2772. Within this fragment was an unique BamHI recognition sequence. In summary, plasmid (pSV865) contained unique BamHI and HindIII recognition sites, allowing insertion of the human EPO gene sequence without restriction and selection in E. coli, and sequences allowing replication in COS-1 cells.

In order to insert the EPO gene into pSV865, plasmid pUC8-HEX was digested with BamHI and HindIII restriction endonucleases, and the 5.6 kb EPO encoding DNA fragment isolated. pSV865 was also digested with BamHI and HindIII and the major 2513 bp fragment isolated (preserving all necessary functions). These fragments were mixed and ligated, creating the final vector "pSV865-EPO".

This vector was propagated in E. coli and form vector DNA isolated. Restriction enzyme analysis was employed to confirm insertion of the EPO gene.

Plasmid pSV865-EPO (pSV865-EPO) was used to express human EPO polypeptide material in COS-1 cells. More specifically, pSV865-EPO DNA was extracted with carrier DNA encoding a repetitive 60 mmol plates of COS-1 cells. As a control, carrier DNA alone was also transfected into COS-1 cells. Cell culture media were sampled five and seven days later and tested for the presence of polypeptides possessing the immunological properties of naturally occurring human EPO.

B. Second EPO Expression System Involving COS-1 Cells

Still another system was designed to provide improved production of human EPO polypeptide material coded by the human genomic DNA EPO clone in COS-1 cells (A.T.C.C. No. CCL-104).

In the second system, EPO was expressed in COS-1 cells using a recombinant expression vector, the 5.6 kb BamHI to HindIII restriction fragment. In the following construction, the EPO gene is altered so that it is expressed using the SV40 human promoter.

More specifically, the cloned 5.6 kb BamHI to HindIII genomic human EPO restriction fragment was modified by the following procedures. Plasmid pUC8-HEX, as described above, was cleaved with BamHI and with HindIII restriction endonucleases. SV40 virus DNA within the 5.6 kb EPO gene was inserted at a position which is 44 base pairs 5' to the initiating ATG coding for the pre-polypeptide and approximately 680 base pairs 5' to the HindIII restriction site. The approximately 4300 base pair fragment was isolated. A synthetic linear DNA fragment, containing SalI and HindIII sticky ends and an internal BamHI recognition site was synthesized and purified. The two fragments were mixed and ligated with plasmid pBR322 which had been cut with SalI and HindIII to produce the intermediate plasmid pSVE75. The genomic human EPO gene can be contained herein at a 4000 base pair BamHI digestion fragment containing the complete structural gene with a single ATG 44 base pairs 5' to BamHI site adjacent to the amino terminal coding region.

This fragment was isolated and inserted into a BamHI fragment from BamHI digest of plasmid pSV861 as described in example 6. The resulting plasmid, pSVE175EPO, as illustrated in FIG. 4, was used to express EPO polypeptide material from COS-1 cells, as described in Examples 6 and 7.

EXAMPLE 8

Culture media from growth of the six immortalized COS-1 cultures of Example 6 were analyzed by radiimmunoassay according to the procedures set forth in Examples 2 and 3.

Each sample was assayed at 250, 50, 25, and 12.5 nanograms of antibody levels. Supernatants from growth of cells are not transfected or transfected with vectors having incorrect EPO gene orientation were undetectably negative for EPO immunoreactivity. For each sample of the two supernatants derived from growth of COS-1 cells transfected with vectors 9 and 1, leaving the EPO DNA in the correct orientation, the percentage inhibition of [125I]EPO binding to antibody ranged from 72 to 88%, which places all values at the top of the standard curve. The exact concentration of EPO in the culture supernatants could not be reliably estimated. A quite conservative estimate of 250 molar units was made, however, from the value calculated at the largest antibody size (526 nanograms).

A representative culture fluid according to Example 6 and five and seven day culture fluids obtained according to Example 7A were assayed in the RIA in order to compare activity of recombinant monkey and human materials to a normally-restricting human EPO standard and the results are set out in graphic form in FIG. 3. Initially, the results unexpectedly revealed that the recombinant monkey EPO significantly competed for anti-human EPO antibody although it was not able to completely inhibit binding under the test conditions. The maximum percent inhibition with ne
for recombinant human EPO however, closely approximated those of the human rheumatoid factor. Therefore, the drug response curves suggested immunological identity of the sequences (epitopes) in common. Prior estimates of monkey EPO in culture fluids were re-evaluated at these higher dilution levels and were found to range from 2.00 to 2.12 U/ml. Estimated human EPO production levels were correspondingly set at 3.92 ml/ml for the five day growth sample and 2.57 ml/ml for the seven day growth sample. Estimated monkey EPO production levels in the Example 78 expression system were on the same order or better.

**EXAMPLE 9**

Culture fluids prepared according to Examples 6 and 7 were subjected to an in vitro assay for EPO activity according to the procedure of Goldwaich, et al., *Endocrinology*, 97, pp. 185–188 (1976). Estimated monkey EPO values for culture fluids tested ranged from 3.2 to 4.3 U/ml. Human EPO culture fluids were also active in this in vitro assay as well. Further, this activity could be neutralized by anti-EPO antibody. The recombinant monkey EPO culture fluids according to Example 6 were also subjected to an assay for in vivo biological activity according to the general procedures of Cotes, et al., *Nature*, 194, pp. 2065–2067 (1961) and Hammond, et al., *Ann. Acad. Sci.,* 149, pp. 316–327 (1968) and activity levels ranged from 0.94 to 1.24 U/ml.

**EXAMPLE 10**

In the previous examples, recombinant monkey or human EPO material was produced from vectors as used in transfected COS-1 cells. These vectors contain COS-1 cells due to the presence of SV40T antigen within the cells and an SV40 origin of replication on the virus. These vectors produce useful quantities of EPO in COS-1 cells, expression is only transient (7 to 40 days) due to the eventual loss of the vector. Additionally, only a small percentage of the cells are positively transfected with the vectors. The present example describes expression systems employing CHO DHFR cells and CHO DHFR cells transfected with the selected DIFR gene. For discussion of eukaryotic expression systems, see U.S. Pat. No. 4,399,216 and European Patent Applications 17688, 17689 and 17706, all published Aug. 29, 1984.4

CHO DHFR cells (DoxxB-D) CHO K1 cells. Ushio, et al., *Proc. Nat. Acad. Sci.,* Vol. 72, 4554 (1985) lack the enzymatic hydrolase reductase (DHFR) due to mutations in the structural genes and therefore require the presence of glycine, cytosamine, and thymidine in the culture media. Plasmid d152US-MDE (Example 6) or pD152US-MDE (Example 7) were transfected along with carrier DNA into CHO DHFR cells growing in media containing hydrolase reductase, thymidine, and glycine in 50 mm culture plates. Plasmid p54514UHPE (Example 7A) was mixed with the plasmid p52G containing a mouse dhfr reductase gene cloned into the bacterial plasmid vector pBR322 (see Gasser, et al., supra). The plasmid mixture and carrier DNA were transfected into CHO DHFR cells (cells which acquire one plasmid and generally also acquire a second plasmid). After three days, the cells were dispersed by centrifugation into several 100 mm culture plates in media lacking hydrolase reductase and thymidine. Only those cells which have been stably transformed with the DHFR gene, and thereby the EPO gene, survive in this media. After 21 days, colonies of surviving cells became apparent. These transformant colonies, after dispersion by centrifugation can be continuously propagated in media lacking hydrolase reductase, thymidine, and glycine in 50 mm culture plates. Cells resistant to EPO were obtained from these transformed colonies by plating in the presence of EPO. The resulting clones were then assayed to determine the EPO activity.

Culture fluids fromCHO pSVghEPO and CHO pD152US-MDE contained EPO with immunological properties like that obtained from COS-1 cells transfected with monoclonal pD152US-MDE. A representative 5 day culture fluid contained EPO at 0.02 U/ml.

Culture fluids from CHO pSVghEPO and CHO pD152US-MDE contained EPO with immunological properties like those obtained with CHO-1 cells transformed with monoclonal pD152US-MDE. A representative 5 day culture fluid contained EPO at 1.02 U/ml. Human EPO activity was tested by RIA.

The quantity of EPO produced by the cell lines described above can be increased by gene amplification giving new cell strains of greater productivity. The enzyme hydrolase reductase (DHFR), which is the product coded for by the DHFR gene can be inhibited by the drug methotrexate (MTX). More specifically, cells propagated in media lacking hydrolase reductase and thymidine are inhibited or killed by MTX. Under the appropriate conditions, (e.g., minimal concentrations of MTX), cells resistant to and able to grow in MTX can be obtained. These cells are found to be resistant to EPO due to an amplification of the number of DHFR genes, resulting in increased production of the EPO enzyme. The surviving cells can, in turn, be treated with increasing concentrations of MTX, resulting in cell strains containing greater numbers of DHFR genes. "Passenger" genes (e.g., EPO) carried on the expression vector along with the DHFR gene are frequently found also to be increased in their gene copy number.

As examples of practice of this amplification system, cell strain CHO pD152US-MDE was subjected to increasing MTX concentrations (0.05, 0.10 and 0.15 mM). Representative 7 day culture media samples from each concentration step were assayed by RIA and determined to contain 0.00, 2.45 and 6.10 U/ml, respectively. Cell strain CHO pD152US-MDE was subjected to a series of increasing MTX concentrations (0.05, 0.10, 0.20, 0.30, 0.5, and 5 mM) MTX. A representative 7 day culture media sample from the 0.10 mM MTX step contained recombinant EPO at 0.069±0.20 as judged by RIA. Representative 7 day culture media samples from the 0.10, 0.20, 0.30, 0.5, and 5 mM MTX were determined to contain, respectively, human EPO at 4.06 and 13.73 U/ml as judged by RIA (average of triplicate assays). In these procedures, 500 ml cultures were plated to 3 x 10<sup>6</sup> cells/ml in 50 mm culture plates. Twenty-four hours later the media were removed and replaced with 5 ml of serum-free media (high glucose DMEM supplemented with 0.1 mM non-essential amino acids and L-glutamine). EPO was allowed to accumulate for 48 hours in the serum-free media. The media was collected for RIA assay and the cells were harvested and counted. The average RIA values of 4.06 U/ml and 13.73 U/ml for cells grown at 0.10 mM and 5 mM MTX, respectively, provided actual yields of 255.2 U/ml and 678.5 U/ml. The average cell number per plate was 1.9 x 10<sup>6</sup> and 3.1 x 10<sup>6</sup> cells, respectively. The effective production rates for these culture conditions were 1264 and 2317 U/ml per cell day, respectively.

The cells in the cultures described immediately above are a genetically heterogeneous population. Standard screening
procedures are being employed in an attempt to isolate genetically homogeneous clones with the highest production capacity. See, Section A, Part 1, of "Points to Consider in the Characterization of Cell Lines Used to Produce Biologics", June 1, 1986, Office of Biologics Research and Review, Center for Drug Evaluation and Research, U.S. Food and Drug Administration.

The productivity of the EPO-producing CHO cell line E25A described above can be improved by appropriate cell culture techniques. The propagation of mammalian cells in culture generally involves the presence of serum in the growth media. A method for production of erythropoietin from CHO cells in medium that does not contain serum greatly facilitates the production of erythropoietin from the culture medium. This method described below is capable of economically producing erythropoietin in serum-free media in large quantities sufficient for production.

Stable CHO pHSV-LGL60E cells, grown in serum-free cell culture conditions, are used to seed sparger cell culture flasks. The cells are propagated in a suspension cell line in the presence of 100 μg/ml of geneticin and a 50-60% mixture of high glucose DMEM and Ham’s F12 supplemented with 5% fetal bovine serum, l-glutamine, penicillin, and streptomycin, 0.05 mM non-essential amino acids and 250 μg/ml of gentamicin. Suspension cell culture allows the EPO-producing CHO cells to be expanded easily to large volumes. CHO cells, grown in suspension, are used to seed roller bottles at an initial seeding density of 1 x 10^5 viable cells per 850 cm^2 roller bottle in 300 ml of medium. The cells are allowed to grow to confluence in an subconfluent cell. After three days, the media used for this phase of the growth is the same as for growth in suspension. At the end of the three-day growth period, the serum-containing media is removed and replaced with 300 ml of medium consisting of 50:50 mixture of high glucose DMEM and Ham’s F12 supplemented with 0.05 mM non-essential amino acids and l-glutamine. The roller bottles are returned to the roller bottle incubator for a period of 1-2 hours and the media again is removed and replaced with 500 ml of fresh serum-free media. The 1-3 hours incubation of the serum-free media reduces the concentration of contaminating serum proteins. The roller bottles are retrieved from the incubator for seven days during which erythropoietin accumulates in the serum-free culture media. At the end of the seven-day production phase, the concentrated media is removed and replaced with fresh serum-free medium for a second production cycle. An example of the procedure of this production system, a representative seven-day, serum-free media, sample collected human erythropoietin at 389.2±6.0 U/ml as judged by the RIA. Based on the estimated cell density of 0.9 to 1.2 x 10^6 cells/ml, each 850 cm^2 roller bottle contained 7.0 x 10^9 to 1.2 x 10^10 cells/ml and thus the rate of production of EPO in the 7-day, 100 ml culture was 750 to 1400 U/ml over 72 hours.

Culture fluid from cell strain CHO pHSV-LGL60E cells grown in 10 mM MTX were subjected to the three assays. A 5.5 day sample contained recombinant human EPO to the media at a level of 18.5 U/ml by RIA assay, 12.5±4.6 U/ml by in vivo assay and 16.2±3.0 U/ml by in vivo assay.

Culture fluid from CHO pHSV-LGL60E cells propagated by aseptically 100 mM TX were subjected to the three assays. A 5.0 day sample contained recombinant human EPO to the media at a level of 159.9±29.9 U/ml by RIA, 15.9±21.5 U/ml by in vivo assay and 29.9±16.9 U/ml by in vivo assay.

Molecular characterization of CHO production revealed an amino acid sequence corresponding to that depicted in FIG. 6.

Cell supernatants from CHO cells transfected with plasmid pDCV1-LGL5 were performed, and the MTX dialyzed out over several days, resulting in media with an EPO activity of 21.4±1.1 U/ml (EPO-CCM). The determination of the in vivo effect of the EPO-CCM upon hemocrit levels in normal Balb/c mice, the following experiment was conducted. Cell conditioned media (1 ml) was transfected into PBS. The CC-CCM was used for the production of 40 units of recombinant human erythropoietin—7 units per injection and 2 units per injection—were employed for the experimental groups (2 mice/group). Over the course of 5 weeks, the mice were injected transtracheally, 3 times per week. After the eighth injection, average hemocrit values for the control group were determined to be 52.4±6.9% for the U group, 53.4±14.6% for the 44 U group, 67.7%.

Maximal cell expression products may be readily recovered in substantially purified form from culture media using HPLC (UV) employing an ethanol gradient, preferably at pH 4.5.

A preliminary attempt was made to characterize recombinant erythropoietin produced in COS-1 and CHO cell lines by comparison to human urinary EPO isolates using both Western blot analysis and SDS-PAGE. These studies indicated that the CHO-produced EPO material had a somewhat higher molecular weight than the COS-1 expression product which, in turn, was slightly larger than the pooled source human urinary extract. The products were identical by SDS-PAGE analysis of the recombinant CHO product and the urinary extract material (to reduce loss due to high molecular weight products in differently reduced solutions).

Purified human urinary EPO and a recombinant CHO cell-produced EPO, both according to the invention, were subjected to carbohydrate analysis using the procedure of M. E. L. H., et al., Methods in Enzymology, Vol. 15, 131-191 (1983) as modified through use of the two-step procedures of Nelson, et al., Anal. Biochem., 142, 18-67 (1984). Experimentally determined carbohydrate content values were obtained as multiples of carbohydrate in the product for the urinary isolate were as follows: Hexoses, 1.73; N-acetylgalactosamine, 1; N-acetylgalactosamine, 0.95; Fucose, 1; and N-acetylglucosamine, 0. Corresponding values for the recombinant product (derived from CHO pHSV-LGL60E) for 3 days, EPO activity at 160 mM MTX are as follows: Hexoses, 1.86; N-acetylgalactosamine, 0.99; Fucose, 1; and N-acetylglucosamine, 1. These findings are consistent with the Western blot and SDS-PAGE analysis described above.
Glycosylation products provided by the present invention are thus comprehensive of products having a primary structural configuration sufficiently different from that of naturally-occurring glycoprotein to allow production of one or more of the biological properties thereof and having an average carbohydrate composition which differs from that of naturally-occurring glycoproteins.

EXAMPLE 11

The present example relates to the overall manufacture by assembly of nucleic bases into two basic constructs - one encoding the human EPO sequence and the other encoding a marker (or gene) for expression in E. coli and yeast (S. cerevisiae) cells. Also disclosed is the construction of genes encoding analogs of human EPO. Briefly stated, the protocol employed was generally as set out in the previously cited disclosure of Alton et al. (WO 83/00453). The genes were designed for initial assembly of component oligonucleotides into multiple duplications which, in turn, were assembled into three discrete sections. These sections were designed for rapid amplification and, upon removal from the amplification system, could be assembled sequentially or through a multiple fragment ligation in a suitable expression vector.

FIGS. 10 through 15 illustrate the design and assembly of a modified gene encoding a human EPO translation product lacking any N-glycans or precursors, but including an initial methionine residue at position 1. Moreover, the gene incorporated the complete pET-11a(+) expression codons and the construction was therefore referred to as the "ECEO" gene.

More particularly, FIG. 10 illustrates oligonucleotides employed to generate the ECEO gene encoding a mature sequence residue to position 45, including the complete pET-11a(+) expression codons and the construction was therefore referred to as the "ECEO" gene.

The manufactured ECEO gene above may be variably modified to encode glycoprotein analogs such as [Asna des-Asn] through [HheI] and [His] through [HheI], as described below.

A) [Asna des-Asn] through [HheI] and [His] through [HheI]

Plasmid 536 carrying the ECEO gene of FIG. 7 was digested with HindIII and EcoRI. The latter endonuclease cuts the ECEO gene at a unique 8-base pair recognition site spanning the last base of the codon encoding Arg iso* through the second base of the Arg iso codon. A Xhol/XhoI "linker" sequence was manufactured having the following sequence:

<table>
<thead>
<tr>
<th>XhoI</th>
<th>+</th>
<th>-</th>
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<tbody>
<tr>
<td>Xhol</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CCGG</td>
<td>TGGG</td>
<td></td>
</tr>
<tr>
<td>GAGT</td>
<td>TCTC</td>
<td></td>
</tr>
<tr>
<td>CHH</td>
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<td></td>
</tr>
<tr>
<td>CHH</td>
<td>CCH</td>
<td></td>
</tr>
</tbody>
</table>

The XhoI/XhoI linker and the Xhol/HindIII ECEO gene sequence fragment were inserted into the larger fragment resulting from Xhol and HindIII digestion of plasmid pCM189 as a derivative of plasmid pCM184 (A.T.C.C. 40707) - as described in co-pending U.S. patent application Ser. No. 102,732, filed Aug. 6, 1984, published EPO Application No. 136,490) by Charles F. Morris. More specifically, plasmid 536 was digested with XhoI and HindIII, and the fragment was isolated and ligated into a two-part ligation with the ECEO gene. Sections 1 (XhoI/HindIII) and 3 (HindIII/HindIII) had been previously assembled in the correct order in M13 and the ECEO gene was isolated therefrom as a single XhoI/HindIII fragment. This fragment contained a portion of the polylinker from M13 and a fragment spanning the SnaBI to HindIII sites. Control of expression was achieved using the resulting expression plasmid, p536, which was of a lambda P1 promoter, which itself may be under control of the CMV enhancer region (such as provided in E. coli strain K12/Mp).
The linker and the XhoI/HindIII PCR sequence fragments were then inserted into pSCM26 to generate a plasmid containing DNA sequence encoding 200 of the expression of the M. genitalium 2004 genome.

Construction of a functional gene ("SCFO") incorporating yeast fermentation in the following Figs. 16 through 21 and 19. As was the case with the other genes, the entire construction involved formation of those genes of oligonucleotides (Figs. 16, 18 and 20) which were then inserted into ligases and assembled into expression vectors (Figs. 17, 19 and 21). Note that this synthesis was facilitated in part by use of some sub-optimal codons in both the SCFO and ECFLO cistrons, i.e., oligonucleotides 7-12 of Section 1 of both genes were identical, as were oligonucleotides 1-4 of Section 2 in each case.

The assembled SCFO cistrons were sequenced at M13 end. Sequences 1, 2 and 3 were identical to the m13, M13/P1Km, M13/P1A, and PHE trials fragments.

The presence of preferred expression system for SCFO gene products in a selection system based on a Saccharomyces cerevisiae strain, as described in the US patent application Ser. No. 487,723, filed Aug. 22, 1985, by Grant A. R. (U.S. Patent Application No. 325,280). Briefly, the system involves constructions wherein DNA encoding the leader sequence of the yeast hexokinase gene, followed immediately to the capping region of the expression gene to be expressed. As a result, the gene product transcribed includes a leader or signal sequence which is "processed off" by an endogenous yeast enzyme in the course of secretion of the remainder of the transect. Because the construction contains some of the leader region of the yeast hexokinase gene, there was no need to provide such a sequence in the polypeptide of the SCFO gene. As may be noted from Fig. 8, the leader (+1) encoding sequence is preceded by a leader sequence allowing for direct insertion into a plasmid containing the DNA for the first 60 residues of the factor leader following the factor gene. Thus, the preferred expression system for SCFO gene expression involved a four-part region encoding the above-named SCFO sequence fragments and the large fragment of HindIII/Sall digestion of plasmid pUC12. From the resulting plasmid pSCM26, the factor promoter and leader sequence and SCFO gene were isolated by digestion with HindIII and ligated into HindIII-cleaved plasmid DPN to form expression plasmid pYE/SCFO.

EXAMPLE 12

The present example reduces expression of recombinant products of the mRNA extracted from human cells. The expression system was designed for the expression of human SCFO and SCFO genes with expression systems of Example 11.

In the expression system designed for use of E. coli, heat cells, plasmid p325 of Example 11 was transformed into AM7 E. coli cells. A suitable host, p325, harbored a "Delta gene". Cultures selected on LB broth (Amipicillin at 50 µg/ml) and kanamycin 5 µg/ml, preferably with 30 mM MgSO4, were incubated at 38°C for 8 hours. Heat shock (20% of culture) at 38°C and then growth of cells in culture to OD_{490}=0.1, ETOH expression was induced by raising the culture temperature to 42°C. Cells grown to about 10 D.U. provided ETOH production (as estimated by gel) of about 5 µg/D.U. liter.

Cells were harvested, lysed, broken with French Press (10,000 psi) and treated with lysostaphin and NP-40 detergent. The pellet resulting from 20,000g centrifugation was washed with sodium bicarbonate and subjected to further purification in a single step by means of C, (Novex) Reverse Phase HPLC (Bond, 0.6 x 30 cm). and then on a ANH prep. HPLC (3.2 x 30 cm). Protein concentration revealed the product to be greater than 95% pure and the product obtained revealed two different amino-terminal peptides, A-P-R-P... and P-R... in a relative quantitative ratio of about 3:1. This latter compound, not [HLO] [HLO] and [HLO] [HLO] [HLO] products indicate that amino terminal "processing" within the host cells serves to remove the terminal methionine and in some instances the initial alanine. Tryptic or nuclease activity for the isolated was at a level of 15,000 to 16,000 U/mg. In vitro assay activity was at a level of 20,000 to 62,000 U/mg. This activity ranged from about 120 to 720 U/mg. (CL, human urinary isolate standard of 70,000 U/mg in each assay.) The dose response curve for the recombinant product in the in vivo assay differed markedly from that of the human urinary standard.

The EPO analog phasmids formed in parts A and B of Example 11 were each transferred into pMV8-transformed AM7 E. coli cells and the cells were cultured as above. Purified isolates were tested in both RIA and in vitro assays RIA and in vitro assay values for [Asp] des-Phe through [HLO] [HLO] expression products were approximately 1,000 U/mg and 5000 U/mg protein, respectively, while the assay values for [HLO] [HLO] were about 41,000 U/mg and 14,000 U/mg protein, respectively, indicating that the analog products were from one-fourth to one-tenth as "active" as the "parent" expression product in the assays.

In the expression system designed for use of S. cerevisiae host cells, plasmid pSV2526CO was transformed into two different strains, YEPD4 (genotype upa-p4-3) and R3E51 (genotype papa-3 upa). Transformed YEPD4 hosts were grown in SD medium (Materials in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 62 (1985)) supplemented with adenine and 0.5% sucrose at 30°C (papa-3 mutants grown at 30°C). A strain was chosen to which the cells had been grown to 10 D.U. and contained ETOH products at levels of about 544 U/D.U (97 µg/D.U. liter by RIA). Transformed VK1 cells grown to either 65 D.U. or 63 D.U. provided results with ETOH concentrations of about 80 µg/ml and 24 µg/ml by RIA. Preliminary results were obtained by the expression system, likely to be due to variations in glycosylation of the expressed protein and relatively high mannose content of the associated carbohydrate.

Plasmids pR5C3 and pYE in MB101 E. coli cells were deposited in accordance with the Rules of Practice of the U.S. Patent Office on Sep. 27, 1994 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. Plasmids pSM526 in AM7 cells, pCM351 in JM103 cells, and pMV8 in MB103 cells were likewise deposited in accordance with the Rules of Practice of the U.S. Patent Office on Sep. 27, 1994 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.
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33 deposited on Nov. 21, 1984 as ATCC 39932, 39934, and 39935, respectively. Saccharomyces cerevisiae strain YPS64 and RX1 were deposited on Nov. 21, 1984 as ATCC 37074 and 37075, respectively.

It should be readily apparent from consideration of the above examples that numerous exceptionally valuable products and processes are provided by the present invention in its many aspects.

Polypeptide products provided by the invention are conspicuously useful materials whether they are microbially expressed products or synthetic products, the primary, secondary or tertiary structural conformation of which was first made known by the present invention.

As previously indicated, recombinant-produced and synthetic products of the invention share, to varying degrees, the in vivo biological activity of EPO isolates from natural sources and consequently are projected to have utility as substituents for EPO isolates in culture media employed for growth of erythroid cell lines, and in fact, to the extent that polypeptide products of the invention share the in vivo activity of natural EPO isolates they are conspicuously useful for use in erythropoietin therapy, processes practiced on mammals included in humans, in order to develop any or all of the effects herein attributed to use in EPO, e.g., stimulation of reticulocyte response, development of hemokinetic effects (such as plasma iron turnover effects and marrow transist time effects), erythropoietin mass changes, stimulation of hematopoietic C synthesis (see, Eichholtz, et al., supra), and, as indicated in Example 10, increasing hematopoietic levels in man animals included within the class of humans treatable with products of the invention are patients generally requiring blood transfusions and including trauma victims, surgical patients, renal dialysis patients, and patients with a variety of blood composition affecting disorders, such as anemia, sickle cell disease, physiologic anemias, and the like. The miniaturization of the need for transfusion therapy through use of EPO therapy can be expected to result in reduced transmission of infectious agents. Products of the invention, by virtue of their properties by recombinant methods, are expected to be free of pyrogens, natural inflammatory substances, and the like, and are thus likely to provide enhanced overall effectiveness in therapeutic processes via via naturally derived products.

Erythropoietin therapy with products of the present invention is also expected to be useful in the enhancement of oxygen carrying capacity of individuals encountering hypoxic environmental conditions and possibly providing beneficial cardiovascular effects.

A preferred method for administration of polypeptide products of the invention is by parenteral (e.g., IV, IM, SC, or IP) routes and the compositions administered would ordinarily include therapeutically effective amounts of product in combination with acceptable diluents, excipients and solvents. Preliminary pharmacokinetic studies indicate a longer half-life in vivo for monkey EPO products when administered IM rather than IV. Effective dosages are expected to vary substantially depending upon the condition treated but hypogonadal doses are generally expected to be in the range of 0.1 to 1.0 micrograms per kg body weight of the active material. Standard diluents such as human serum albumin are recommended for pharmaceutical compositions of the invention, as are standard carriers such as saline.

Adjuvant materials suitable for use in compositions of the invention include compounds independently noted for erythropoietic stimulatory effects, such as testosterone, progenitor cell stimulation, insulin-like growth factor, protocidins, steroids, cyclic AMP, prostaglandin,

34 as well as agonists generally deployed in treatment of anemic states such as metanephrine, aracnid and aldolasebromodin, (e.g., Resta, et al., "Proteinase Medius," 55:1, 1981; McGlash, et al., "Kidney Int.," 26(2), 437-447 (1984); Pasley, et al., "Exp. Hematol.," Supp. 8, 783-591 (1980) and Kunz, "FEBBS Letters," 114(1), 105-108 (1982)]. Also contemplated as adjuvants are substances reported to enhance the effects of, or synergize, erythropoietic and/or EPO, such as the vasopressin agonists, thyroid hormones, androgens and PTH, as well as "cytoprotectants" (as described by Carson, et al., in Abstract 394, Proceedings, 7th International Congress of Endocrinology (Quebec City, Quebec, June 1-7, 1984); Carson, "Biochem. Biophys. Res. Comm.," 115(2), 447-452 (1983) and Carson, Annu. Rev. Biomed., 141, 438-472 (1980) and "cytoprotective" [as described in Hoffman, et al., J. Exp. Med., 101, 105-106 (1949)]. Preliminary screenings designed to measure erythropoietic responses of ex-hypoxic polychemo-treatment pretreated with either 5- or 6-dehydroandrosterone or testosterone and then given erythropoietin in the present invention have generated encouraging results.

Diagnostic uses of polypeptides of the invention are similarly extensive and include use in labeling and unlabeled forms in a variety of immunosassay techniques including RIA's ELISA's and the like, as well as in a variety of in vivo and in vivo activity assays. See, e.g., Dianz, et al., "Exp. Hematol.," 11(7), 580-603 (1983); Gibson, et al., "Hypoth. SOL, 155-156 (1984); and "Hypoth. SOL, 117, 468-469 (1983); Sabin, et al., "Exp. Hematol.," 117, 21-28 (1983); and, various references pertaining to assays referred to therein. Polyepitides of the invention, including synthetic peptides comprising sequences of residues of EPO first revealed herein, also provide highly useful pure materials for generating polyclonal antibodies and "hetero" polyclonal antibodies specific for differing continuous and discontinuous epitopes of EPO. As one example, preliminary analysis of the amino acid sequences of FIG 6 in the context of hydrophobicity, according to Lippe, et al., "Pf. A. L. (U.L.A.)," RY, 1369-1420 (1981) and of tertiary structures according to Choo, et al., "Ann. Rev. Biophys. Chem.," 47, p. 251 (1976) revealed that synthetic peptides protective of continuous sequences of residues spanning positions 41-57 inclusive, 100-138 inclusive and 144-176 inclusive are likely to produce a highly specific response against polyclonal, monoclonal and polyclonal antibodies immunoreactive with both the synthetic peptide and the entire protein. Such antibodies are expected to be useful in the detection and affinity purification of EPO and EPO-related products.

Illustratively, the following three synthetic peptides were prepared:


employing the above noted polypeptides have revealed a relatively weak positive response to EPO 45-57. Of great "appreciable" response to EPO 116-28, and a strong positive response to EPO 144.166, as measured by the capacity of rabbit serum antibodies to immunoprecipitate the peptide EPO 235-374. Preliminary in vivo activity studies on the three polypeptides revealed no significant activity either alone or in combination.

While the deduced sequences of amino acid residues of mammalian EPO provided by the illustrative examples necessitate the primary structure DNA generation of mature EPO, it will be understood that the specific sequence of various amino acid residues of mammalian EPO in FIG. 5 and the 166 residues of human Epo (5) in FIG. 6 do not limit the scope of useful polypeptides provided by the invention. Comprehended by the present invention are those various naturally occurring alleles of EPO with partial or full expression into biologically active mammalian polypeptides such as human y-interferring species. It is possible for an endonuclease to cleave a nucleotide residue at position No. 140 in EPO, but it is possible that the residues reported to have glutamine at position No. 140 in Gray, et al., Nature, 255, pp. 509-505 (1972). Data species are characterized as constituting "narrow" human y-interferring sequences. Allude forms of mature EPO polypeptides may vary from each other from the sequence of amino acids 5 and 6 to terms of length of sequence and/or in terms of deletion, substitution, insertion or addition of amino acids in the sequence, with the potential for variations in the capacity for glycosylation. As noted previously, one putative allelic form of human specimens EPO is intended to illustrate a nucleotide residue at position 146. In general, naturally occurring allelic forms of EPO encoding DNA generation and DNA sequences are also likely to occur which code for the above noted allelic polypeptides or simply employ differing codons for designation of the same polypeptides as specified.

In addition to naturally occurring allelic forms of mature EPO, the present invention also embraces other "EPO products" and polypeptide analogs of EPO and Fragments of "natural" EPO. Following the procedures of the above noted published application by Akamia, et al. (WO 86/000163) (p) may readily distinguish mammalian species codons for mature expression of polypeptides having primary conformational characteristics which differ from those herein specified for mature EPO in terms of the identity of location of one or more residues (e.g., substituents, terminal and intermediate additions and deletions). Alternatively, modifications of CDNA and genomic EPO genes may be readily accomplished by well-known Sitedirected mutagenesis techniques and employed to generate analogs and derivatives of EPO. Such EPO products would share at least one of the biological properties of EPO but may differ in others. As examples, protected EPO products of the invention, include those which are functionally modified by, e.g., deletion [po, e.g., 3-42, conventional "5-85" EPO], [po, e.g., 3-42, conventional "5-85" EPO], the latter having the residues deleted for by an endonuclease (and, therefore, may have some biological activity), non-natural occurring EPO); or which have been altered to encode one or more potential sites for glycosylation (which may result in higher activity for biological properties); or which have one or more cysteine residues deleted or replaced by, e.g., histidine or serine residues found in the analogous [His-Met-Cys] and are potentially more easily isolated in active form from microbial systems or which have one or more tyrosine residues replaced by phenylalanine (such as the analogs [Phy-158EPO, [Phy-158EPO, and [Phy-158EPO] and may bind more or less readily to EPO receptors on larger cells. Also contemplated are polypeptides fragments containing only a part of the mammalian amino acid sequence or secondary conformations within mature EPO, which fragments may possess one activity of EPO (e.g., receptor binding) and not others (e.g., erythropoietic activity). Especially significant in this regard are those polypeptides or fragments of EPO which are isolated upon consideration of the human genetic DNA sequence of FIG. 6, and "fragments" of the total continuous EPO sequence which are delineated by linear sequences and which may constitute distinct "domains" of biological activity. It is noteworthy that the absence of any activity for any one or more of the "EPO products" of the invention is not wholly preclusive of therapeutic utility (see, Weiland, et al., supra) or activity in other contexts, such as may be of use in treatment of the defects associated with an overexpression of EPO (see, e.g., Adamec, Terminology, Practice, 19:92, 69-70 (1989), and Haffman, et al., Clin. Lab. Haematol., 3, 335-342 (1983).

According to another aspect of the present invention, the disclosed DNA sequences described herein which encode human and rhesus monkey EPO polypeptides are enzymatically valuable for the information which they provide concerning the amino acid sequence of mammalian hypoxia-inducible gene which has been determined to be valuable despite decades of analytical processing of isolates of naturally-occurring products. The DNA sequences are also commercially valuable as products useful in effecting the large scale microbial synthesis of hypoxia-inducible gene by a variety of recombinant techniques. Put together, DNA sequences provided by the invention are useful in generating and useful viral and eukaryotic plasmid DNA generation and expression vectors, and transfected mammalian and eukaryotic cells (including bacterial and yeast cells and mammalian cells grown in culture) and new and useful methods for cultured growth of such microbial and cell capable of expression of EPO and EPO products. DNA sequences of the invention are also useful as probes or reagents for use as labels or probes in isolating EPO and related protein encoding, cDNA or genomic DNA sequences of mammalian species other than human and rhesus monkey species human specifically isolated. The extent to which DNA sequences of the invention will have use in various alternative methods of protein synthesis (e.g., in insect cell) or in macromolecular synthesis, and other means for isolating the DNA sequences of the invention, is expected to be useful in identifying transgenic mammalian species which may serve as "transgenic "hosts" for production of hypoxia-inducible gene and hypoxia-inducible gene products in quantity. See, generally, Balachandran, et al., Science, 222:64621, 809-814 (1983).

Viewed in this light, the specific disclosures of the illustrative examples are not limited to the specific examples presented in the illustrative examples. The invention is intended to be Unit and the present invention is not intended to be limited to the specific examples presented in the illustrative examples. The invention is intended to be Unit and the present invention is not intended to be limited to the specific examples presented in the illustrative examples. The invention is intended to be Unit and the present invention is not intended to be limited to the specific examples presented in the illustrative examples.
cases of naturally-occurring EPO but not share others (or possess others to different degrees).

DNA sequences provided by the present invention are thus not seen to comprise all DNA sequences suitable for use in hybridizing to a protein or eukaryotic host cell of a polypeptide product having at least a half of the amino acid content and one or more of the biological properties of erythropoietin, and selected from among (a) the DNA sequences set out in FIGS. 5 and 6; (b) DNA sequences which hybridize to the DNA 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) and (b). It is noteworthy in this regard, for example, that existing a lethal monkey and human EPO gene sequences and other mammalian species gene sequences are expected to hybridize to the sequences of FIGS. 5 and 6 or to fragments thereof. Further, but for the degeneracy of the genetic code, the SCFPO and ECPO genes and the mammalian or murine/ratized DNA or genomic DNA sequences encoding various EPO fragments and analogs would also hybridize to the above-mentioned DNA sequences. Such hybridizations could be carried out under the hybridization conditions described herein with respect to the initial isolation of the monkey and human EPO encoding DNA or more stringent conditions, if desired to reduce background hybridization.

In a like manner, while the above examples illustrate the invention of cDNA expression of EPO proteins in the context of mammalian cell expression of DNA inserted in a hybrid vector of bacterial plasmid and viral genome origins, a wide variety of expression systems are within the contemplation of the invention. Completely comprehended are expression systems involving vectors of heterologous origins applied to a variety of bacterial, yeast, and mammalian cells in culture as well as to expression systems not involving vectors (such as calcium phosphate transfection of cells). In this regard, it will be understood that expression of, e.g., monkey or rat DNA in a monkey host cells in culture and human host cells in culture, actually constitutes instances of "exogenous" DNA expression similar to the EPO DNA whose high level expression is taught would not have its origins in the genome of the host. Expression systems of the invention further contemplate these machines resulting in cytoplasmic formation of EPO proteins and accumulation of glycosylated and non-glycosylated EPO proteins in host cell cytoplasm or membranes (e.g., accumulation in bacterial periplasmic spaces) or in culture medium supernatants as above illustrative or in similar mammalian systems such as Rat-2 expression systems (described in Guy, et al., Biotechnology, 2, pp. 165-168 (1984)).

Improved hybridization methodologies of the invention, while illustratively applied above at DNA/DNA hybridization screening are readily applicable to RNA/DNA and RNA/DNA screening. Mixed probe techniques as herein illustrated generally constitute a number of improvements in hybridization processes allowing for more rapid and reliable polynucleotide isolations. These include individual processing improvements such as improved colony transfer and maintenance procedures, use of non-denaturing filters such as GeneScreen, and GeneScreen Plus to allow reprobing with same filters and repeated use of the filters. Application of novel probe treatments (compared, e.g., to Tran, et al., Anal. Biochem., 120, pp. 225-238 (1982)) use of very low stringency conditions (on the order of 0.2 Ss (2xSSC)) of a large number of mixed probes (e.g., rabbit, in excess of 50); and, performing hybridization and post-hybridization

...
9. A pharmaceutical composition comprising an effective amount of a glycoprotein product effective for erythropoietin therapy according to claim 1, 2, 3, 4, 5 or 6 and a pharmaceutically acceptable diluent, adjuvant or carrier.

10. A method for providing erythropoietin therapy to a mammal comprising administering an effective amount of a pharmaceutical composition of claim 9.

11. A method for treating a kidney dialysis patient which comprises administering a pharmaceutical composition of claim 9 in an amount effective to increase the hemoglobin level of said patient.

12. A pharmaceutical composition comprising an effective amount of a glycoprotein product effective for erythropoietin therapy according to claim 7 and a pharmaceutically acceptable diluent, adjuvant or carrier.

13. A method for providing erythropoietin therapy to a mammal comprising administering an effective amount of a pharmaceutical composition of claim 12.

14. A method for treating a kidney dialysis patient which comprises administering a pharmaceutical composition of claim 12 in an amount effective to increase the hemoglobin level of said patient.
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,547,933
DATED : August 20, 1996
INVENTOR(S) : Fu-Kuen Lin

It is notified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the References, page 1, column 2, line 3
"012394" should be -- 0123294 --.

In Other Publications, page 2, column 1, line 60,
Chia Reference, "(1992)" should -- (1982) --.

In Other Publications, page 5, column 2, line 38,
Konwiainka Reference, "Human" should be -- Human --.

In Other Publications, page 6, column 1, line 51,
Lee-Huang Reference, "620, 622 and 624" should be -- 620-624 --.

In Other Publications, page 7, column 1, lines 7-8,
Miran Reference, "94, 96, 98, 100, 102, 104, 106" should be -- 94-106 --.

In Other Publications, page 7, column 1, line 16,
Montgomery Reference, "673-680" should be -- 673-680 --.

In Other Publications, page 9, column 1, line 9,
Storing Reference, "458-459" should be -- 458-459 --.

In Other Publications, page 9, column 2, line 58, Wallace Reference insert (1981) after 3647-3657 but before ID.
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.: 5,547,333
DATED: August 20, 1996
INVENTOR(S): Fu-Kuen Lin

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Figures, Figure 5C, line 18,
GTCGTGCAACGAGGAGGTGCAGCCTGGACATCTCCGAGCTGCAGCAGC should be
GTCGTGCAACGAGGAGGTGCAGCCTGGACATCTCCGAGCTGCAGC

In the Figures, Figure 13. line 4
GTGCAAC should be GTAAC
CAACCTAGG

In the Figures, Figure 17, line 2
AATCGAAGCTTGGATA should be AATCGAAGCTTGGATA
G TCTGAACTAT

In the Figures, Figure 18, line 9
AAGCTTTTCGACGAGCTGCAGCCT should be AAGCTTTTCGACGAGCTGCAGCCT

In the Figures, Figure 21, line 6, 5th grouping
CAGGTTTAC should be CAGGTTTAC
GTCTCAATTC

In the Figures, Figures 21, line 8, 4th grouping
TACACCGGTCG should be TACACCGGTCG
ATCTGGCCAC

In the Figures, Figure 21, line 10, 3rd grouping
GACTGATAAC should be GACTGATAAC
CTGACTATTG
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,547,933
DATED : August 20, 1996
INVENTOR(S) : Fu-Kuen Lin

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

column 1, line 60, "uracil" should be -- uracil (U) --.
column 1, line 62, "sequences" should be -- sequences --.
column 1, line 62, "message" should be -- "message,".
column 4, line 15, "DnAs" should be -- DNAs --.
column 4, line 25, "Wallace" should be -- Wallace --.
column 4, line 25, "[1981," should be -- (1981); --.
column 4, line 31, "Wallace," should be -- Wallace, --.
column 4, line 61, "off" should be -- of --.
column 5, line 18, "amine" should be -- amino --.
column 5, line 31, "amine" should be -- amino --.
column 5, line 36, "amine" should be -- amino --.
column 5, line 50, "and" should be -- and --.
column 6, line 27, "antibodies" should be -- antibodies --.
column 10, line 21, "avian" should be -- avian --.
column 10, line 46, "100 U" should be -- 100U --.
column 10, line 47, "500 U" should be -- 500U --.
UNIVERSAL S M T H E S R T T A T E N A T P R E N T A N D T R A D E M A R K O F F I C E
CERTIFICATE OF CORRECTION

PATENT NO. : 5,547,833
DATED : August 20, 1996
INVENTOR(S) : Fu-Kuen Lin

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

. column 11, line 41, "DN" should be -- DNA --.
. column 14, line 10, "17 mer" should be -- 17-mer --.
. column 16, line 5, "128-mer" should be -- 128 20-mer --.
. column 16, line 46, "25" should be -- 25% --.
. column 16, line 58, "32P labelled" should be -- 32P-labelled --.
. column 16, line 58, after "added," insert -- and --.
. column 17, line 40, after "procedures," insert -- (i) --.
. column 17, line 62, "EODA" should be -- EDTA --.
. column 18, line 16, "micromoles" should be -- micromoles --.
. column 18, line 33, "EcoR I" should be -- EcoRI --.
. column 18, line 44, "EcoRI/Hindill" should be -- EcoRI/HindIII --.
. column 20, line 7, "(NACl)" should be -- (NaCl) --.
. column 20, line 17, "EPY" should be -- EPV --.
. column 21, line 67, coRI should be -- EcoRI --.
. column 22, line 19, "vector s" should be -- vector's --.
. column 22, line 49, "suu" should be -- supra --.
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,547,933
DATED : August 20, 1996
INVENTOR(S) : Fu-Kuen Lin

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

column 23, line 43, "Salt" should be -- Sall --.
column 23, line 46, "Salt" should be -- Sall --.
column 24, line 51, "H and L" should be -- (H and L) --.
column 24, line 62, "he" should be -- the --.
column 26, line 6, "pDSVL-KEPO" should be -- pDSVL-MKEPO --.
column 26, line 8, "pDSVL-KEPO" should be -- pDSVL-MKEPO --.
column 26, line 14, "pDSVL" should be -- pDSVL --.
column 26, line 22, "[OHRF]^*" should be --[DHFR] -*.
column 28, line 29, "NTX" should be -- MTX --.
column 28, line 30, "CHFR" should be -- DHFR --.
column 28, line 31, "CHFR" should be -- DHFR --.
column 28, line 32, "NTX" should be -- MTX --.
column 28, line 35, after "CHFR" should be -- DHFR --.
column 28, line 35, after "CHFR" should be -- DHFR --.
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

Page 6 of 7

PATENT NO.: 5,547,933
DATED: August 20, 1996
INVENTOR(S): Fu-Kuen Lin

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

column 26, line 39, "pDSVL-NkE" should be --"pDSVL-MkE" --.

column 26, line 39, "NTX" should be -- MTX --.

column 26, line 44, "NTX" should be -- MTX --.

column 20, line 45, "nN" should be -- mM --.

column 27, line 18, "pDSVL-gHuEPO" should be -- pDSVL-gHuEPO --.

column 27, line 30, "lie" should be -- line --.

column 27, line 32, "use" should be -- used --

column 27, line 34, "replace" should be -- replaced --.

column 27, line 36, "m" should be -- mM --.

column 28, line 5, "pDSVL-gHuEPO" should be pDSVL-gHuEPO --.

column 28, line 7, "TX" should be -- MTX --.

column 29, line 56, "KEqI" should be -- KeqI --.

column 31, line 9, "Xhol" should be -- XhoI --.

column 31, line 26, "indIII" should be -- HindIII --.

column 31, line 26, "Kpnl/BgIII" should be -- Kpnl/BgIII --.
It is certified that an error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

- Column 31, line 53: "DYE" should be -- PYE --.
- Column 32, line 47: after "genotype" but before "pep4-3" insert -- a --.
- Column 32, line 65: "C34" should be -- (34 --.
- Column 33, line 60: "7500 U) g/kg" should be -- 7000U) g/kg --.
- Column 34, line 55: "an" should be -- and --.
- Column 35, line 21: "interferon" should be -- interferon --.
- Column 36, line 19: after "such as" insert -- in EPO assays or EPO antagonism: Antagonists of erythropoetin --.
- Column 37, line 10: "DA" should be -- DNA --.
- Column 37, line 10: second occurrence of "DA" should be -- DNA --.
- Column 37, line 50: "P. aeruginosa" should be -- P. aeruginosa --.

In the Claims, column 39, line 2: after "amount" insert -- of --.

Signed and Sealed this Twenty-fourth Day of March, 1998

[Signature]

Attorney

[Signature]

Attending Examiner

Commissioner of Patents and Trademarks