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. CRL-1650).

In the immediately preceding system, EPO was expressed in COS-1 cells using its own promoter which is within 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 late promoter.

More specifically, the cloned 5.6 Kb BamHI to HindIII genomic human EPO restriction fragment was modified by the following procedures. Plasmid pUC8-HuE, as described above, was cleaved with BamHI and with BstEII restriction endonucleases. BstEII cleaves within the 5.6 Kb EPO gene at a position which is 44 base pairs 5′ to the initiating ATG coding for the prepeptide and approxImately 680 base pairs 3′ to the HindIII restriction site. The approximately 4900 base pair fragment was isolated. A synthetic linker DNA fragment, containing SalI and BstEII 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 BamHI to produce the intermediate plasmid pBRgHE. The genomic human EPO gene can be isolated therefrom as a 4900 base pair BamHI digestion fragment carrying the complete structural gene with a single ATG 44 base pairs 3′ to BamHI site adjacent the amino terminal coding region.

This fragment was isolated and inserted as a BamHI fragment into BamHI cleaved expression vector plasmid pDSVL1 (described in Example 6). The resulting plasmid, pSVLgHuEPO, as illustrated in FIG. **4**, was used to express EPO polypeptide material from COS-1 cells, as described in Examples 6 and 7A.

EXAMPLE 8

Culture media from growth of the six transfected COS-1 cultures of Example 6 were analyzed by radioimmunoassay according to the procedures set forth in Example 2, Part B. Each sample was assayed at 250, 125, 50, and 25 microliter aliquot levels. Supernatants from growth of cells mock transfected or transfected with vectors having incorrect EPO gene orientation were unambiguously negative for EPO immunoreactivity. For each sample of the two supernatants derived from growth of COS-1 cells transfected with vectors (H and L) having the EPO DNA in the correct orientation, the % inhibition of ^{125}I-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 supernatant could not then reliably be estimated. A quite conservative estimate of 300 mU/ml was made, however, from the value calculation of the largest aliquot size (250 microliter).

A representative culture fluid according to Example 6 and five and seven day culture fluids obtained according to Example 7A were tested in the RIA in order to compare activity of recombinant monkey and human EPO materials to a naturally-occurring human EPO standard and the results are set out in graphic form in FIG. **1**. Briefly, the results expectedly 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 values for recombinant human EPO, however, closely approximated those of the human EPO standard. The parallel nature of the dose response curves suggests 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 2.91 to 3.12 U/ml. Estimated human EPO production levels were correspondingly set at 392 mU/ml for the five-day growth sample and 567 mU/ml for the seven day growth sample. Estimated monkey EPO production levels in the Example 7B 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 Goldwasser, et al., *Endocrinology,* 97, 2, pp. 315–323 (1975). 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 and, 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,* 191, pp. 1065–1067 (1961) and Hammond, et al., *Ann.N.Y.Acad.Sci.,* 149, pp. 516–527 (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 used to transfect COS-1 cells. These vectors replicate in COS-1 cells due to the presence of SV40 T antigen within the cell and an SV40 origin of replication on the vectors. Though these vectors produce useful quantities of EPO in COS-1 cells, expression is only transient (7 to 14 days) due to the eventual loss of the vector. Additionally, only a small percentage of COS-1 became productively transfected with the vectors. The present example describes expression systems employing Chinese hamster ovary (CMO) DHFR− cells and the selectable marker, DHFR. [For discussion of related expression systems, see U.S. Pat. No. 4,399,216 and European Patent Application Nos. 117058, 117059 and 117060, all published Aug. 29, 1984.]

CHO DHFR− cells (DuX-B11) CHO K1 cells, Urlaub, et al., *Proc. Nat. Acad. Sci.* (*U.S.A.*), Vol. 77, 4461 (1980) lack the enzyme dihydrofolate reductase (DHFR) due to mutations in the structural genes and therefore require the presence of glycine, hypoxanthine, and thymidine in the culture media. Plasmids pDSVL-MkE (Example 6) or pDSVL-gHuEPO (Example 7B) were transfected along with carrier DNA into CHO DHFR− cells growing in media containing hypoxanthine, thymidine, and glycine in 60 mm culture plates. Plasmid pSVgHuEPO (Example 7A) was mixed with the plasmid pMG2 containing a mouse dihydrofolate reductase gene cloned into the bacterial plasmid vector pBR322 (per Gasser, et al., supra.) The plasmid mixture and carrier DNA was transfected into CHO DHFR− cells. (Cells which acquire one plasmid will generally also acquire a second plasmid). After three days, the cells were dispersed by trypsinization into several 100

mm culture plates in media lacking hypoxanthine and thymidine. Only those cells which have been stably transformed with the DHFR gene, and thereby the EPO gene, survive in this media. After 7–21 days, colonies of surviving cells became apparent. These transformant colonies, after dispersion by trypsinization can be continuously propagated in media lacking hypoxanthine and thymidine, creating new cell strains (e.g., CHO pDSVL-MkEPO, CHO pSVgHuEPO, CHO-pDSVL-gHuEPO).

Culture fluids from the above cell strains were tested in the RIA for the presence of recombinant monkey or human EPO. Media for strain CHO pDSVL-MkEPO contained EPO with immunological properties like that obtained from COS-1 cells transfected with plasmid pDSVL-MkEPO. A representative 65 hour culture fluid contained monkey EPO at 0.60 U/ml.

Culture fluids from CHO pSVgHuEPO and CHO pDSVL-gHuEPO contained recombinant human EPO with immunological properties like that obtained with COS-1 cells transfected with plasmid pSVgHuEPO or pDSVL-gHuEPO. A representative 3 day culture fluid from CHO pSVgHuEPO contained 2.99 U/ml of human EPO snd a 5.5 day sample from CHO pDSVL-gHuEPO had 18.2 U/ml of human EPO as measured by the RIA.

The quantity of EPO produced by the cell strains described above can be increased by gene amplification giving new cell strains of greater productivity. The enzyme dihydrofolate 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 hypoxanthine 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 resistent to MTX due to an amplification of the number of their DHFR genes, resulting in increased production of DHFR enzyme. The surviving cells can, in turn, be treated with increasing concentrations of MTX, resulting in cell strains containing greater numbers of DMFR genes. "Passenger genes" (e.g., EPO) carried on the expression vector along with the DHFR gene or transformed 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 CMO pDSVL-MkE was subjected to increasing MTX concentrations (0 nM, 30 nM and 100 nM). Representative 65-hour culture media samples from each amplification step were assayed by RIA and determined to contain 0.60, 2.45 and 6.10 U/ml, respectively. Cell strain CHO pDSVL-gHuEPO was subjected to a serles of increasing MTX concentrations of 30 nM, 50 nM, 100 nM, 200 nM, 1 μM, and 5 μM MTX. A representative 3-day culture media sample from the 100 nM MTX step contained human EPO at 3089 ± 129 u/ml as judged by RIA. Representative 48 hour cultural medium samples from the 100 nM and 1 μM MTX steps contained, respectively, human EPO at 466 and 1352 U/ml as judged by RIA (average of triplicate assays). In these procedures, 1×10^6 cells were plated in 5 ml of media in 60 mm culture dishes. 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 trypsinized and counted. The average RIA values of 467 U/ml and 1352 U/ml for cells grown at 100 nM and 1 μM MTX, respectively, provided actual yields of 2335 U/plate and 6750 U/-plate. The average cell numbers per plate were 1.94×10^6 and 3.12×10^6 cells, respectively. The effective production rates for these culture conditions were thus 1264 and 2167 U/10^6 cells/48 hours.

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 hemogeneous clones with the highest production capacity. See, Section A, Part 2, of "Points to Consider in the Characterization of Cell Lines Used to Produce Biologics", June 1, 1984, Office of Biologics Research Review, Center for Drugs and Biologics, U.S. Food and Drug Administration.

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

Strain CHO pDSVL-gHuEPO cells, grown in standard cell culture conditions, are used to seed spinner cell culture flasks. The cells are propagated as a suspension cell line in the spinner cell culture flask in media consisting of a 50—50 mixture of high glucose DMEM and Ham's F12 supplemented with 5% fetal calf serum, L-glutamine, Penicillin and Streptomycin, 0.05 mM non-essential amino acids and the appropriate concentration of methotrexate. 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 1.5×10^7 viable cells per 850 cm^2 roller bottle in 200 ml of media. The cells are allowed to grow to confluency as an adherent cell line over a three-day period. The media used for this phase of the growth is the same as used for growth in suspension. At the end of the three-day growth period, the serum containing media is removed and replaced with 100 ml of serum-free media; 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 1–3 hours and the media again is removed and replaced with 100 ml of fresh serum-free media. The 1–3 hour incubation of the serum-free media reduces the concentration of contaminating serum proteins. The roller bottles are returned to 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 conditioned media is removed and replaced with fresh serum-free medium for a second production cycle. As an example of the practice of this production system, a representative seven-day, serum-free media sample contained human erythropoietin at 3892 ± 409 U/ml as judged by the RIA. Based on an estimated cell density of 0.9 to 1.8×10^5 cells/cm^2, each 850 cm^2 roller bottle contained from 0.75 to 1.5×10^8 cells and thus the rate of production of EPO in the 7-day, 100 ml culture was 750 to 1470 U/10^6 cells/48 hours.

Culture fluids from cell strain CMO pDSVL-MkEPO carried in 10 nM MTX were subjected to RIA in vitro and in vivo EPO activity assays. The conditioned media sample contained 41.2±1.4 U/ml of MkEPO as measured by the RIA, 41.2±0.064 U/ml as measured by the in vitro biological activity assay and 42.5±5 U/ml as measured by the in vivo biological activity assay. Amino acid sequencing of polypeptide products revealed the presence of EPO products, a principle species having 3 residues of the "leader" sequence adjacent the putative amino terminal alanine. Whether this is the result of incorrect membranc processing of the polypeptide in CHO cells or reflects a difference in structure of the amino terminus of monkey EPO vis-a-vis human EPO, is presently unknown.

Culture fluids from cell strain CHO pDSVL-gHuEPO were subjected to the three assays. A 5.5 day sample contained recombinant human EPO in the media at a level of 18.2 U/ml by RIA assay, 15.8±4.6 U/ml by in vitro assay and 16.8±3.0 U/ml by in vivo assay.

Culture fluid from CHO pDSVL-gHuEPO cells prepared amplified by stepwise 100 nM MTX were subjected to the three assays. A 3.0 day sample contained recombinant human EPO at a level of 3089±129 U/ml by RIA, 2589±71.5 U/ml by in vitro assay, and 2040±160 U/ml by in vivo assay. Amino acid sequencing of this product reveals an amino terminal corresponding to that designated in FIG. **6**.

Cell conditioned media from CHO cells transfected with plasmid pDSVL-MkE in 10 nM MTX were pooled, and the MTX dialyzed out over several days, resulting in media with an EPO activity of 221±5.1 U/ml (EPO-CCM). To determine the in vivo effect of the EPO-CCM upon hematocrit levels in normal Balb/C mice, the following experiment was conducted. Cell conditioned media from untransfected CHO cells (CCM) and EPO-CCM were adjusted with PBS. CCM was used for the control group (3 mice) and two dose levels of EPO-CCM—4 units per injection and 44 units per injection—were employed for the experimental groups (2 mice/group). Over the course of 5 weeks, the seven mice were injected intraperitoneally, 3 times per week. After the eighth injection, average hematocrit values for the control group were determined to be 50.4%; for the 4U group, 55.1%; and, for the 44U group, 67.9%.

Mammalian cell expression products may be readily recovered in substantially purified form from culture media using HPLC (C_4) employing an ethanol gradient, preferably at pH7.

A preliminary attempt was made to characterize recombinant glycoprotein products from conditioned medium of COS-1 and CHO cell expression of the human EPO gene in 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. All products were somewhat heterogeneous. Neuraminidase enzyme treatment to remove sialic acid resulted in COS-1 and CHO recombinent products of approximately equal molecular weight which were both nonetheless larger than the resulting asialo human urinary extract. Endoglycosidase F enzyme (EC 3.2.1) treatment of the recombinant CHO product and the urinary extract product (to totally remove carbohydrate from both) resulted in substantially homogeneous products having essentially identical molecular weight characteristics.

Purified human urinary EPO and a recombinant, CHO cell-produced, EPO according to the invention were subjected to carbohydrate analysis according to the procedure of Ledeen, et al. *Methods in Enzymology,* 83(*Part D*), 139–191 (1982) as modified through use of the hydrolysis procedures of Nesser, et al., *Anal.Biochem.,* 142, 58–67 (1984). Experimentally determined carbohydrate constitution values (expressed as molar ratios of carbohydrate in the product) for the urinary isolate were as follows: Hexoses, 1.73, N-acetylglucosamine, 1; N-acetylneuraminic acid, 0.93; Fucose, 0; and N-acetylgalactosamine, 0. Corresponding values for the recombinant product (derived from CHO pDSVL-gHuEPO 3-day culture media at 100 nM MTX) were as follows: Hexoses, 15.09; N-acetylglucosamine, 1; N-acetylneuraminic acid, 0.998; Fucose, 0; and N-acetylgalactosamine, 0. These findings are consistent with the Western blot and SDS-PAGE analysis described above.

Glycoprotein products provided by the present invention are thus comprehensive of products 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.

EXAMPLE 11

The present example relates to the total manufacture by assembly of nucleotide bases of two structural genes encoding the human species EPO sequence of FIG. **6** and incorporating, respectively "preferred" codons for expression in *E.coli* and yeast (*S.cerevisiae*) cells. Also described is the construction of genes encoding analogs of human EPO. Briefly stated, the protocol employed was generally as set out in the previously noted disclosure of Alton, et al. (WO 83/04053). The genes were designed for initial assembly of component oligonucleotides into multiple duplexes which, in turn, were assembled into three discrete sections. These sections were designed for ready 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** and **7** illustrate the design and assembly of a manufactured gene encoding a human EPO translation product lacking any leader or presequence but including an initial methionine residue at position −1. Moreoever, the gene incorporated in substantial part *E.coli* preference codons and the construction was therefore referred to as the "ECEPO" gene

More particularly, FIG. **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 FIG. **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 iso-

lating 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 (FIGS. **13** and **15**) were constructed in a similar manner from the oligonucleotides of FIGS. **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 FIG. **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 (FIG. **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 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 Ser. No. 636,727, filed Aug. 6, 1984, (published EPO Application No. 136,490) 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 FIG. 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		XhoI
5'-CTAG	ATG	GCT	AAT	TGC	GAC-3'		
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 Ser. No. 636,727, filed Aug. 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 FIGS. **16** through **21** and **8**. As was the case with the ECEPO gene, the entire construction involved formation of three sets of oligonucleotides (FIGS. **16**, **18** and **20**) which were formed into duplexes and assembled into sections (FIGS. **17**, **19** and **21**). Note that synthesis was facilitated in part by use of some sub-optimal codons in both the SCEPO and ECEPO constructions, i.e., oligonucleotides 7–12 of Section 1 of both genes were identical, as were oligonucleotides 1–6 of Section 2 in each 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 Ser. No.* 487,753, filed Apr. 22, 1983, by Grant A. Bitter, published Oct. 31, 1984 as European Patent Application No. 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 FIG. **8**, 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 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 μg/ml and kanamycin 5 μg/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 EPG standard.

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 trpl) and RK81 (genotype ααpep4-3 trpl). 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 μg/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 μg/OD liter by RIA). Preliminaly 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 PαC3 and pYE in HB101 *E.coli* cells were deposited in accordance with the Rules of Practice of the U.S. Patent Office on Sept. 27, 1984, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., 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 cells were likewise deposited on Nov. 21, 1984 as A.T.C.C. 33932, 33934, and 33933, respectively. *Saccharomyces cerevisiae* strains YSPD4 and RK81 were deposited on Nov. 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 reguiring 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 use-

ful 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) μg/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, cyclic AMP, prolactin and triiodothyzonine, 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,* 14*a*(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);l 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); Cingote, *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 erythlopoietin 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 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 FIG. **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.

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 string 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 and the 166 residues of human species EPO 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 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 No. 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 FIG. **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 8 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 a 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 [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 polypeplide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within mature EPO, which fragments may possess one activlty 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, 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 theretofore 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 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 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 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 noteworthly 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 FIGS. **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 really 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 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 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 membrances (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 dissociation temperature of any of the mixed probes employed. These improvements combine to 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.

What is claimed is:

1. A purified and isolated DNA sequence encoding erythropoietin, said DNA sequence selected from the group consisting of:

(a) the DNA sequences set out in FIGS. **5** and **6** or their complementary strands; and

(b) DNA sequences which hybridize under stringent conditions to the DNA sequences defined in (a).

2. A purified and isolated DNA sequence consisting essentially of a DNA sequence encoding human erythropoietin.

3. A purified and isolated DNA sequence consisting essentially of a DNA sequence encoding monkey erythropoietin.

4. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to claim **1**, **2** or **3** in a manner allowing the host cell to express erythropoietin.

5. A biologically functional circular plasmid or viral DNA vector including a DNA sequence according to claim **1**, **2**, or **3**.

6. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to claim **5**.

7. A purified and isolated DNA sequence consisting essentially of a DNA sequence encoding a polypeptide having an amino acid sequence sufficiently duplicative of that of erythropoietin to allow possession of the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells, and to increase hemoglobin synthesis or iron uptake.

8. A cDNA sequence according to claim **7**.

9. A monkey species erythropoietin coding DNA sequence according to claim **8**.

10. A DNA sequence according to claim **9** and including the protein coding region set forth in FIG. **5**.

11. A genomic DNA sequence according to claim **7**.

12. A human species erythropoietin coding DNA sequence according to claim **11**.

13. A DNA sequence according to claim **12** and including the protein coding region set forth in FIG. **6**.

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

15. A DNA sequence according to claim **14**, coding for expression of human species erythropoietin.

16. A DNA sequence according to claim **15** including the protein coding region set forth in FIG. **7**.

17. A DNA sequence according to claim **7** and including one or more codons preferred for expression in yeast cells.

18. A DNA sequence according to claim **17**, coding for expression of human species erythropoietin.

19. A DNA sequence according to claim **18** including the protein coding region set forth in FIG. **8**.

20. A DNA sequence according to claim **7** covalently associated with a detectable label substance.

21. A DNA sequence according to claim **20** wherein the detectable label is a radiolabel.

22. A single-strand DNA sequence according to claim **20**.

23. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to claim **7**, **8**, or **11** in a manner allowing the host cell to express said polypeptide.

24. A transformed or transfected host cell according to claim **23** which host cell is capable of glycosylating said polypeptide.

25. A transformed or transfected mammalian host cell according to claim **24**.

26. A transformed or transfected COS cell according to claim **25**.

27. A transformed or transfected CHO cell according to claim **25**.

28. A biologically functional circular plasmid or viral DNA vector including a DNA sequence according to claim 7.

29. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to claim 28.

30. A DNA sequence according to claim 7 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.

31. A purified and isolated DNA sequence as set out in FIGS. 5 or 6 or the complementary strand of such a sequence.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008
DATED : October 27, 1987
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 18, line 54, after "83", please insert

--, deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., under deposit accession No. A.T.C.C. 67545 on October 20, 1987--

Column 21, line 25, after "λHE1"
please insert

--, deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., under deposit accession No. A.T.C.C. 40381 on October 20, 1987--

Signed and Sealed this

Fourteenth Day of November, 1989

Attest:

JEFFREY M. SAMUELS

Attesting Officer *Acting Commissioner of Patents and Trademarks*

UNITED STATES PATE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008
DATED : October 27, 1987
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:

Abstract, line 22, "heterologus" should be --heterologous--

Col. 1, line 17, "erythropoletin" should be --erythropoietin--

Col. 1, line 33, "nucleotlde" should be --nucleotide--

Col. 1, line 55, "smallRNA" should be --small RNA--

Col. 1, line 62, "grouplngs" should be --groupings--

Col. 1, line 67, "promoter" should be --Promoter--

Col. 2, line 6, "sequenoes" should be --sequences--

Col. 2, line 36, "amolification" should be --amplification--

Col. 2, line 37, please insert "the" after "in"

Col. 2, line 41, "whioh" should be --which--

Col. 2, line 46, please insert ")" after "heterologous"

Col. 2, line 51, "restoratlon" should be --restoration--

Col. 2, line 60, "frequentiy" should be --frequently--

Col. 3, line 24, "WO83/0405" should be --WO83/04053--

Col. 3, line 31, "sequenoes" should be --sequences--

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008

DATED : October 27, 1987

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:

Col. 3, line 59, "singlestranded" should be --single-stranded--

Col. 3, line 63, "tc" should be --to--

Col. 3, line 64, "provlded" should be --provided--

Col. 4, line 12, '"techniques:" should be --techniques;--

Col. 4, line 43, "32 member" should be --32-member--

Col. 4, line 47, "DMA" should be --DNA--

Col. 4, line 57, "DMA" should be --DNA--

Col. 5, line 4, please delete the second occurrence of "the"

Col. 5, line 9, "80pp." should be --80, pp.--

Col. 5, line 14, "panoreatic" should be --pancreatic--

Col. 5, line 32, "librales" should be --libraries--

Col. 5, line 51, "polypeptide" should be --Polypeptide--

Col. 5, line 64, please insert "but" after "components"

Col. 5, lines 66-67, "Carbohydrate" should be --carbohydrate--

Col. 6, lines 2-3, "Tbis normai" should be --This normal--

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008

DATED : October 27, 1987 Page 3 of 8

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:

Col. 6, line 23, "Exp.Mematol." should be --Exp.Hematol.--

Col. 6, line 27, "Expt.Hematol." should be --Exp.Hematol.--

Col. 6, line 28, "1980:" should be --(1980);--

Col. 6, line 30, please insert a space before "1832"

Col. 6, line 33, "Desspyris" should be --Dessypris--

Col. 6, line 50, "alo" should be --also--

Col. 7, line 6, plese delete the character "I" between "1106" and "(1983)"

Col. 7, line 10, "erythropoletin" should be --erythropoietin--

Col. 7, line 25, "urin" should be --urine--

Col. 7, line 40, please insert a quotation mark (") after "effects"

Col. 7, line 47, please insert a close parenthesis ")" after "propagation"

Col. 7, line 65, "erythlopoietin" should be --erythropoietin--

Col. 8, line 43, "moiecular" should be --molecular--

Col. 9, lines 14-15, "erythlopoietin" should be --erythropoietin--

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008

DATED : October 27, 1987 Page 4 of 8

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:

Col. 10, line 19, "Whlle" should be --While--

Col. 10, line 50, "characterired" should be --characterized--

Col. 10, line 64, "mammallan" should be --mammalian--

Col. 11, line 2, "prlmary" should be --primary--

Col. 11, line 11, "whioh" should be --which--

Col. 11, line 46, "polypep-tides" should be --polypeptides--

Col. 12, line 10, "analoqs" should be --analogs--

Col. 12, line 17, "ccnformation" should be --conformation--

Col. 12, line 18, "propezties" should be --properties--

Col. 12, line 26, "DMA" should be --DNA--

Col. 12, line 20, "Tables V and VI" should be --Figures 5 and 6--

Col. 12, line 39, "Table VI" should be --Figure 6--

Col. 12, line 63, "DMA" should be --DNA--

Col. 12, line 64, "neiqhboring" should be --neighboring--

Col. 13, line 67, "construction" should be --constructions--

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008
DATED : October 27, 1987 Page 5 of 8
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:

Col. 14, line 12, "bioloqical" should be --biological--

Col. 14, line 45, "homoloqy" should be --homology--

Col. 14, line 47, "fraqments" should be --fragments--

Col. 14, line 62, "immunoloqical" should be --immunological--

Col. 14, line 64, "lis" should be --is--

Col. 14, line 65, "fraqments" should be --fragments--

Col. 15, lines 29-30, "Example" should be --Examples--

Col. 15, line 35, "genomlc" should be --genomic--

Col. 15, line 36, "CMO" should be --CHO--

Col. 15, line 38, "charactezization" should be --characterization--

Col. 16, line 43, please insert a parenthesis "(" before "Gln"

Col. 16, line 56, "qamma" should be --gamma--

Col. 17, line 15, please insert "of" before "either"

Col. 17, line 52, please delete the comma after "Springs"

Col. 21, line 25, "(designated λλHE1)" should be --(designated λHE1)--

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008

DATED : October 27, 1987

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:

Col. 21, line 27, "The" should be --the--

Col. 21, line 41, "glutamine" should be --glutamic acid--

Col. 21, lines 56-57, "Table VI" should be --Figure 6--

Col. 22, line 36, "l.e.," should be --i.e.,--

Col. 22, line 49, "Iinker" should be --linker--

Col. 22, line 52, "BamHMI" should be --BamHI--

Col. 23, line 34, "(DMFR)" should be --(DHFR)--

Col. 23, line 43, "litaged" should be --ligated--

Col. 24, line 39, "EcoRl" should be --EcoRI--

Col. 24, line 52, "BamHl" should be --BamHI--

Col. 24, line 55, "BamHl" should be --BamHI--

Col. 24, line 60, "angalysis" should be --analysis--

Col. 25, line 23, "approxImately" should be --approximately--

Col. 25, line 34, "44" should be --53--

Col. 26, line 46, "(CMO)" should be --(CHO)--

Col. 27, line 24, "snd" should be --and--

Col. 27, line 42, "DMFR" should be --DHFR--

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008

DATED : October 27, 1987 Page 7 of 8

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:

Col. 27, line 54, "serles" should be --series--

Col. 28, line 12, "hemogeneous" should be --homogeneous--

Col. 29, line 1, "CMO" should be --CHO--

Col. 30, line 54, please insert a period "." after "gene"

Col. 32, line 50, "KpnI/BgLII" should be --KpnI/BglII--

Col. 33, line 51, "EPG" should be --EPO--

Col. 34, line 54, "reguiring" should be --requiring--

Col. 35, line 49, "Cingote" should be --Congote--

Col. 35, line 56, "erythlopoietin" should be --erythropoietin--

Col. 35, line 60, "lablled" should be --labelled--

Col. 36, line 42, "Table V" should be --Figure 5--

Col. 37, line 20, please delete "8" and insert a bracket "[" immediately before "Asn^2"

Col. 37, line 26, please delete "a"

Col. 37, line 39, "activlty" should be --activity--

Col. 37, line 44, "Table VI" should be --Figure 6--

Col. 38, line 61, "really" should be --readily--

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,703,008

DATED : October 27, 1987

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:

Col. 39, line 20, "membrances" should be --membranes--

Col. 42, line 3, please insert a bracket "]" after "Arg^{166}"
i.e., Arg^{166}]hEPO

Signed and Sealed this

Fourteenth Day of June, 1988

Attest:

Attesting Officer

DONALD J. QUIGG

Commissioner of Patents and Trademarks

UNITED STATES PATE

CERTIFICATE OF

PATENT NO. : 4,703,008
DATED : October 27, 1987
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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 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 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 dissociation temperature of any of the mixed probes employed. These improvements combine to 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.

What is claimed is:

1. A process for the production of a glycosylated erythropoietin polypeptide having the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells comprising the steps of:

(a) growing, under suitable nutrient conditions, mammalian host cells transformed or transfected with an isolated DNA sequence encoding human erythropoietin; and

(b) isolating said glycosylated erythropoietin polypeptide therefrom.

2. The process according to claim **1** wherein said host cells are CHO cells.

3. The process according to claim **1** wherein said host cells are COS cells.

4. The process according to claim **1** wherein said DNA is cDNA.

5. The process according to claim **1** wherein said DNA is genomic DNA.

* * * * *

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 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 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 allelic 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 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 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 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 dissociation temperature of any of the mixed probes employed. These improvements combine to 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.

What is claimed is:

1. A process for the preparation of an in vivo biologically active erythropoietin product comprising the steps of:

(a) growing, under suitable nutrient conditions, host cells transformed or transfected with an isolated DNA sequence selected from the group consisting of (1) the DNA sequences set out in FIGS. **5** and **6**, (2) the protein coding sequences set out in FIGS. **5** and **6**, and (3) DNA sequences which hybridize under stringent conditions to the DNA sequences defined in (1) and (2) or their complementary strands; and

(b) isolating said erythropoietin product therefrom.

2. A process for the preparation of an in vivo biologically active erythropoietin product comprising the steps of transforming or transfecting a host cell with an isolated DNA sequence encoding the mature erythropoietin amino acid sequence of FIG. **6** and isolating said erythropoietin product from said host cell or the medium of its growth.

3. The process according to claim **1** or **2** wherein said host cells are mammalian cells.

4. A process for the production of a glycosylated erythropoietin polypeptide having the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells comprising the steps of:

a) growing, under suitable nutrient conditions, vertebrate cells comprising promoter DNA, other than human erythropoietin promoter DNA, operatively linked to DNA encoding the mature erythropoietin amino acid sequence of FIG. **6**; and

b) isolating said glycosylated erythropoietin polypeptide expressed by said cells.

5. The process of claim **4** wherein said promoter DNA is viral promoter DNA.

6. A process for the production of a glycosylated erythropoietin polypeptide having the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells comprising the steps of:

a) growing, under suitable nutrient conditions, vertebrate cells comprising amplified DNA encoding the mature erythropoietin amino acid sequence of FIG. **6**; and

b) isolating said glycosylated erythropoietin polypeptide expressed by said cells.

7. The process of claim **6** wherein said vertebrate cells further comprise amplified marker gene DNA.

8. The process of claim **7** wherein said amplified marker gene DNA is Dihydrofolate reductase (DHFR) gene DNA.

9. The process according to claims **2**, **4** and **6** wherein said cells are mammalian cells.

* * * * *

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 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 allelic 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 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 mammalain 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 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 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.

What is claimed is:

1. A pharmaceutical composition comprising a therapeutically effective amount of human erythropoietin and a pharmaceutically acceptable diluent, adjuvant or carrier, wherein said erythropoietin is purified from mammalian cells grown in culture.

2. A pharmaceutically-acceptable preparation containing a therapeutically effective amount of erythropoietin wherein human serum albumin is mixed with said erythropoietin.

* * * * *

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. 15 161–165 (1984)).

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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 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.

What is claimed is:

1. Vertebrate cells which can be propagated in vitro and which are capable upon growth in culture of producing erythropoietin in the medium of their growth in excess of 100 U of erythropoietin per 10^6 cells in 48 hours as determined by radioimmunoassay, said cells comprising non-human DNA sequences which control transcription of DNA encoding human erythropoietin.

2. Vertebrate cells according to claim **1** capable of producing in excess of 500 U erythropoietin per 10^6 cells in 48 hours.

3. Vertebrate cells according to claim **1** capable of producing in excess of 1000 U erythropoietin per 10^6 cells in 48 hours.

4. Vertebrate cells which can be propagated in vitro which comprise transcription control DNA sequences, other than human erythropoietin transcription control sequences, for production of human erythropoietin, 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.

5. Vertebrate cells according to claim **4** capable of producing in excess of 500 U erythropoietin per 10^6 cells in 48 hours.

6. Vertebrate cells according to claim **4** capable of producing in excess of 1000 U erythropoietin per 10^6 cells in 48 hours.

7. A process for producing erythropoietin comprising the step of culturing, under suitable nutrient conditions, vertebrate cells according to claim **1**, **2**, **3**, **4**, **5** or **6**.

* * * * *

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 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 FIGS. **5** and **6**; (b) ONA 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 FIGS. **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 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 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 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 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.

What is claimed is:

1. An isolated erythropoietin glycoprotein having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of FIG. **6** and has glycosylation which differs from that of human urinary erythropoietin.

2. An isolated erythropoietin glycoprotein having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of FIG. **6** and is not isolated from human urine.

3. A non-naturally occurring erythropoietin glycoprotein having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of FIG. **6**.

4. A pharmaceutical composition comprising a therapeutically effective amount an erythropoietin glycoprotein product according to claim **1**, **2** or **3**.

5. A method for providing erythropoietin therapy to a mammal comprising administering an effective amount of a pharmaceutical composition of claim **4**.

6. A method for treating a kidney dialysis patient which comprises administering a pharmaceutical composition of claim **4** in an amount effective to increase the hematocrit level of said patient.

7. An isolated polypeptide product characterized by being the product of the expression by a procaryotic host cell of an exogenous DNA sequence encoding the mature erythropoietin amino acid sequence of FIG. **6**.

* * * * *

erties 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 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 FIGS. **5** and **6**; (b) DA sequences which hybridize to the DA 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 noteworthly 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 FIGS. **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 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 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.aerginosa* 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 the mixed probes employed. These improvements combine to 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.

What is claimed is:

1. A non-naturally occurring erythropoietin glycoprotein product having the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and having glycosylation which differs from that of human urinary erythropoietin.

2. The non-naturally occurring EPO glycoprotein product according to claim **1** wherein said product has a higher molecular weight than human urinary EPO as measured by SDS-PAGE.

3. A non-naturally occurring glycoprotein product of the expression in a mammalian host cell of an exogenous DNA sequence comprising a DNA sequence encoding human erythropoietin said product possessing the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells.

4. A non-naturally occurring human erythropoietin glycoprotein possessing the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells which is the product of the process comprising the steps of:

(a) growing, under suitable nutrient conditions, mammalian host cells transformed or transfected with an isolated DNA sequence encoding the human erythropoietin amino acid sequence set out in FIG. **6** or a fragment thereof; and

(b) isolating a glycosylated erythropoietin polypeptide therefrom.

5. A non-naturally occurring human erythropoietin glycoprotein possessing the in vivo biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells which is the product of the process comprising the steps of:

(a) growing, under suitable nutrient conditions, mammalian host cells transformed or transfected with an isolated DNA sequence comprising a sequence encoding the leader sequence of human erythropoietin set out in FIG. **6**; and

(b) isolating a glycosylated erythropoietin polypeptide therefrom.

6. A non-naturally occurring glycoprotein product of the expression in a non-human eucaryotic host of an exogenous DNA sequence comprising a DNA sequence encoding human erythropoietin, said product possessing the in vivo biological property of causing human bone marrow cells to increase production of reticulocytes and red blood cells and having an average carbohydrate composition which differs from that of naturally occurring erythropoietin.

7. The glycoprotein product according to claim **3**, **4**, **5** or **6** wherein the host cell is a non-human mammalian cell.

8. The glycoprotein product according to claim **7** wherein the non-human mammalian cell is a CHO cell.

9. A pharmaceutical composition comprising an effective amount 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 hematocrit 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 hematocrit level of said product.

* * * * *

EXHIBIT 3

Second Edition

BIOCHEMISTRY

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PRODUCTION COORDINATOR: *William Murdock*
COMPOSITOR: *York Graphic Services*
PRINTER AND BINDER: *Arcata Book Group*

Library of Congress Cataloging in Publication Data

Stryer, Lubert.
Biochemistry.

Includes bibliographies and index.
1. Biological chemistry. I. Title. [DNLM: 1. Biochemistry. QU4 S928b]
QP514.2.S66 1981 574.19'2 80-24699
ISBN 0-7167-1226-1

Printed in the United States of America

8 9 KP 1 0 8 9 8 7 6 5 4

damental alphabet of proteins is at least two billion years old. The remarkable range of functions mediated by proteins results from the diversity and versatility of these twenty kinds of building blocks. In subsequent chapters, we will explore ways in which this alphabet is used to create the intricate three-dimensional structures that enable proteins to participate in so many biological processes.

Let us look at this repertoire of amino acids. The simplest one is glycine, which contains a hydrogen atom as its side chain (Figure 2-8). Alanine has a methyl group as its side chain. The other amino

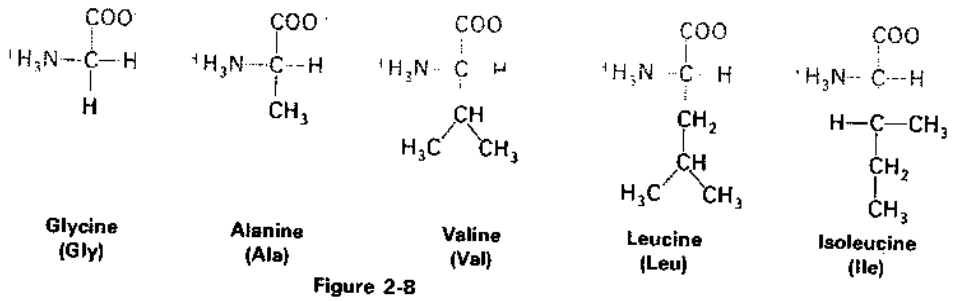


Figure 2-8
Amino acids having aliphatic side chains.

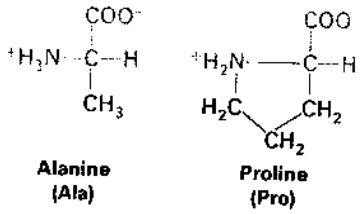


Figure 2-9
Proline differs from the other common amino acids in that it has a secondary amino group.

acids that have hydrocarbon side chains are valine, leucine, isoleucine, and proline. However, proline differs from the other amino acids in the basic set of twenty in that it contains a secondary rather than a primary amino group (Figure 2-9). Strictly speaking, proline is an imino acid rather than an amino acid. The side chain of proline is bonded to both the amino group and the α-carbon, which results in a cyclic structure.

Two amino acids, serine and threonine, contain aliphatic hydroxyl groups (Figure 2-10).

There are three common aromatic amino acids: phenylalanine, tyrosine, and tryptophan (Figure 2-11).

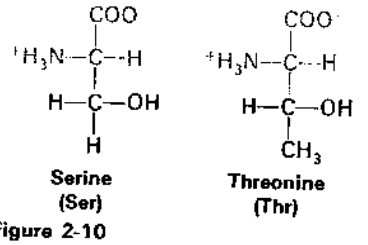


Figure 2-10
Serine and threonine have aliphatic hydroxyl side chains.

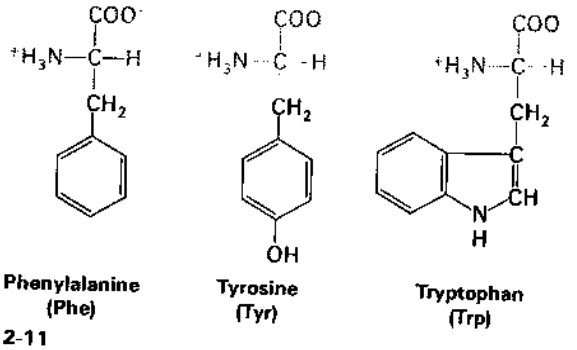
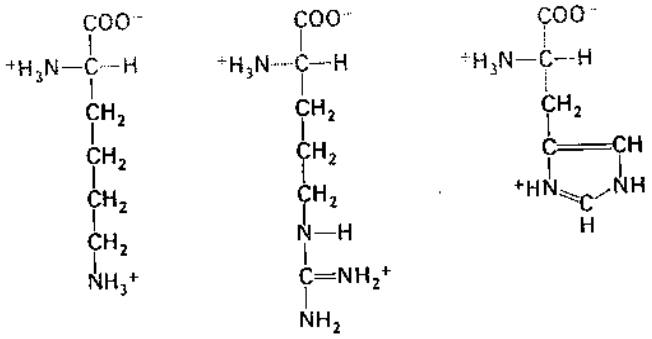


Figure 2-11
Phenylalanine, tyrosine, and tryptophan have aromatic side chains.

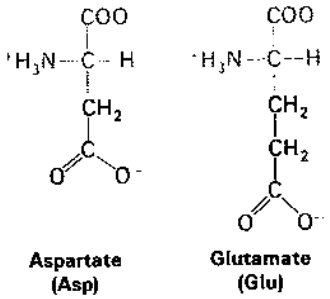
The side chains of the amino acids mentioned so far are uncharged at physiological pH. We turn now to some charged side chains. Lysine and arginine are positively charged at neutral pH, whereas whether histidine is positively charged or neutral depends on its local environment. These basic amino acids are shown in Figure 2-12. The negatively charged side chains are those of glu-



Lysine (Lys) **Arginine (Arg)** **Histidine (His)**

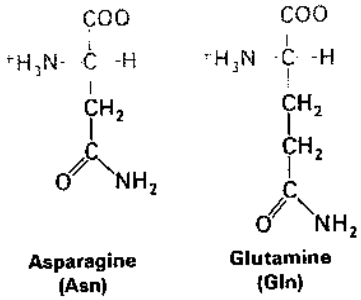
Figure 2-12
Lysine, arginine, and histidine have basic side chains.

tamic acid and aspartic acid (Figure 2-13). These amino acids will be called glutamate and aspartate to emphasize the fact that they are negatively charged at physiological pH. The uncharged derivatives of glutamate and aspartate are glutamine and asparagine (Figure 2-14), each of which contains a terminal amide group rather than a carboxylate. Finally, there are two amino acids whose side chains contain a sulfur atom: methionine and cysteine (Figure 2-15). As will be discussed shortly, cysteine plays a special role in some proteins by forming disulfide cross-links.



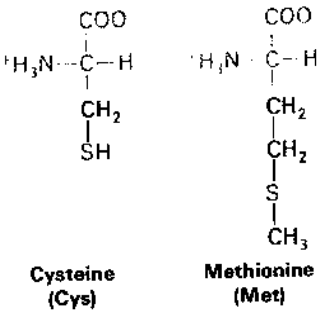
Aspartate (Asp) **Glutamate (Glu)**

Figure 2-13
Aspartate and glutamate have acidic side chains.



Asparagine (Asn) **Glutamine (Gln)**

Figure 2-14
Asparagine and glutamine have amide side chains.



Cysteine (Cys) **Methionine (Met)**

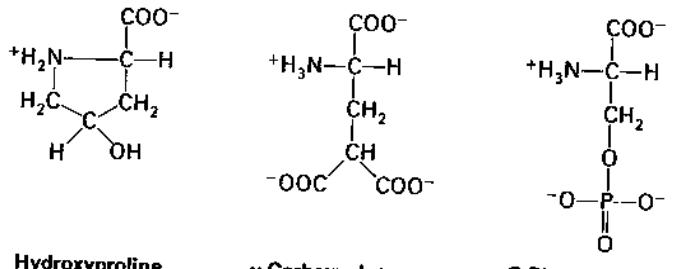
Figure 2-15
Cysteine and methionine have sulfur-containing side chains.

Table 2-1
Abbreviations for amino acids

Amino acid	*Three-letter abbreviation*	*One-letter symbol*
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

SPECIAL AMINO ACIDS SUPPLEMENT THE BASIC SET OF TWENTY

Some proteins contain special amino acids that are formed by modification of a common amino acid following its incorporation into the polypeptide chain. For example, collagen contains hydroxyproline, a hydroxylated derivative of proline (Figure 2-16). The added



Hydroxyproline **γ-Carboxyglutamate** ***O*-Phosphoserine**

Figure 2-16
Some modified amino acid residues in proteins: hydroxyproline, γ-carboxyglutamate, and phosphoserine. Groups added after the polypeptide chain is synthesized are shown in red.