EXHIBIT I
Part 2 of 2

TABLE XII

ECEPO SECTION 3

1. GATCCAGATCTCTGACTACTCTGC
2. ACGCAGCAGAGTAGTCAGAGATCTG
3. TGCGTGCTCTGGGTGCAGAGAAAGAGG
4. GATAGCCTCTTTCTGTGCACCCAGAGC
5. CTATCTCTCCGCCGGATGCTGCATCT
6. CAGCAGATGCAGCATCCGGCGGAGA
7. GCTGCACCGCTGCGTAGCATCACTG
8. ATCAGCAGTGATGCTACGCAGCGGTG
9. CTGATACCTTCCGCAAACTGTTTCG
10. ATACACGAAACAGTTTGCGGAAGGT
11. TGTATACTCTTAACTTCCTGCGTGGTA
12. CAGTTTACCACGCAGGAAGTTAGAGT
13. AACTGAAACTGTATACTGGCGAAGC
14. GGCATGCTTCGCCAGTATACAGTTT
15. ATGCCGTACTGGTGACCGCTAATAG
16. TCGACTATTAGCGGTCACCAGTAC

TABLE XIII

ECEPO SECTION 3

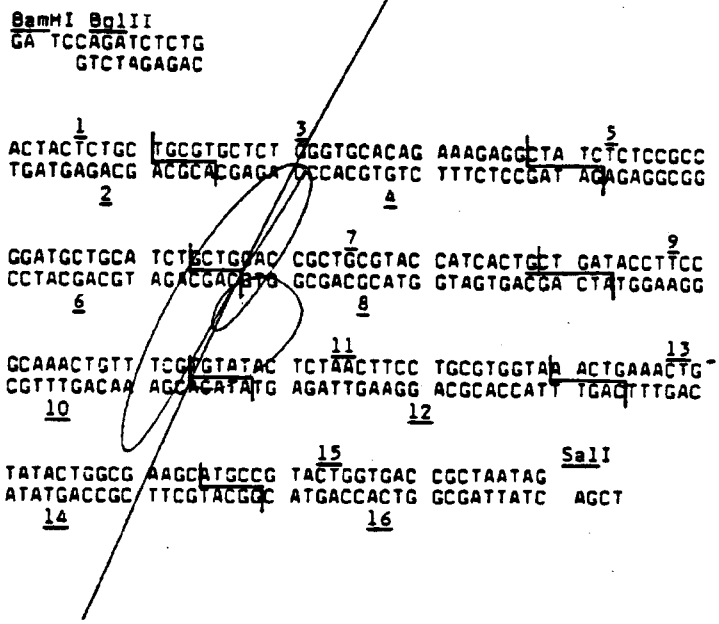
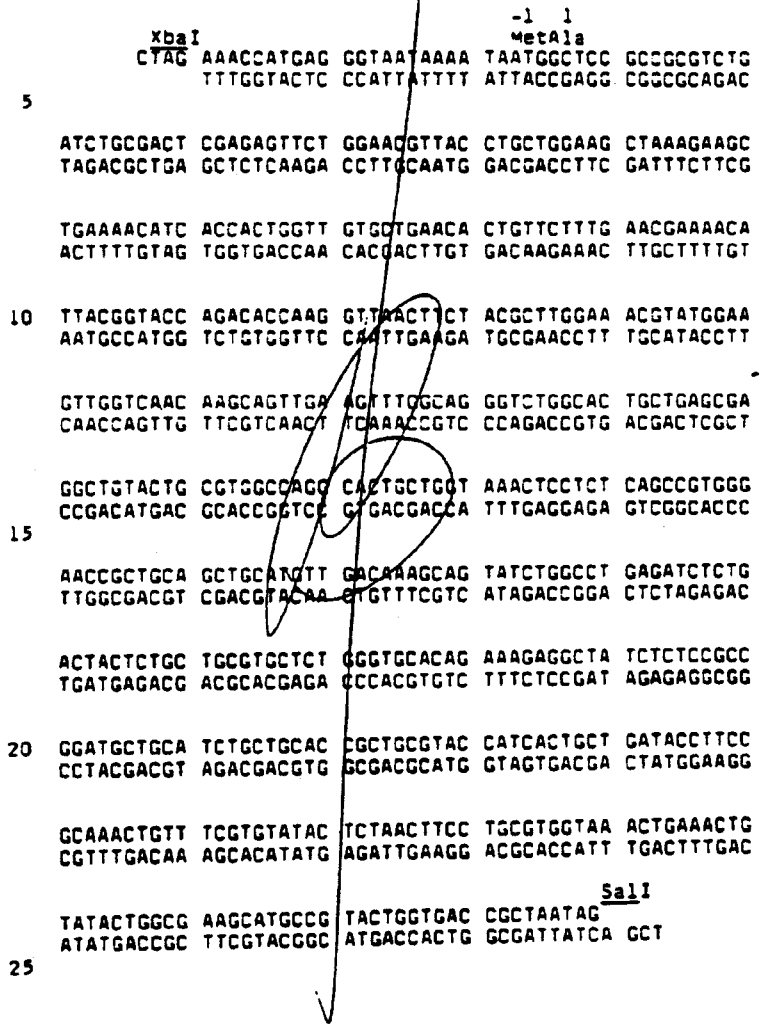


TABLE XIV

ECEPO GENE



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More particularly, Figure 10 illustrates oligonucleotides employed to generate the Section 1 of the ECEPO gene encoding amino terminal residues of the human species polypeptide. Oligonucleotides were assembled into duplexes (1 and 2, 3 and 4, etc.) and the duplexes were then ligated to provide ECEPO Section 1 as in Figure 11. Note that the assembled section includes respective terminal EcoRI and BamHI sticky ends, that "downstream" of the EcoRI sticky end is a XbaI restriction enzyme recognition site; and that "upstream" of the BamHI sticky end is a KpnI recognition site. Section 1 could readily be amplified using the M13 phage vector employed for verification of sequence of the section. Some difficulties were encountered in isolating the section as an XbaI/KpnI fragment from RF DNA generated in E.coli, likely due to methylation of the KpnI recognition site bases within the host. Single-stranded phage DNA was therefore isolated and rendered into double-stranded form in vitro by primer extension and the desired double-stranded fragment was thereafter readily isolated.

ECEPO gene Sections 2 and 3 (Figures 13 and 15) were constructed in a similar manner from the oligonucleotides of Figures 12 and 14, respectively. Each section was amplified in the M13 vector employed for sequence verification and was isolated from phage DNA. As is apparent from Figure 13, ECEPO Section 2 was constructed with EcoRI and BamHI sticky ends and could be isolated as a KpnI/BglII fragment. Similarly, ECEPO Section 3 was prepared with BamHI and SalI sticky ends and could be isolated from phage RF DNA as a BglII/SalI fragment. The three sections thus prepared can readily be assembled into a continuous DNA sequence (Figure 7) encoding the entire human species EPO polypeptide with an amino terminal methionine codon (ATG) for E.coli translation initiation. Note also that "upstream" of the initial ATG is a series of base pairs substantially

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duplicating the ribosome binding site sequence of the highly expressed OMP-f gene of E.coli.

Any suitable expression vector may be employed to carry the ECEPO. The particular vector chosen for expression of the ECEPO gene as the "temperature sensitive" plasmid pCFM536 -- a derivative of plasmid pCFM414 (A.T.C.C. 40076) -- as described in co-pending U.S. Patent Application Serial No. 636,727, filed August 6, 1984, by Charles F. Morris. More specifically, pCFM536 was digested with XbaI and HindIII; the large fragment was isolated and employed in a two-part ligation with the ECEPO gene. Sections 1 (XbaI/KpnI), 2 (KpnI/BglII) and 3 (BglII/SalI) had previously been assembled in the correct order in M13 and the EPO gene was isolated therefrom as a single XbaI/HindIII fragment. This fragment included a portion of the polylinker from M13 mp9 phage spanning the SalI to HindIII sites therein. Control of expression in the resulting expression plasmid, p536, was by means of a lambda P_L promoter, which itself may be under control of the C_{I857} repressor gene (such as provided in E.coli strain K12ΔHtrp).

The manufactured ECEPO gene above may be variously modified to encode erythropoietin analogs such as [Asn^2, des-Pro^2 through Ile^6]hEPO and [His^7]hEPO, as described below.

A. [Asn^2, des-Pro^2 through Ile^6]hEPO

Plasmid 536 carrying the ECEPO manufactured gene of ~~Table XIV~~ Figure 7 as a XbaI to HindIII insert was digested with HindIII and XhoI. The latter endonuclease cuts the ECEPO gene at a unique, 6 base pair recognition site spanning the last base of the codon encoding Asp^8 through the second base of the Arg^{10} codon. A XbaI/XhoI "linker" sequence was manufactured having the following sequence:

```
XbaI          +1  2   7   8   9
      Met Ala Asn Cys Asp
5'-CTAG ATG GCT AAT TGC GAC-3'  XhoI
   3'-TAC CGA TTA ACG CTG AGCT-5'
```

The XbaI/XhoI linker and the XhoI/HindIII ECEPO gene sequence fragment were inserted into the large fragment resulting from XbaI and HindIII digestion of plasmid pCFM526 -- a derivative of plasmid pCFM414 (A.T.C.C. 40076) -- as described in co-pending U.S. Patent Application Serial No. 636,727, filed August 6, 1984, by Charles F. Morris, to generate a plasmid-borne DNA sequence encoding E.coli expression of the Met^{-1} form of the desired analog.

B. [His^{7}]hEPO

Plasmid 536 was digested with HindIII and XhoI as in part A above. A XbaI/XhoI linker was manufactured having the following sequence:

```
XbaI      +1  2   3   4   5   6   7   8   9      XhoI
      Met Ala Pro Pro Arg Leu Ile His Asp
5'-CTAG ATG GCT CCG CCA CGT CTG ATC CAT GAC-3'
   3'-TAC CGA GGC GGT GCA GAC TAG GTA CTG  AGCT-5'
```

The linker and the XhoI/HindIII ECEPO sequence fragment were then inserted into pCFM526 to generate a plasmid-borne DNA sequence encoding E.coli expression of the Met^{-1} form of the desired analog.

Construction of a manufactured gene ("SCEPO") incorporating yeast preference codons is as described in the following ~~Tables XV through XXI~~ Figures 16 through 21 and 8. As was the case with the ECEPO gene, the entire construction involved formation of three sets of oligonucleotides (~~Tables XV, XVII and XIX~~ Figures 16, 18 and 20) which were formed into duplexes and assembled into sections (~~Tables XVI, XVIII and XX~~ Figures 17, 19 and 21). Note that synthesis was facilitated in part by use of some sub-optimal codons in both the SCEPO and ECEPO construc-

tions, i.e., oligonucleotides 7-12 of Section 1 of both genes were identical, as were oligonucleotides 1-6 of Section 2 in each gene.

TABLE XV

SCEPO SECTION 1 OLIGONUCLEOTIDES

1. AATTCAAGCTTGGATAAAAGAGCT
2. GTGGAGCTCTTTTATCCAAGCTTG
3. CCACCAAGATTGATCTGTGACTC
4. TCTCGAGTCACAGATCAATCTTG
5. GAGAGTTTTGGAAAGATACTTGTTG
6. CTTCCAACAAGTATCTTTCCAAAAC
7. GAAGCTAAAGAAGCTGAAAACATC
8. GTGGTGATGTTTTCAGCTTCTTTAG
9. ACCACTGGTTGTGGTGAACACTGTTC
10. CAAAGAACAGTGTTCAGCACAACCA
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTTCGTT

TABLE XVI

SCEPO SECTION 1

```
    EcoRI  HindIII 1
    AATTCA AGCTTGGATA
          GT TCGAACCTAT
                    2

                  3
    AAAGAGCTCC ACCAAGATTG ATCTGTGACT CGAGAGTTTT
    TTTCTCGAGG TGGTTCTAAC TAGACACTGA GCTCTCAAAA
                       4

       5                        7
    GGAAAGATAC TTGTTGCAAG CTAAAGAAGC TGAAAACATC ACCACTGGTT
    CCTTTCTATG AACAACCTTC GATTTCTTCG ACTTTTGTAG TGGTGACCAA
       6                        8

          9                        11        KpnI    BamHI
    GTGCTGAACA CTGTTCTTTG AACGAAAACA TTACGGTACC G
    CACGACTTGT GACAAGAAAC TTGCTTTTGT AATGCCATGG CCTAG
                                12
```

TABLE XVII

SCEPO SECTION 2 OLIGONUCLEOTIDES

	1.	AATTCGGTACCAGACACCAAGGT
	2.	GTTAACCTTGGTGTCTGGTACCG
	3.	TAACTTCTACGCTTGGAAACGTAT
	4.	TTCCATACGTTTCCAAGCGTAGAA
	5.	GGAAGTTGGTCAACAAGCAGTTGAAGT
	6.	CCAAACTTCAACTGCTTGTTGACCAAC
	7.	TTGGCAAGGTTGCCCTTGTTATCTG
	8.	GCTTCAGATAACAAGCCCAAACCTTG
	9.	AAGCTGTTTTGAGAGGTCAAGCCT
	10.	AACAAGCCTTGACCTCTCAAAACA
	11.	TGTTGGTTAACTCTTCTCAACCATGGG
	12.	TGGTTCCCATGGTTGAGAAGAGTTAACC
	13.	AACCATTGCAATTGCACGTCGAT
	14.	CTTTATCGACGTGCAATTGCAA
	15.	AAAGCCGTCTCTGGTTTGAGATCTG
	16.	GATCCAGATCTCAAACCAGAGACGG

TABLE XVIII

SCEPO SECTION 2

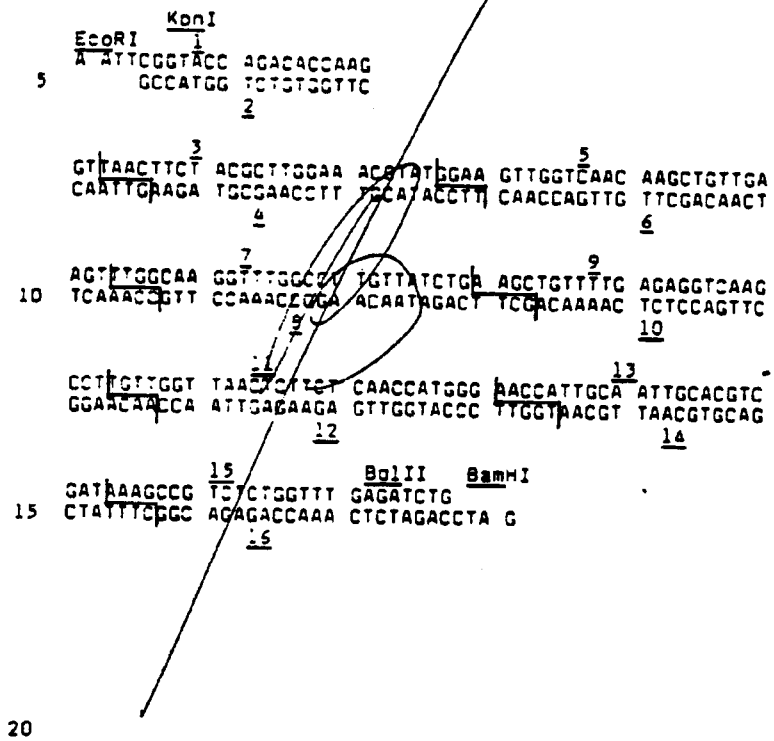

TABLE XIX

SCEPO SECTION 3 OLIGONUCLEOTIDES

	1.	GATCCAGATCTTTGACTACTTTGTT
5	2.	TCTCAACAAAGTAGTCAAAGATCTG
	3.	GAGAGCTTTGGGTGCTCAAAAGGAAG
	4.	ATGGCTTCCTTTTGAGCACCCAAAGC
	5.	CCATTTCCCCACCAGACGCTGCTT
	6.	GCAGAAGCAGCGTCTGGTGGGGAA
10	7.	CTGCCGCTCCATTGAGAACCATC
	8.	CAGTGATGGTTCTCAATGGAGCG
	9.	ACTGCTGATACCTTCAGAAAGTT
	10.	GAATAACTTTCTTAAGGTATCAG
	11.	ATTCAGAGTTTACTCCAACTTCT
15	12.	CTCAAGAAGTTGGAGTAAACTCT
	13.	TGAGAGGTAAATTGAAGTTGTACAC
	14.	ACCGGTGTACAACTTCAATTTACCT
	15.	CGGTGAAGCCTGTAGAACTGGT
	16.	CTGTCACCAGTTCTACAGGCTTC
20	17.	GACAGATAAGCCCGACTGATAA
	18.	GTTGTTATCAGTCGGGCTTAT
	19.	CAACAGTGTAGATGTAACAAAG
	20.	TCGACTTTGTTACATCTACACT

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TABLE xx

SCEPO SECTION 3

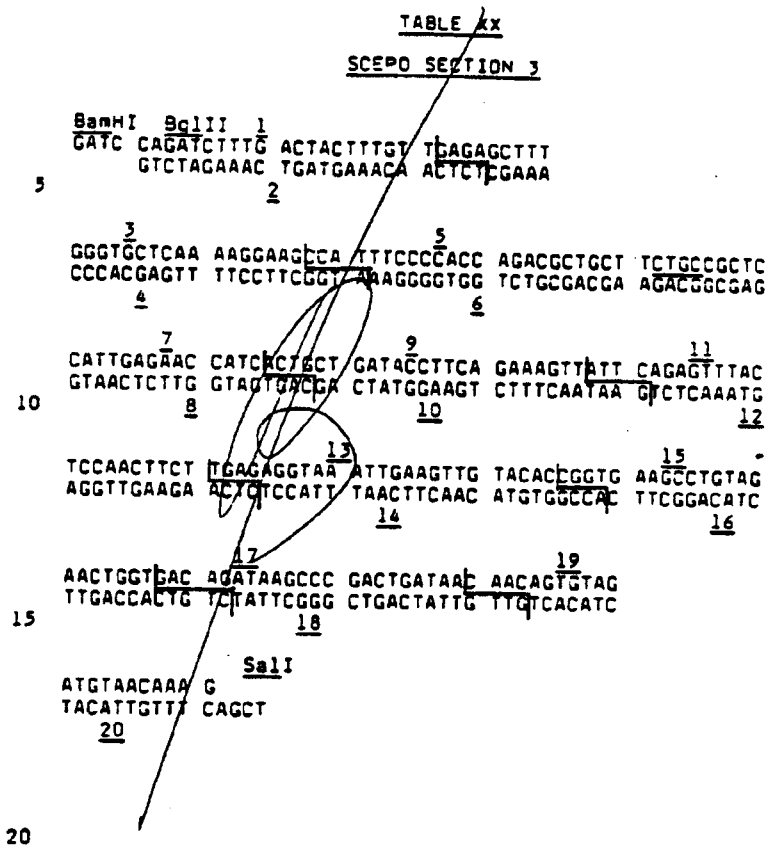
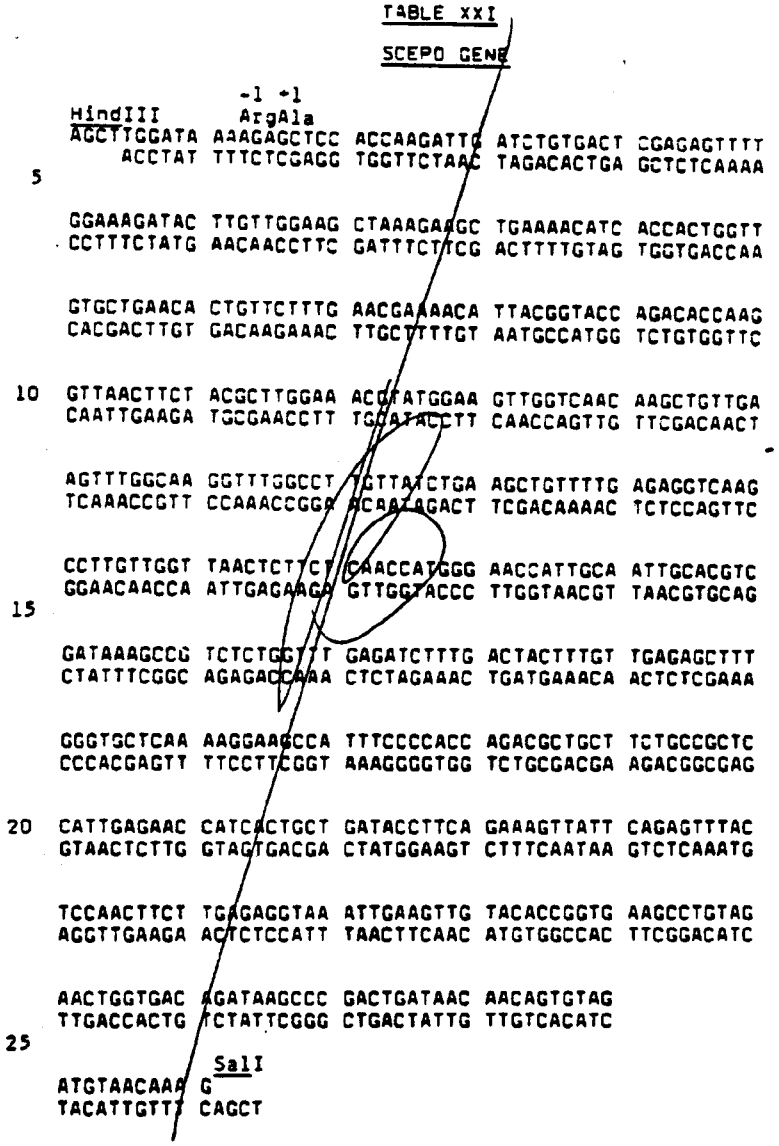


TABLE XXI

SCEPO GENE



The assembled SCEPO sections were sequenced in M13 and Sections 1, 2 and 3 were isolatable from the phage as HindIII/KpnI, KpnI/BglII, and BglII/SalI fragments.

The presently preferred expression system for SCEPO gene products is a secretion system based on S.cerevisiae α-factor secretion, as described in co-pending U.S. Patent Application Serial No. 487,753, filed April 22, 1983, by Grant A. Bitter, published October 31, 1984 as European Patent Application 0 123,294. Briefly put, the system involves constructions wherein DNA encoding the leader sequence of the yeast α-factor gene product is positioned immediately 5' to the coding region of the exogenous gene to be expressed. As a result, the gene product translated 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 product. Because the construction makes use of the α-factor translation initiation (ATG) codon, there was no need to provide such a codon at the -1 position of the SCEPO gene. As may be noted from ~~Table XXI~~ figure 2, the alanine (+1) encoding sequence is preceded by a linker sequence allowing for direct insertion into a plasmid including the DNA for the first 80 residues of the α-factor leader following the α-factor promoter. The specific preferred construction for SCEPO gene expression involved a four-part ligation including the above-noted SCEPO section fragments and the large fragment of HindIII/SalI digestion of plasmid pαC3. From the resulting plasmid pαC3/SCEPO, the α-factor promoter and leader sequence and SCEPO gene were isolated by digestion with BamHI and ligated into BamHI digested plasmid pYE to form expression plasmid pYE/SCEPO.

EXAMPLE 12

The present example relates to expression of

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recombinant products of the manufactured ECEPO and SCEPO genes within the expression systems of Example 11.

In use of the expression system designed for use of E.coli host cells, plasmid p536 of Example 11 was transformed into AM7 E.coli cells previously transformed with a suitable plasmid, pMW1, harboring a C_{I857} gene. Cultures of cells in LB broth (Ampicillin 50 ug/ml and kanamycin 5 ug/ml, preferably with 10 mM $MgSO_4$) were maintained at 28°C and upon growth of cells in culture to $O.D._{600}$ = 0.1, EPO expression was induced by raising the culture temperature to 42°C. Cells grown to about 40 O.D. provided EPO production (as estimated by gel) of about 5 mg/OD liter.

Cells were harvested, lysed, broken with French Press (10,000 psi) and treated with lysozyme and NP-40 detergent. The pellet resulting from 24,000 xg centrifugation was solubilized with guanidine HCl and subjected to further purification in a single step by means of C_4 (Vydac) Reverse Phase HPLC (EtOH, 0-80%, 50 mM NH_4Ac, pH 4.5). Protein sequencing revealed the product to be greater than 95% pure and the products obtained revealed two different amino terminals, A-P-P-R... and P-P-R... in a relative quantitative ratio of about 3 to 1. This latter observation of hEPO and [des Ala^1]hEPO products indicates that amino terminal "processing" within the host cells serves to remove the terminal methionine and in some instances the initial alanine. Radioimmunoassay activity for the isolates was at a level of 150,000 to 160,000 U/mg; in vitro assay activity was at a level of 30,000 to 62,000 U/mg; and in vivo assay activity ranged from about 120 to 720 U/mg. (Cf., 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 EPO standard.

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The EPO analog plasmids formed in parts A and B of Example 11 were each transformed into pMW1-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 [Asn^2, des-Pro^2 through Ile^6]hEPO expression products were approximately 11,000 U/mg and 6,000 U/mg protein, respectively, while the assay values for [His^7]hEPO 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 pYE/SCEPO was transformed into two different strains, YSDP4 (genotype α pep4-3 trp1) and RK81 (genotype αα pep4-3 trp1). Transformed YSDP4 hosts were grown in SD medium (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 62 (1983) supplemented with casamino acids at 0.5%, pH 6.5 at 30°C. Media harvested when the cells had been grown to 36 O.D. contained EPO products at levels of about 244 U/ml (97 ug/OD liter by RIA). Transformed RK81 cells grown to either 6.5 O.D. or 60 O.D. provided media with EPO concentrations of about 80-90 U/ml (34 ug/OD liter by RIA). Preliminary analyses reveal significant heterogeneity in products produced by the expression system, likely to be due to variations in glycosylation of proteins expressed, and relatively high mannose content of the associated carbohydrate.

Plasmids PaC3 and pYE in HB101 E.coli cells were deposited in accordance with the Rules of Practice of the U.S. Patent Office on September 27, 1984, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, under deposit numbers A.T.C.C. 39881 and A.T.C.C. 39882, respectively. Plasmids pCFM526 in AM7 cells, pCFM536 in JM103 cells, and pMW1 in JM103

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cells were likewise deposited on November 21, 1984 as A.T.C.C. ~~39932~~, ~~39934~~, and ~~39935~~, respectively. Saccharomyces cerevisiae strains YSPD4 and RK81 were deposited on November 21, 1984 as A.T.C.C. 20734 and 20733, respectively.

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

Polypeptides 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 vitro biological activity of EPO isolates from natural sources and consequently are projected to have utility as substitutes for EPO isolates in culture media employed for growth of erythropoietic cells in culture. Similarly, to the extent that polypeptide products of the invention share the in vivo activity of natural EPO isolates they are conspicuously suitable for use in erythropoietin therapy procedures practiced on mammals, including humans, to develop any or all of the effects herefore attributed in vivo to EPO, e.g., stimulation of reticulocyte response, development of ferrokinetic effects (such as plasma iron turnover effects and marrow transit time effects), erythrocyte mass changes, stimulation of hemoglobin C synthesis (see, Eschbach, et al., supra) and, as indicated in Example 10, increasing hematocrit levels in mammals. 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 disease patients including dialysis patients, and

patients with a variety of blood composition affecting disorders, such as hemophilia, sickle cell disease, physiologic anemias, and the like. The minimization 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 production by recombinant methods, are expected to be free of pyrogens, natural inhibitory substances, and the like, and are thus likely to provide enhanced overall effectiveness in therapeutic processes vis-a-vis 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 in 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, carriers and/or adjuvants. 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 therapeutic doses are presently expected to be in the range of 0.1 (~7U) to 100 (~7000U) ug/kg body weight of the active material. Standard diluents such as human serum albumin are contemplated 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 testosterones, progenitor cell stimulators, insulin-like growth factor, prostaglandins, serotonin,

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cyclic AMP, prolactin and triiodothyronine, as well as agents generally employed in treatment of aplastic anemia, such as methenolene, stanozolol and nandrolone [see, e.g., Resegotti, et al., Panminerva Medica, 23, 243-248 (1981); McGonigle, et al., Kidney Int., 25(2), 437-444 (1984); Pavlovic-Kantera, et al., Expt.Hematol., 8(Supp. 8), 283-291 (1980); and Kurtz, FEBS Letters, 144(1), 105-108 (1982)]. Also contemplated as adjuvants are substances reported to enhance the effects of, or synergize, erythropoietin or asialo-EPO, such as the adrenergic agonists, thyroid hormones, androgens and BPA [see, Dunn, "Current Concepts in Erythropoiesis", John Wiley and Sons (Chichester, England, 1983); Weiland, et al., Blut, 44(3), 173-175 (1982); Kalmanti, Kidney Int., 22, 383-391 (1982); Shahidi, New.Eng.J.Med., 289, 72-80 (1973); Fisher, et al., Steroids, 30(6), 833-845 (1977); Urabe, et al., J.Exp.Med., 149, 1314-1325 (1979); and Billat, et al., Expt.Hematol., 10(1), 133-140 (1982)] as well as the classes of compounds designated "hepatic erythropoietic factors" [see, Naughton, et al., Acta.Haemat., 69, 171-179 (1983)] and "erythrotropins" [as described by Congote, et al. in Abstract 364, Proceedings 7th International Congress of Endocrinology (Quebec City, Quebec, July 1-7, 1984); Congote, Biochem.Biophys.Res.Comm., 115(2), 447-483 (1983) and Congote, Anal.Biochem., 140, 428-433 (1984)] and "erythrogenins" [as described in Rothman, et al., J.Surg.Oncol., 20, 105-108 (1982)]. Preliminary screenings designed to measure erythropoietic responses of ex-hypoxic polycythemic mice pre-treated with either 5-α-dihydrotestosterone or nandrolone and then given erythropoietin of the present invention have generated equivocal results.

Diagnostic uses of polypeptides of the invention are similarly extensive and include use in labelled and unlabelled forms in a variety of immunoassay techniques

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including RIA's, ELISA's and the like, as well as a variety of in vitro and in vivo activity assays. See, e.g., Dunn, et al., Expt.Hematol., 11(7), 590-600 (1983); Gibson, et al., Pathology, 16, 155-156 (1984); Krystal, Expt.Hematol., 11(7), 649-660 (1983); Saito, et al., Jap.J.Med., 23(1), 16-21 (1984); Nathan, et al., New Eng.J.Med., 308(9), 520-522 (1983); and various references pertaining to assays referred to therein. Polypeptides 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 "banks" of monoclonal antibodies specific for differing continuous and discontinuous epitopes of EPO. As one example, preliminary analysis of the amino acid sequences of ~~Table VI~~ Figure 6 in the context of hydropathicity according to Hopp, et al., P.N.A.S. (U.S.A.), 78, pp. 3824-3828 (1981) and of secondary structures according to Chou, et al., Ann.Rev.Biochem., 47, p. 251 (1978) revealed that synthetic peptides duplicative of continuous sequences of residues spanning positions 41-57 inclusive, 116-128 inclusive and 144-166 inclusive are likely to produce a highly antigenic response and generate useful 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:

(1) hEPO 41-57, V-P-D-T-K-V-N-F-Y-A-W-K-R-M-E-V-G;
(2) hEPO 116-128, K-E-A-I-S-P-P-D-A-A-S-A-A;
(3) hEPO 144-166, V-Y-S-N-F-L-R-G-K-L-K-L-Y-T-G-E-A-C-R-T-G-D-R.

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Preliminary immunization studies employing the above-noted polypeptides have revealed a relatively weak positive response to hEPO 41-57, no appreciable response to hEPO 116-128, and a strong positive response to hEPO 144-166, as measured by capacity of rabbit serum antibodies to immunoprecipitate ^{125}I-labelled human urinary EPO isolates. Preliminary in vivo activity studies on the three peptides 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 essentially define the primary structural conformation of mature EPO, it will be understood that the specific sequence of 165 amino acid residues of monkey species EPO in ~~Table V~~ Figure 5 and the 166 residues of human species EPO in ~~Table VI~~ Figure 6 do not limit the scope of useful polypeptides provided by the invention. Comprehended by the present invention are those various naturally-occurring allelic forms of EPO which past research into biologically active mammalian polypeptides such as human γ interferon indicates are likely to exist. (Compare, e.g., the human immune interferon species reported to have an arginine residue at position No. 140 in EPO published application 0 077 670 and the species reported to have glutamine at position No. 140 in Gray, et al., Nature, 295, pp. 503-508 (1982). Both species are characterized as constituting "mature" human γ interferon sequences.) Allelic forms of mature EPO polypeptides may vary from each other and from the sequences of ~~Tables V and VI~~ Figures 5 and 6 in terms of length of sequence and/or in terms of deletions, substitutions, insertions or additions of amino acids in the sequence, with consequent potential variations in the capacity for glycosylation. As noted previously, one putative allelic form of human species EPO is believed to include a methionine residue at position 126. Expectedly, naturally-occurring allelic forms

of EPO-encoding DNA genomic and cDNA sequences are also likely to occur which code for the above-noted types of 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" such as polypeptide analogs of EPO and fragments of "mature" EPO. Following the procedures of the above-noted published application by Alton, et al. (WO/83/04053) one may readily design and manufacture genes coding for microbial expression of polypeptides having primary conformations which differ from that herein specified for mature EPO in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions). Alternately, modifications of cDNA and genomic EPO genes may be readily accomplished by well-known site-directed 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, projected EPO products of the invention include those which are foreshortened by e.g., deletions [Asn^2, des-Pro^2 through Ile^6]hEPO, [des-Thr^{163} through Arg^{166}]hEPO and "Δ27-55hEPO", the latter having the residues coded for by an entire exon deleted; or which are more stable to hydrolysis (and, therefore, may have more pronounced or longer lasting effects than naturally-occurring EPO); or which have been altered to delete one or more potential sites for glycosylation (which may result in higher activities for yeast-produced products); or which have one or more cystein residues deleted or replaced by, e.g., histidine or serine residues (such as the analog [His^7]hEPO) 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

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[Phe^{15}]hEPO, [Phe^{49}]hEPO, and [Phe^{145}]hEPO) and may bind more or less readily to EPO receptors on target cells. Also comprehended are polypeptide fragments duplicating only a part of the continuous 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 potential fragments of EPO which are elucidated upon consideration of the human genomic DNA sequence of ~~Table VI~~ Table 6, i.e., "fragments" of the total continuous EPO sequence which are delineated by intron sequences and which may constitute distinct "domains" of biological activity. It is noteworthy that the absence of *in vivo* 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 of utility in other contexts, such as in EPO assays or EPO antagonism. Antagonists of erythropoietin may be quite useful in treatment of polycythemias or cases of overproduction of EPO [see, e.g., Adamson, Hosp.Practice, 18(12), 49-57 (1983), and Hellmann, et al., Clin.Lab.Haemat., 5, 335-342 (1983)].

According to another aspect of the present invention, the cloned DNA sequences described herein which encode human and monkey EPO polypeptides are conspicuously valuable for the information which they provide concerning the amino acid sequence of mammalian erythropoietin which has heretofore been unavailable despite decades of analytical processing of isolates of naturally-occurring products. The DNA sequences are also conspicuously valuable as products useful in effecting the large scale microbial synthesis of erthropoietin by a variety of recombinant techniques. Put another way, DNA sequences provided by the invention are useful in generating new and useful viral and circular plasmid DNA vectors, new and useful transformed and transfected

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microbial procaryotic and eucaryotic host cells (including bacterial and yeast cells and mammalian cells grown in culture), and new and useful methods for cultured growth of such microbial host cells capable of expression of EPO and EPO products. DNA sequences of the invention are also conspicuously suitable materials for use as labelled probes in isolating EPO and related protein encoding cDNA and genomic DNA sequences of mammalian species other than human and monkey species herein specifically illustrated. The extent to which DNA sequences of the invention will have use in various alternative methods of protein synthesis (e.g., in insect cells) or in genetic therapy in humans and other mammals cannot yet be calculated. DNA sequences of the invention are expected to be useful in developing transgenic mammalian species which may serve as eucaryotic "hosts" for production of erythropoietin and erythropoietin products in quantity. See, generally, Palmiter, et al., Science, 222(4625), 809-814 (1983).

Viewed in this light, therefore, the specific disclosures of the illustrative examples are clearly not intended to be limiting upon the scope of the present invention and numerous modifications and variations are expected to occur to those skilled in the art. As one example, while DNA sequences provided by the illustrative examples include cDNA and genomic DNA sequences, because this application provides amino acid sequence information essential to manufacture of DNA sequence, the invention also comprehends such manufactured DNA sequences as may be constructed based on knowledge of EPO amino acid sequences. These may code for EPO (as in Example 12) as well as for EPO fragments and EPO polypeptide analogs (i.e., "EPO Products") which may share one or more biological properties of naturally-occurring EPO but not share others (or possess others to different degrees).

DNA sequences provided by the present invention are thus seen to comprehend all DNA sequences suitable

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for use in securing expression in a procaryotic or eucaryotic host cell of a polypeptide product having at least a part of the primary structural conformation and one or more of the biological properties of erythropoietin, and selected from among: (a) the DNA sequences set out in ~~Tables V and VI~~ Figures 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 allelic monkey and human EPO gene sequences and other mammalian species gene sequences are expected to hybridize to the sequences of ~~Tables V and VI~~ Figures 5 and 6 or to fragments thereof. Further, but for the degeneracy of the genetic code, the SCEPO and ECEPO genes and the manufactured or mutagenized cDNA or genomic DNA sequences encoding various EPO fragments and analogs would also hybridize to the above-mentioned DNA sequences. Such hybridizations could readily 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 microbial expression of EPO products in the context of mammalian cell expression of DNA inserted in a hybrid vector of bacterial plasmid and viral genomic origins, a wide variety of expression systems are within the contemplation of the invention. Conspicuously comprehended are expression systems involving vectors of homogeneous origins applied to a variety of bacterial, yeast and ~~mammalian~~ mammalian cells in culture as well as to expression systems not involving vectors (such as calcium phosphate transfection of cells). In

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this regard, it will be understood that expression of, e.g., monkey origin DNA in monkey host cells in culture and human host cells in culture, actually constitute instances of "exogenous" DNA expression inasmuch as the EPO DNA whose high level expression is sought would not have its origins in the genome of the host. Expression systems of the invention further contemplate these practices resulting in cytoplasmic formation of EPO products and accumulation of glycosylated and non-glycosylated EPO products in host cell cytoplasm or membranes (e.g., accumulation in bacterial periplasmic spaces) or in culture medium supernatants as above illustrated, or in rather uncommon systems such as P.aeruginosa expression systems (described in Gray, et al., Biotechnology, 2, pp. 161-165 (1984)).

Improved hybridization methodologies of the invention, while illustratively applied above to DNA/DNA hybridization screenings are equally applicable to RNA/RNA 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 many individual processing improvements include: improved colony transfer and maintenance procedures; use of nylon-based filters such as GeneScreen and GeneScreen Plus to allow reprobing with same filters and repeated use of the filter, application of novel protease treatments [compared, e.g., to Taub, et al. Anal.Biochem., 126, pp. 222-230 (1982)]; use of very low individual concentrations (on the order of 0.025 picomole) of a large number of mixed probes (e.g., numbers in excess of 32); and, performing hybridization and post-hybridization steps under stringent temperatures closely approaching (i.e., within 4°C and preferably within 2°C away from) the lowest calculated dissocation temperature of any of the mixed probes employed. These improvements combine to

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provide results which could not be expected to attend their use. This is amply illustrated by the fact that mixed probe procedures involving 4 times the number of probes ever before reported to have been successfully used in even cDNA screens on messenger RNA species of relatively low abundancy were successfully applied to the isolation of a unique sequence gene in a genomic library screening of 1,500,000 phage plaques. This feat was accomplished essentially concurrently with the publication of the considered opinion of Anderson, et al., supra, that mixed probe screening methods were "...impractical for isolation of mammalian protein genes when corresponding RNA's are unavailable.

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WHAT IS CLAIMED IS:

1. A purified and isolated polypeptide having part or all of the primary structural conformation and one or more of the biological properties of naturally-occurring erythropoietin and characterized by being the product of procaryotic or eucaryotic expression of an exogenous DNA sequence.

2. A polypeptide according to claim 1 further characterized by being free of association with any mammalian protein.

3. A polypeptide according to claim 1 wherein the exogenous DNA sequence is a cDNA sequence.

4. A polypeptide according to claim 1 wherein the exogenous DNA sequence is a manufactured DNA sequence.

5. A polypeptide according to claim 1 wherein the exogenous DNA sequence is a genomic DNA sequence.

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

7. A polypeptide according to claim 1 possessing part or all of the primary structural conformation of human erythropoietin as set forth in Table VI or any naturally occurring allelic variant thereof.

8. A polypeptide according to claim 1 possessing part or all of the primary structural conformation of monkey erythropoietin as set forth in Table V or any naturally occurring allelic variant thereof.

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9. A polypeptide according to claim 1 which has the immunological properties of naturally-occurring erythropoietin.

10. A polypeptide according to claim 1 which has the in vivo biological activity of naturally-occurring erythropoietin.

11. A polypeptide according to claim 1 which has the in vitro biological activity of naturally-occurring erythropoietin.

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

13. A polypeptide according to claim 12 wherein said detectable label is a radiolabel.

14. A DNA sequence for use in securing expression in a procaryotic or eucaryotic host cell of a polypeptide product having at least a part of the primary structural conformation and one or more of the biological properties of naturally-occurring erythropoietin, said DNA sequence selected from among:

(a) the DNA sequences set out in Tables V and VI or their complementary strands;

(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).

15. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according

to claim 14 in a manner allowing the host cell to express said polypeptide product.

16. A polypeptide product of the expression of a DNA sequence of claim 14 in a procaryotic or eucaryotic host.

17. A purified and isolated DNA sequence coding for procaryotic or eucaryotic host expression of a polypeptide having part or all of the primary structural conformation and one or more of the biological properties of erythropoietin.

18. A cDNA sequence according to claim 17.

19. A monkey species erythropoietin coding DNA sequence according to claim 18.

20. A DNA sequence according to claim 19 and including the protein coding region set forth in Table V.

21. A genomic DNA sequence according to claim 17.

22. A human species erythropoietin coding DNA sequence according to claim 21.

23. A DNA sequence according to claim 22 and including the protein coding region set forth in Table VI.

24. A manufactured DNA sequence according to claim 14.

25. A manufactured DNA sequence according to claim 24 and including one or more codons preferred for expression in E.coli cells.

26. A manufactured DNA sequence according to claim 25, coding for expression of human species erythropoietin.

27. A manufactured DNA sequence according to claim 26 including the protein coding region set forth in Table XIV.

28. A manufactured DNA sequence according to claim 24 and including one or more codons preferred for expression in yeast cells.

29. A manufactured DNA sequence according to claim 28, coding for expression of human species erythropoietin.

30. A manufactured DNA sequence according to claim 29 including the protein coding region set forth in Table XXI.

31. A DNA sequence according to claim 17 covalently associated with a detectable label substance.

32. A DNA sequence according to claim 31 wherein the detectable label is a radiolabel.

33. A single-strand DNA sequence according to claim 31.

34. A DNA sequence coding for a polypeptide fragment or polypeptide analog of naturally-occurring erythropoietin.

35. A DNA sequence coding for [Phe^{15}]hEPO, [Phe^{49}]hEPO, [Phe^{145}]hEPO, [His^{7}]hEPO, [Asn^{2} des-Pro^{2} through Ile^{6}]hEPO, [des-Thr^{163} through Arg^{166}]hEPO, or [Δ27-55]hEPO.

36. A DNA sequence according to claim 34 which is a manufactured sequence.

37. A biologically functional circular plasmid or viral DNA vector including a DNA sequence according to either of claims 14, 17, 34 or 35.

38. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to claim 37.

39. A polypeptide product of the expression in a procaryotic or eucaryotic host cell of a DNA sequence according to claims 17 or 34.

40. A glycoprotein product having a primary structural conformation sufficiently duplicative of that of a naturally-occurring 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 erythropoietin.

41. A glycoprotein product having a primary structural conformation sufficiently duplicative of that of a naturally-occurring 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 human erythropoietin.

42. Vertebrate cells 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 U of erythropoietin per 10^6 cells in 48 hours as determined by radioimmunoassay.

43. Vertebrate cells according to claim 42 capable of producing in excess of 500 U erythropoietin per 10^6 cells in 48 hours.

44. Vertebrate cells according to claim 42 capable of producing in excess of 1,000 U erythropoietin per 10^6 cells in 48 hours.

45. Vertebrate cells according to claim 42 which are mammalian or avian cells.

46. Vertebrate cells according to claim 45 which are COS-1 cells or CHO cells.

47. A synthetic polypeptide having part or all of the amino acid sequence as set forth in ~~Table V~~ Figure 5 and having one or more of the in vivo or in vitro biological activities of naturally-occurring monkey erythropoietin.

48. A synthetic polypeptide having part or all of the amino acid sequence set forth in ~~Table VI~~, Figure 6, other than a sequence of residues entirely within the sequence numbered 1 through 20, and having a biological property of naturally-occurring human erythropoietin.

49. A synthetic polypeptide having part or all of the secondary conformation of part or all of the amino acid sequence set forth in ~~Table VI~~, Figure 6, other than a sequence of residues entirely within the sequence numbered 1 through 20, and having a biological property of naturally-occurring human erythropoietin.

50. A process for the production of a polypeptide having part or all of the primary structural conformation and one or more of the biological properties of naturally-occurring erythropoietin, said process comprising:

growing, under suitable nutrient conditions, procaryotic or eucaryotic host cells transformed or transfected with a DNA vector according to claim 37 and isolating desired polypeptide products of the expression of DNA sequences in said vector.

51. An antibody substance characterized by immunoreactivity with erythropoietin and with a synthetic polypeptide having a primary structural conformation substantially duplicative of a continuous sequence of amino acid residues extant in naturally-occurring erythropoietin except for any polypeptide comprising a sequence of amino acid residues entirely comprehended within sequence,
A-P-P-R-L-I-C-D-S-R-V-L-E-R-Y-L-L-E-A-K.

52. An antibody according to claim 51, which is a monoclonal antibody.

53. An antibody according to claim 51, which is a polyclonal antibody.

54. An antibody according to claim 51, which is immunoreactive with erythropoietin and a synthetic polypeptide having the sequence selected from the sequences:
V-P-D-T-K-V-N-F-Y-A-W-K-R-M-E-V-G,
K-E-A-I-S-P-P-D-A-A-S-A-A, and
V-Y-S-N-F-L-R-G-K-L-K-L-Y-T-G-E-A-C-R-T-G-D-R.

55. A pharmaceutical composition comprising an effective amount of a polypeptide according to claims 1, 16, 39, 40 or 41 and a pharmaceutically acceptable diluent, adjuvant or carrier.

56. A method for providing erythropoietin therapy to a mammal comprising administering an effective amount of a polypeptice according to claims 1, 16, 39, 40 or 41.

57. A method according to claim 56 wherein the therapy comprises enhancing hematocrit levels.

58. A purified and isolated DNA sequence as set out in Table V or VI or a fragment thereof or the complementary strand of such a sequence or fragment.

59. A polypeptide product of the expression of a DNA sequence according to claim 58 in a procaryotic or eucaryotic host cell.

60. An improvement in the method for detection of a specific single stranded polynucleotide of unknown sequence in a heterogeneous cellular or viral sample including multiple single-stranded polynucleotides wherein:

(a) a mixture of labelled single-stranded polynucleotide probes is prepared having uniformly varying sequences of bases, each of said probes being potentially specifically complementary to a sequence of bases which is putatively unique to the polynucleotide to be detected,

(b) the sample is fixed to a solid substrate;

(c) the substrate having the sample fixed thereto is treated to diminish further binding of polynucleotides thereto except by way of hybridization to polynucleotides in said sample,

(d) the treated substrate having the sample fixed thereto is transitorily contacted with said mixture of labelled probes under conditions facilitative of hybridization only between totally complementary polynucleotides, and,

(e) the specific polynucleotide is detected by monitoring for the presence of a hybridization reaction between it and a totally complementary probe within said mixture of labelled probes, as evidenced by the presence of a higher density of labelled material on the substrate at the locus of the specific polynucleotide in comparison to a background density of labelled material resulting from non-specific binding of labelled probes to the substrate,

said improvement comprising using in excess of 32 mixed probes and performance of one or more of the following:

(1) employing a nylon-based paper as said solid substrate;

(2) treating with a protease in step (c);

(3) employing individual labelled probe concentrations of approximately 0.025 picomoles; and

(4) employing as one of the hybridization conditions in step (d) stringent temperatures approaching to with 4°C away from the lowest calculated Td of any of the probes employed.

add C3

add m1

Add D1, F1, I1

add I15

add K1

add L1

ABSTRACT

"PRODUCTION OF ERYTHROPOIETIN"

Disclosed are novel polypeptides possessing part or all of the primary structural conformation and one or more of the biological properties of mammalian erythropoietin ("EPO") which are characterized in preferred forms by being the product of procaryotic or eucaryotic host expression of an exogenous DNA sequence. Illustratively, genomic DNA, cDNA and manufactured DNA sequences coding for part or all of the sequence of amino acid residues of EPO or for analogs thereof are incorporated into autonomously replicating plasmid or viral vectors employed to transform or transfect suitable procaryotic or eucaryotic host cells such as bacteria, yeast or vertebrate cells in culture. Upon isolation from culture media or cellular lysates or fragments, products of expression of the DNA sequences display, e.g., the immunological properties and in vitro and in vivo biological activities of EPO of human or monkey species origins. Disclosed also are chemically synthesized polypeptides sharing the biochemical and immunological properties of EPO. Also disclosed are improved methods for the detection of specific single stranded polynucleotides in a heterologous cellular or viral sample prepared from, e.g., DNA present in a plasmid or viral-borne cDNA or genomic DNA "library".

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name. I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "PRODUCTION OF ERYTHROPOIETIN"

the specification of which (check one): ☒ is attached hereto; ☐ was filed on _____ as Application Serial No. _____ and was amended on (or amended through) _____ (if applicable). I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☒ Yes	☐ No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status - Patented, Pending or Abandoned)
561,024	December 13, 1983	Pending
582,185	February 21, 1984	Pending
655,841	September 28, 1984	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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William A. Marshall (17,023)
Jerome B. Klauss (17,104)
Basil P. Maas (18,464)

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State or Country: California	State or Country: California
Date: November 29, 1984	Signature: Fu-Kuen Lin

☐ See attached page for additional joint inventors

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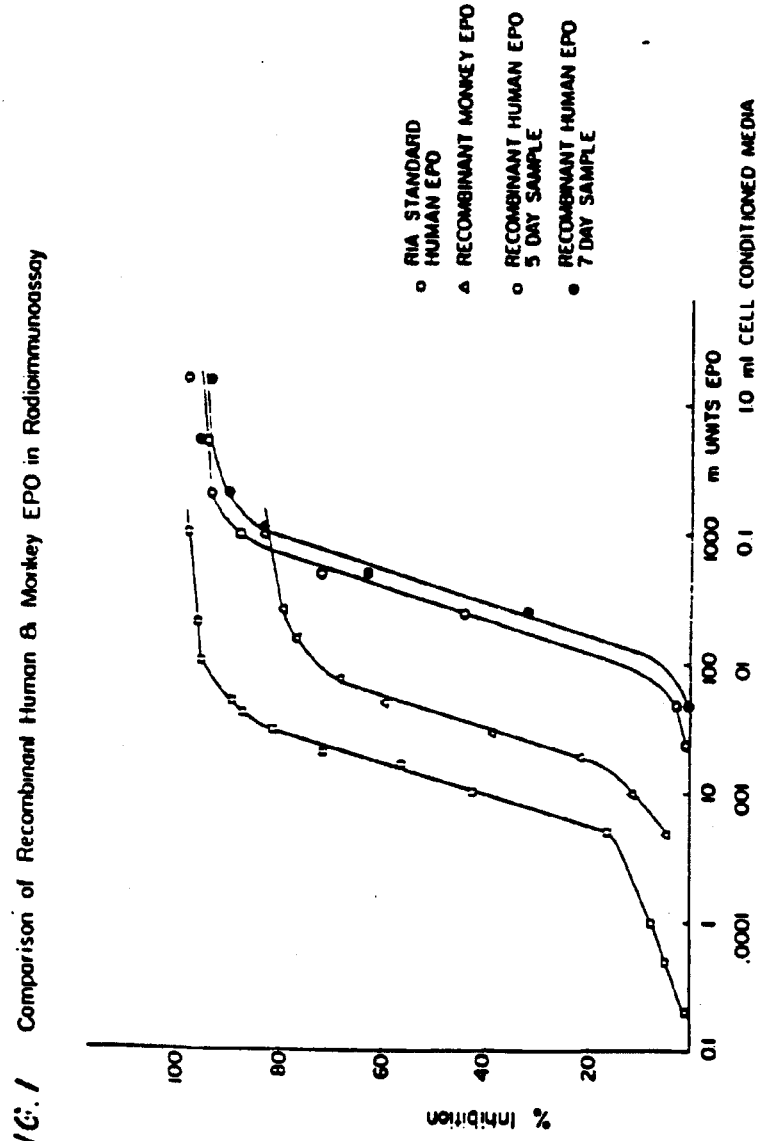

FIG. 1 Comparison of Recombinant Human & Monkey EPO in Radioimmunoassay

FIG. 2

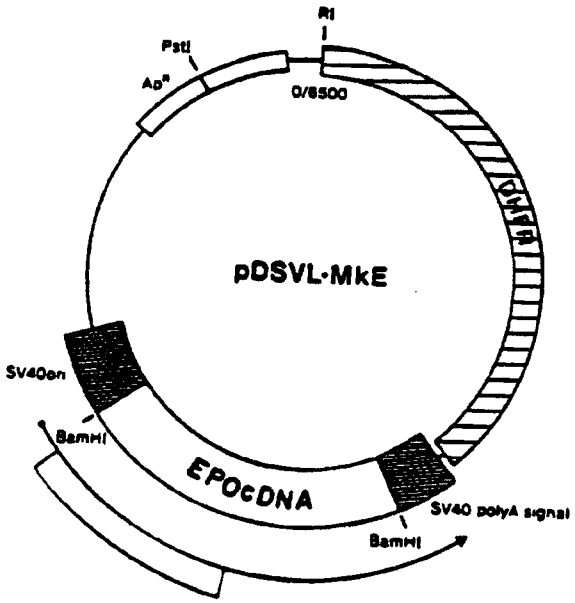
RI
PstI
Ap^R
0/6500
pDSVL-MkE
SV40en
BamHI
EPOcDNA
SV40 polyA signal
BamHI

FIG. 3

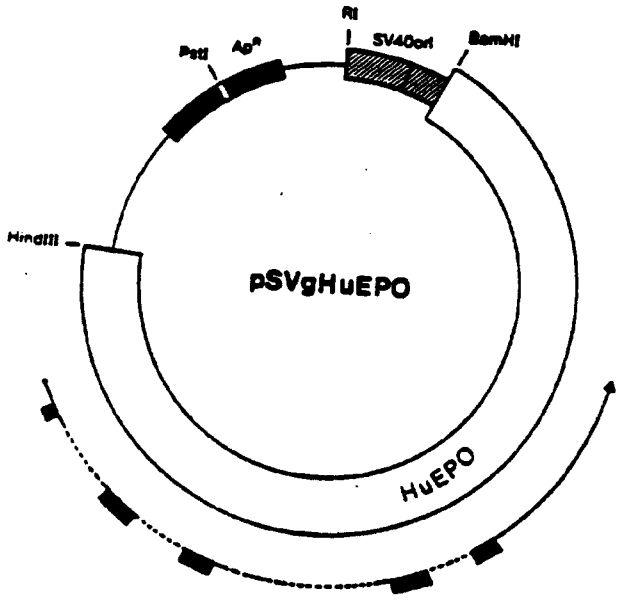

FIG. 4

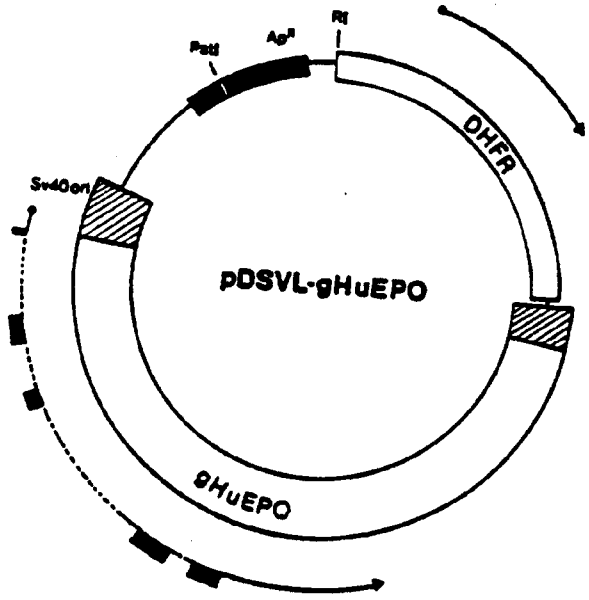

FIG.5A

Translation of Monkey EPO cDNA

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Sau3A
GATCCCGCCGCCCCCTGGACAGCCGCCCTCTCCTCCAGGCCCCGTGGGGCTGGCCCTGCCC

CGCTGAACTTCCCGGGATGAGGACTCCCGGTGTGGTCACCGCGCGCCTAGGTCGCTGAG

                                  -27                         -20
                          Met Gly Val His Glu Cys Pro Ala Trp
GGACCCCGGCCAGGCGCGGAGATG 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 GGC 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                  *       40
Gly Cys Ser Glu Ser Cys Ser Leu Asn Glu Asn Ile Thr Val Pro
GGC TGT TCC GAA AGC TGC AGC TTG AAT GAG AAT ATC ACC GTC CCA
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Print of Drawing As Originally Filed

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FIG.5B

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                                         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 GGG

         60                                      70
Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu
CAG CAG GCT GTA GAA GTC TGG CAG GGC CTG GCC CTG CTC TCA GAA

                             80          *
Ala Val Leu Arg Gly Gln Ala Val Leu Ala Asn Ser Ser Gln Pro
GCT GTC CTG CGG GGC CAG GCC GTG TTG GCC AAC TCT TCC CAG CCT

         90                                      100
Phe Glu 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 GGC CTT

                             110
Arg Ser Ile Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Glu Ala
CGC AGC ATC ACC ACT CTG CTT CGG GCG CTG GGA GCC CAG GAA GCC

         120                                     130
Ile Ser Leu Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile
ATC TCC CTC CCA GAT GCG GCC TCG GCT GCT CCA CTC CGA ACC ATC

                             140
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 TAC TCC AAT TTC
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11/3/05

FIG.5C

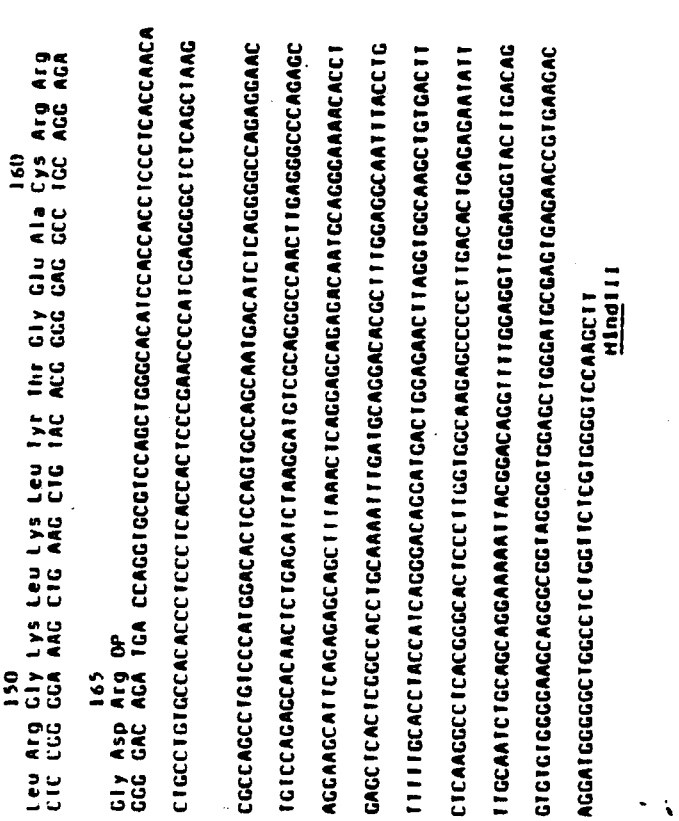


FIG.6A

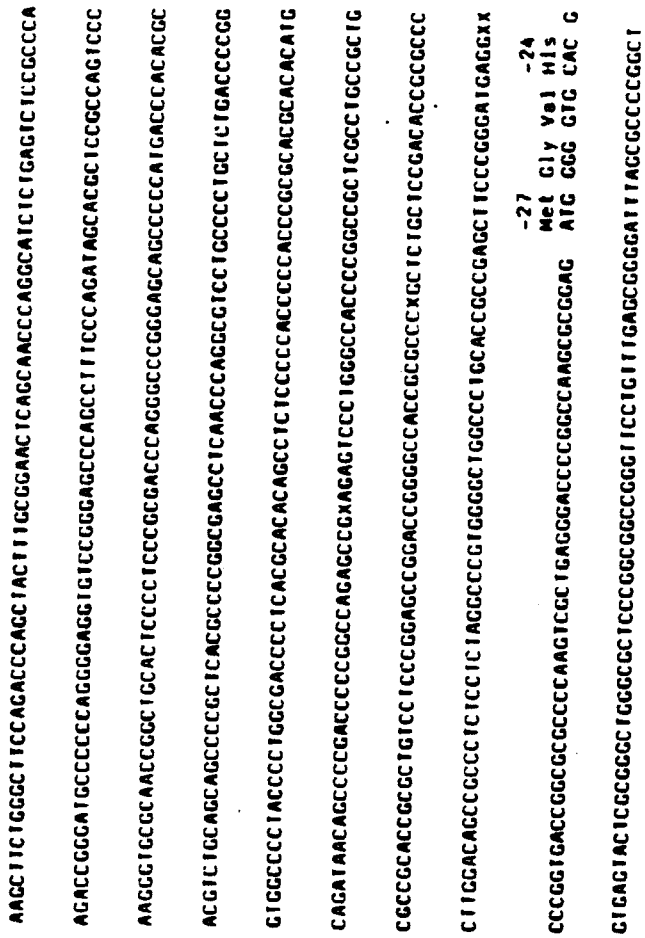
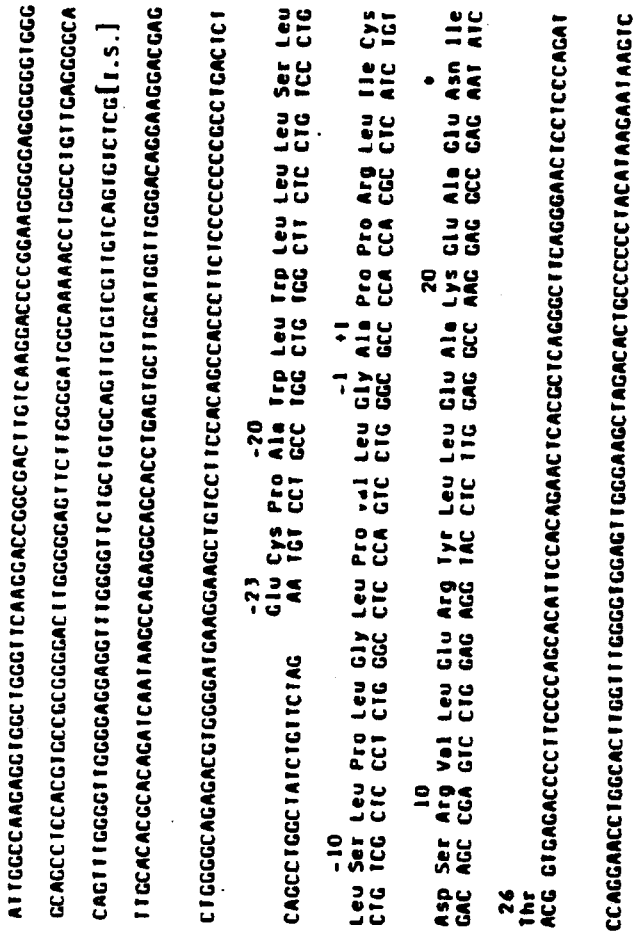


FIG.6B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CAGTTTGGGGTTGGGGAGGAGGTTTGGGGTTCTGCTGTGCAGTTGTCGTTCAGTGTCTCGG[n.s.]
TTGCACACGCCACAGATCAATAAGCCAGAGGCAGCACCTGAGTGCTTGCATGGTTGGGACAGGAAGGACGAG
CTGGGGCAGAGACGTGGGGATGAAGGAAGCTGTCCTTCCACAGCCACCCTTCTCCCCCCCCGCCTGACTCT
-23 -20
Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
CAGCCTGGCTATCTGTTCTAG AA TGT CCT GCC TGG CTG CTT CTC CTG TCC CTG
-10 -1 +1
Leu Ser Leu Pro Leu Gly Leu Pro val Leu Gly Ala Pro Pro Arg Leu Ile Cys
CTG TCG CTC CCT CTG GGC CTC CCA GTC CTG GGC GCC CCA CGC CTC ATC TGT
10 20
Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Ala Lys Glu Ala Glu Asn Ile
GAC AGC CGA GTC CTG GAG AGG TAC CTC TTG GAG GCC GCC AAG GAG GCC GAG AAT ATC
26
Thr
ACG GTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTCAGGGCTTCAGGGAACTCCTCCCAGAT
CCAGGAACCTGGCACTTGGTTGGGGTGGAGTTGGGAAGCTAGACACTGCCCCCCCTACATAAGAATAAGTC

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FIG.6C

```
TGGTGGCCCCAAACCATACCTGAAACTAGGCAAGGAGCAAAGCCAGCAGATCCTACGCCTGTGGCCAGGG

                                                   27              30
                                                   Thr Gly Cys Ala Glu
CCAGAGCCTTCAGGGACCCTTGACTCCCCGGGCTGTGTGCATTTCAG    ACG GGC TGT GCT GAA

                        •       40
His Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr
CAC TGC AGC TTG AAT GAG AAT ATC ACT GTC CCA GAC ACC AAA GTT AAT TTC TAT

50                  55
Ala Trp Lys Arg Met Glu
GCC TGG AAG AGG ATG GAG GTGAGTTCCTTTTTTTTTTTTTTTTTTTCCTTTCTTTTGGAGAATCTCATT

TGCCGAGCCTGATTTTGGATGAAAGGGAGAATGATCGGGGGAAAGGTAAAATGCAGCAGCAGAGATGAGCCT

GCCTGGGCCGCAGAGGCTCACGTCTATAATCCCAGGCTGAGATGGCCGAGATGGGAGAATTGCTTGAGCCCT

GGAGTTTCAGACCAACCTAGGCAGCATAGTGAGATCCCCCATCTCTACAAACATTTAAAAAAATTAGTCAG

GTGAAGTGGTGCATGGTGGTAGTCCCAGATATTTGGAAGGCTGAGGCGGGAGGATCGCTTGAGCCCAGGAA

TTTGAGGCTGCAGTGAGCTGTGATCACACCACTGCACTCCAGCCTCAGTGACAGAGTGAGGCCCTGTCTCA
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AM670156390

AM-ITC 00941057

FIG.6D

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AAAAAGAAAACAAAAAAGCAAAAATAATGAGGGCTGTATGGAATACATTCATTATTCATTCACTCACTCACT

CACTCATTCATTCATTCATTCAACAAGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGCTTGG

GGCTGCTGAGGGCCAGGAGGGAGAGGGTGACATGGGTCAGCTCGACTCCCAGAGTCCACTCCCTGTAG

 56                  60                  70
Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala
GTC GGG CAG CAG GCC GTA GAA GTC TGG CAG GGC CTG GCC CTG CTG TCG GAA GCT

                    80                  90
Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu
GTC CTG CGG GGC CAG GCC CTG TTG GTC AAC TCT TCC CAG CCG TGG GAG CCC CTG

                   100
Gln Leu His Val Asp Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu
CAG CTG CAT GTG GAT AAA GCC GTC AGT GGC CTT CGC AGC CTC ACC ACT CTG CTT

110                 115
Arg Ala Leu Gly Ala Gln
CGG GCT CTG GGA GCC CAG GTGAGTAGGAGCCCACACTCTGCTTGCCCCTTTCTGTGGCACTGCAGCGACCTCCT

                   116                 120
GAAGGGTCTTGCTAAGGAGTACAGGAACTGTCCGTATTCCTTCCCTTTCTGTGGCACTGCAGCGACCTCCT

                   116                 120
GTTTTCTCCTTGGCAG    AAG GAA GCC ATC TCC CCT CCA GAT GCG GCC TCA GCT GCT
```

FIG. 6E

```
     130                                          140
Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser
CCA CTC CGA ACA ATC ACT GCT GAC ACT TTC CGC AAA CTC TTC CGA GTC TAC TCC

            150                                     160
Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly
AAT TTC CTC CGG GGA AAG CTG AAG CTG TAC ACA GGG GAC GCC TGC AGG ACA GGG

    166
Asp Arg OP
GAC AGA TGA CCAGGTGTGTCCACCTGGGCATATCCACCACCTCCCTCACCAACATTGCTTGTGCCACA

CCCTCCCCCGCCACTCCTGAACCCCGTCGAGGGGCTCTCAGCTCAGCGCCAGCCTGTCCCATGGACACTCC

AGTGCCAGCAATGACATCTCAGGGGCCAGAGGAACTGTCCAGAGAGCAACTCTGAGATCTAAGGATGTCAC

AGGGCCAACTTGAAGGGCCCAGAGCAGGAAGCATTCAGAGAGCAGCTTTAAACTCAGGGACAGAGCCATGC

TGGGAAGACGCCTGAGCTCACTCGGCACCCTGCAAAATTTGATGCCAGGACACGCTTTGGAGGCGATTTAC

CTGTTTTCGCACCTACCATCAGGGACAGGATGACCTGGAGAACTTAGGTGGCAAGCTGTGACTTCTCCAGG

TCTCACGGGCATGGGCACTCCCTTGGTGGCAAGAGCCCCCTTGACACCGGGGTGGTGGGAACCATGAAGAC

AXGATXGGGGCTGGCCTCTGGCTCTCATGGGGTCCAAGTTTTGTGTATTCTCAACCTATTGACAGACTGAA

ACACAATATGAC
```

FIG.7

ECEPO GENE

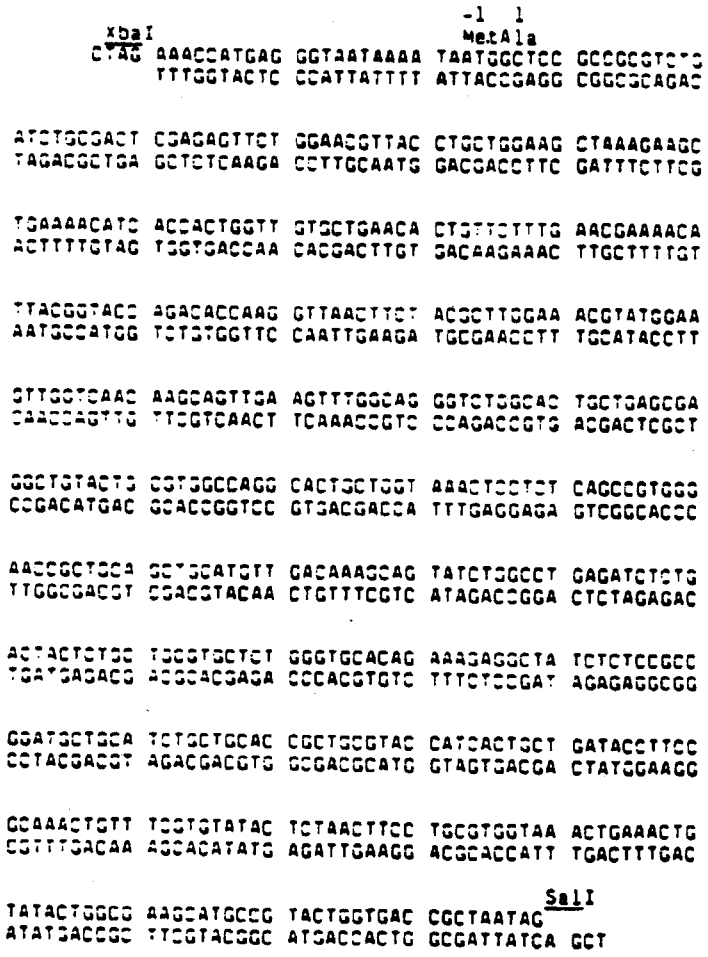

FIG.8

SCEPC GENE

```
                  -1 +1
HindIII           ArgAla
AGCTTGGATA AAAGAGCTCC ACCAAGATTG ATCTGTGACT CGAGAGTTTT
    ACCTAT TTTCTCGAGG TGGTTCTAAC TAGACACTGA GCTCTCAAAA

GGAAAGATAC TTGTTGGAAG CTAAAGAAGC TGAAAACATC ACCACTGGTT
CCTTTCTATG AACAACCTTC GATTTCTTCG ACTTTTGTAG TGGTGACCAA

GTGCTGAACA CTGTTCTTTG AACGAAAACA TTACGGTACC AGACACCAAG
CACGACTTGT GACAAGAAAC TTGCTTTTGT AATGCCATGG TCTGTGGTTC

GTTAACTTCT ACGCTTGGAA ACGTATGGAA GTTGGTCAAC AAGCTGTTGA
CAATTGAAGA TGCGAACCTT TGCATACCTT CAACCAGTTG TTCGACAACT

AGTTTGGCAA GGTTTGGCCT TGTTATCTGA AGCTGTTTTG AGAGGTCAAG
TCAAACCGTT CCAAACCGGA ACAATAGACT TCGACAAAAC TCTCCAGTTC

CCTTGTTGGT TAACTCTTCT CAACCATGGG AACCATTGCA ATTGCACGTC
GGAACAACCA ATTGAGAAGA GTTGGTACCC TTGGTAACGT TAACGTGCAG

GATAAAGCCG TCTCTGGTTT GAGATCTTTG ACTACTTTGT TGAGAGCTTT
CTATTTCGGC AGAGACCAAA CTCTAGAAAC TGATGAAACA ACTCTCGAAA

GGGTGCTCAA AAGGAAGCCA TTTCCCCACC AGACGCTGCT TCTGCCGCTC
CCCACGAGTT TTCCTTCGGT AAAGGGGTGG TCTGCGACGA AGACGGCGAG

CATTGAGAAC CATCACTGCT GATACCTTCA GAAAGTTATT CAGAGTTTAC
GTAACTCTTG GTAGTGACGA CTATGGAAGT CTTTCAATAA GTCTCAAATG

TCCAACTTCT TGAGAGGTAA ATTGAAGTTG TACACCGGTG AAGCCTGTAG
AGGTTGAAGA ACTCTCCATT TAACTTCAAC ATGTGGCCAC TTCGGACATC

AACTGGTGAC AGATAAGCCC GACTGATAAC AACAGTGTAG
TTGACCACTG TCTATTCGGG CTGACTATTG TTGTCACATC

                  SalI
ATGTAACAAA G
TACATTGTTT CAGCT
```

<u>Comparison of Human and Monkey EPO Polypeptides</u>

```
                -20          -10          +1         10          20          30          40
Human    MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLEAKEAENITTGCAEHCSLNENITVPDTK
         **************** ******** ************************* * ** * *************
Monkey   MGVHECPAWLWLLLSLVSLPLGLPVPGAPPRLICDSRVLERYLLEAKEAENVTMGCSESCSLNENITVPDTK

            50        60           70          80          90         100        110
Human    VNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKE
         ********************************** * ***** ****** *** ***** ********** *
Monkey   VNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQAVLANSSQPFEPLQLHMDKAISGLRSITTLLRALGAQ-E

         120       130        140        150        160
Human    AISPPDAASAAPLRTITADTFRKLFRVYSNFLRGKLKLYTGEACRTGDR
         *** ***************** *********************** ***
Monkey   AISLPDAASAAPLRTITADTFCKLFRVYSNFLRGKLKLYTGEACRRGDR
```

FIG. 9

ECEPO SECTION 1 OLIGONUCLEOTIDES

1. AATTCTAGAAACCATGAGGGTAATAAAATA
2. CCATTATTTTATTACCCTCATGGTTTCTAG
3. ATGGCTCCGCCGCGTCTGATCTGCGAC
4. CTCGAGTCGCAGATCAGACGCGGCGGAG
5. TCGAGAGTTCTGGAACGTTACCTGCTG
6. CTTCCAGCAGGTAACGTTCCAGAACT
7. GAAGCTAAAGAAGCTGAAAACATC
8. GTGGTGATGTTTTCAGCTTCTTTAG
9. ACCACTGGTTGTGCTGAACACTGTTC
10. CAAAGAACAGTGTTCAGCACAACCA
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTTCGTT

FIG. 10

11311K

ECEPO SECTION 1

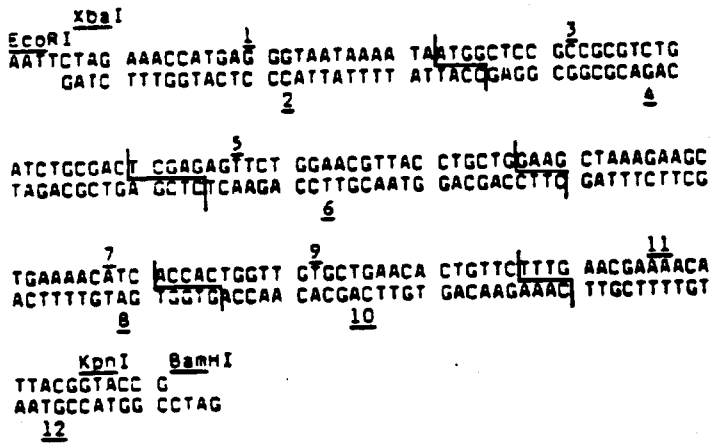

FIG. 11

As Original Filed

ECEPO SECTION 2 OLIGONUCLEOTIDES

1. AATTCGGTACCAGACACCAAGGT
2. GTTAACCTTGGTGTCTGGTACCG
3. TAACTTCTACGCTTGGAAACGTAT
4. TTCCATACGTTTCCAAGCGTAGAA
5. GGAAGTTGGTCAACAAGCAGTTGAAGT
6. CCAAACTTCAACTGCTTGTTGACCAAC
7. TTGGCAGGGTCTGGCACTGCTGAGCG
8. GCCTCGCTCAGCAGTGCCAGACCCTG
9. AGGCTGTACTGCGTGGCCAGGCA
10. GCAGTGCCTGGCCACGCAGTACA
11. CTGCTGGTAAACTCCTCTCAGCCGT
12. TTCCCACGGCTGAGAGGAGTTTACCA
13. GGGAACCGCTGCAGCTGCATGTTGAC
14. GCTTTGTCAACATGCAGCTGCAGCGG
15. AAAGCAGTATCTGGCCTGAGATCTG
16. GATCCAGATCTCAGGCCAGATACT

FIG. 12

FIG. 13

ECEPO SECTION 3

1. GATCCAGATCTCTGACTACTCTGC
2. ACGCAGCAGAGTAGTCAGAGATCTG
3. TGCGTGCTCTGGGTGCACAGAAAGAGG
4. GATAGCCTCTTTCTGTGCACCCAGAGC
5. CTATCTCTCCGCCGGATGCTGCATCT
6. CAGCAGATGCAGCATCCGGCGGAGA
7. GCTGCACCGCTGCGTACCATCACTG
8. ATCAGCAGTGATGGTACGCAGCGGTG
9. CTGATACCTTCCGCAAACTGTTTCG
10. ATACACGAAACAGTTTGCGGAAGGT
11. TGTATACTCTAACTTCCTGCGTGGTA
12. CAGTTTACCACGCAGGAAGTTAGAGT
13. AACTGAAACTGTATACTGGCGAAGC
14. GGCATGCTTCGCCAGTATACAGTTT
15. ATGCCGTACTGGTGACCGCTAATAG
16. TCGACTATTAGCGGTCACCAGTAC

FIG. 14

ECEPO SECTION 3

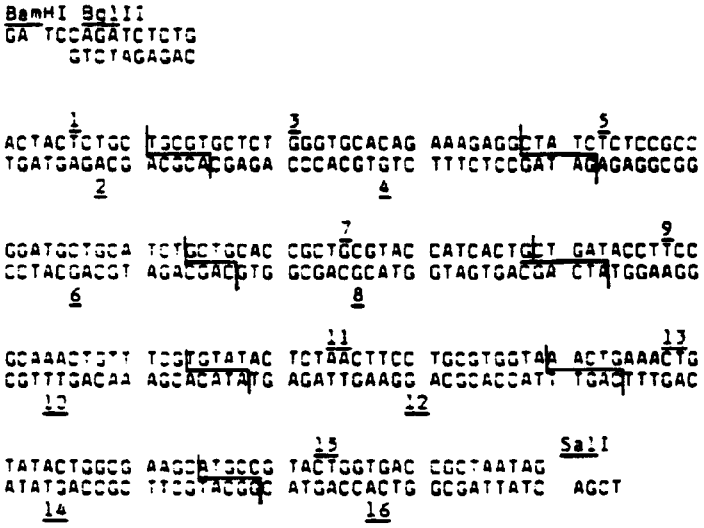

FIG. 15

SCEPO SECTION 1 OLIGONUCLEOTIDES

1. AATTCAAGCTTGGATAAAAGAGCT
2. GTGGAGCTCTTTTATCCAAGCTTG
3. CCACCAAGATTGATCTGTGACTC
4. TCTCGAGTCACAGATCAATCTTG
5. GAGAGTTTTGGAAAGATACTTGTTG
6. CTTCCAACAAGTATCTTTCCAAAAC
7. GAAGCTAAAGAAGCTGAAAACATC
8. GTGGTGATGTTTTCAGCTTCTTTAG
9. ACCACTGGTTGTGCTGAACACTGTTC
10. CAAAGAACAGTGTTCAGCACAACCA
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTTCGTT

FIG. 16

SCEPO SECTION 1

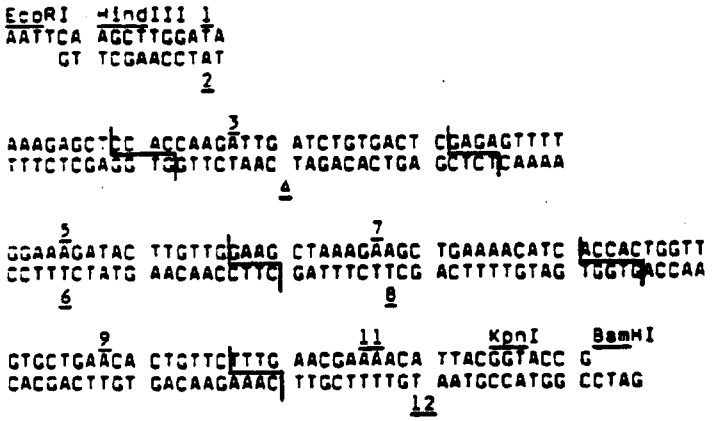

FIG. 17

SCEPO SECTION 2 OLIGONUCLEOTIDES

1. AATTCGGTACCAGACACCAAGGT
2. GTTAACCTTGGTGTCTGGTACCG
3. TAACTTCTACGCTTGGAAACGTAT
4. TTCCATACGTTTCCAAGCGTAGAA
5. GGAAGTTGGTCAACAAGCAGTTGAAGT
6. CCAAACTTCAACTGCTTGTTGACCAAC
7. TTGCCAAGGTTTGGCCTTGTTATCTG
8. GCTTCAGATAACAAGGCCAAACCTTG
9. AAGCTGTTTTGAGAGGTCAAGCCT
10. AACAAGGCTTGACCTCTCAAAACA
11. TGTTGGTTAACTCTTCTCAACCATGGG
12. TGGTTCCCATGGTTGAGAAGAGTTAACC
13. AACCATTGCAATTGCACGTCGAT
14. CTTTATCGACGTGCAATTGCAA
15. AAAGCCGTCTCTGGTTTGAGATCTG
16. GATCCAGATCTCAAACCAGAGACGG

FIG. 18

SCEPO SECTION 2

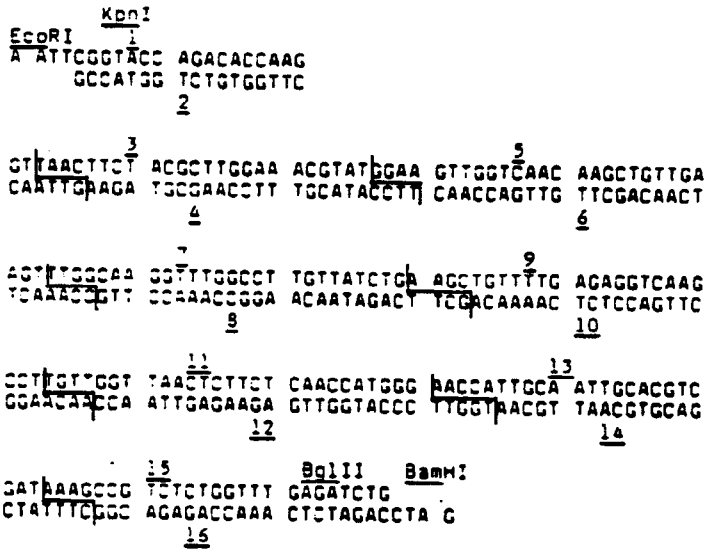

FIG. 19

11398

SCEPO SECTION 3 OLIGONUCLEOTIDES

1. GATCCAGATCTTTGACTACTTTGTT
2. TCTCAACAAAGTAGTCAAAGATCTG
3. GAGAGCTTTGGGTGCTCAAAAGGAAG
4. ATGGCTTCCTTTTGAGCACCCAAAGC
5. CCATTTCCCCACCAGACGCTGCTT
6. GCAGAAGCAGCGTCTGGTGGGGAA
7. CTGCCGCTCCATTGAGAACCATC
8. CAGTGATGGTTCTCAATGGAGCG
9. ACTGCTGATACCTTCAGAAAGTT
10. GAATAACTTTCTGAAGGTATCAG
11. ATTCAGAGTTTACTCCAACTTCT
12. CTCAAGAAGTTGGAGTAAACTCT
13. TGAGAGGTAAATTGAAGTTGTACAC
14. ACCGGTGTACAACTTCAATTTACCT
15. CGGTGAAGCCTGTAGAACTGGT
16. CTGTCACCAGTTCTACAGGCTTC
17. GACAGATAAGCCCGACTGATAA
18. GTTGTTATCAGTCGGGCTTAT
19. CAACAGTGTAGATGTAACAAAG
20. TCGACTTTGTTACATCTACACT

FIG. 20

As Original Filed

SCEPO SECTION 3

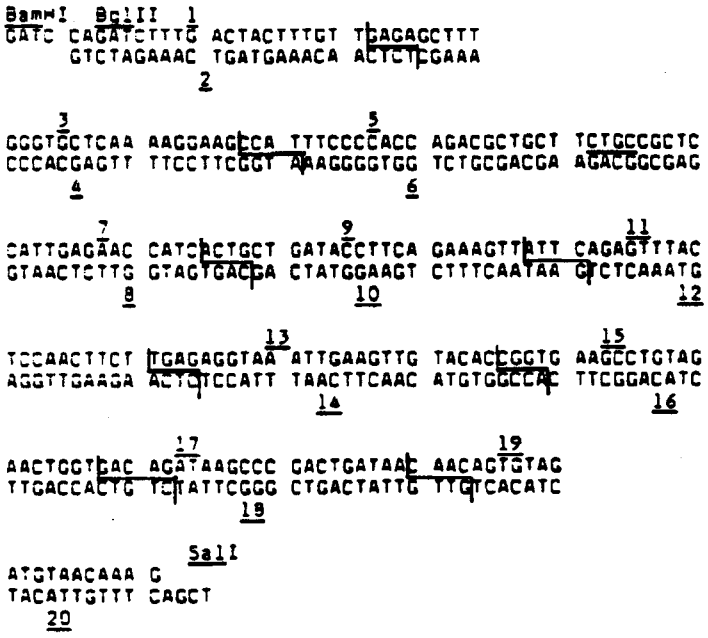

FIG. 21

113178

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

Case Docket No. D-8273

Anticipated Classification
Class ___ Subclass ___

THE HON. COMMISSIONER OF PATENTS
AND TRADEMARKS
Washington, DC 20231

Prior Application:
Examiner Tanenholtz
Art Unit 127

Sir:

This is a request for filing a

[X] Continuation

application under 37 CFR 1.60.

[ ] Divisional

of pending prior application Serial No. 675,298 filed on November 30, 1984 (date) of Fu-Kuen Lin (inventor)

for "PRODUCTION OF ERYTHROPOIETIN" (title of invention)

1. [X] Enclosed is a true copy of the prior application, including the oath or declaration as originally filed.

2. [X] The filing fee is calculated below:

	Claims as Filed, Less Any Claims Cancelled by Amendment Below			
For	Number Filed	Number Extra	Rate	Basic Fee
				340.00
Total claims--------	29 -20=	9	x 12.00	$ 108.00
Independent claims--	4 - 3=	1	x 34.00	$ 34.00
Multiple Dependent Claim			x 110.00	110.00
			Total filing fee	$ 492.00

3. [X] The Commissioner is hereby authorized to charge any fees which may be required, or to credit any overpayment to Account No. 13-2855. A duplicate copy of this sheet is enclosed.

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4. [X] A check in the amount of $ 492.00 is enclosed.

5. [X] Cancel claims 14, 15, 17-38, 42-46, 50-54 and 58-60.

6. [ ] Amend the specification by inserting before the first line the sentence: --This is a [ ] continuation, [ ] division of application Serial No. _____, filed _____.--

7. [ ] Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate of this sheet is enclosed for filing in the prior application file.

8. [X] New formal drawings, or [ ] informal drawings are enclosed.

9. [ ] Priority of application Serial No. _____ filed on _____, in _____ is claimed under 35 U.S.C. 119. The certified copy(s) have been filed in prior application Serial No. _____, filed _____.

10. [X] The prior application is assigned of record to Kirin-Amgen, Inc.

11. [X] The power of attorney in the prior application includes:

William E. Dominick (~~15,~~286)
Albert W. Bicknell (15,389)
Jerome B. Klose (17,104)
Basil P. Mann (18,464)
Alvin D. Shulman (19,412)
Donald J. Brott (19,490)
Owen J. Murray (22,111)
Allen H. Gerstein (22,218)
Nate F. Scarpelli (22,320)
Edward M. O'Toole (22,477)
Michael F. Borun (25,447)
Carl E. Moore, Jr. (26,487)
Lewis S. Gruber (30,060)
Terrence W. McMillan (30,476)
Richard A. Schnurr (30,890)
Christine A. Dudzik (31,245)
Kevin D. Hogg (~~31,~~839)
Jeffrey S. Sharp (31,879)

(a) [X] The power appears in the original papers of the prior application.

(b) ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

(c) ☐ Address all future communications to Michael F. Borun (25,447) MARSHALL, O'TOOLE ET AL. Two First National Plaza, Suite 2100 Chicago, Illinois 60603 (name, Reg. No., and Address)

12. ☒ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

13. ☒ I hereby verify that the attached papers are a true copy of prior application Serial No. 675,298 as originally filed on November 30, 1987 (date).

14. ☐ A declaration claiming Small Entity Status under 37 C.F.R. 1-9(f) and 1.27(c) has been filed in the prior application

The undersigned declare further that all statements made herein of his or her own knowledge are true and that all statements made or information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

October 23 1987 (date)

(signature) M. F. Borun

Address of signator:
Marshall, O'Toole et al.
Two First National Plaza
Chicago, Illinois 60603

☐ Inventor
☐ Assignee of complete interest
☒ Attorney or agent of record Reg. No. 25,447
☐ Filed under §1.34(a)

.60 Continuation Application of: FU-KUEN LIN

Title: "PRODUCTION OF ERYTHROPOIETIN"

Based on U.S. Serial No. 675,298, filed 11/30/84

Attorney D-8273

Mailing Certification for: Application Under 37 C.F.R. 1.60 including copy of prior Application and Declaration and new Formal Drawings

EXPRESS MAIL" mailing label No. B 61711198

Date of Deposit:

October 23, 1987

I hereby certify that this paper (or fee) is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C., 20231

Michael F. Borun (25,447)

Rule 1.60 Continuation Application of: FU-KUEN LIN

Title: "PRODUCTION OF ERYTHROPOIETIN"

Based on U.S. Serial No. 675,298, filed 11/30/84

Attorney D-8273

Mailing Certification for: Payment of fees for filing of new Rule 1.60 Continuation Patent Application
Check No: 3467

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Michael F. Borun (25,447)

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