TABLE XVIII

SCEPO SECTION 2

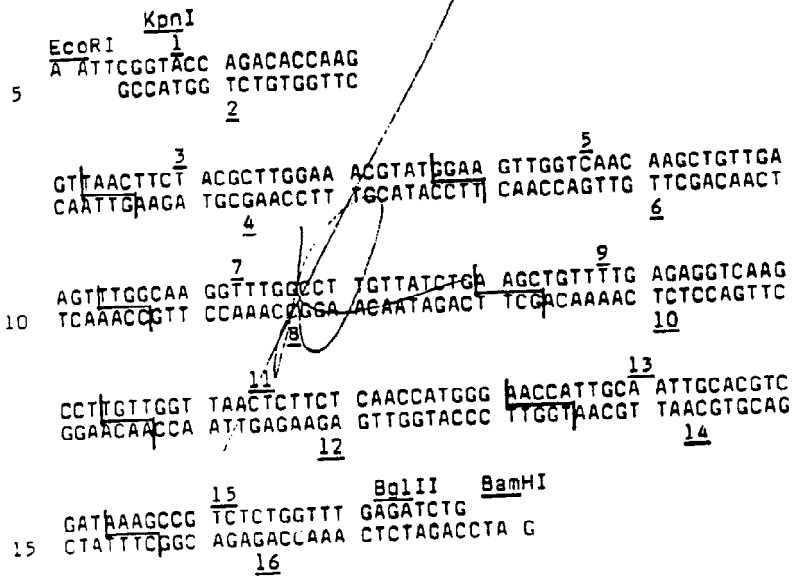

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TABLE XIX

SCEPO SECTION 3 OLIGONUCLEOTIDES

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

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TABLE Xx

SCEPO SECTION 3

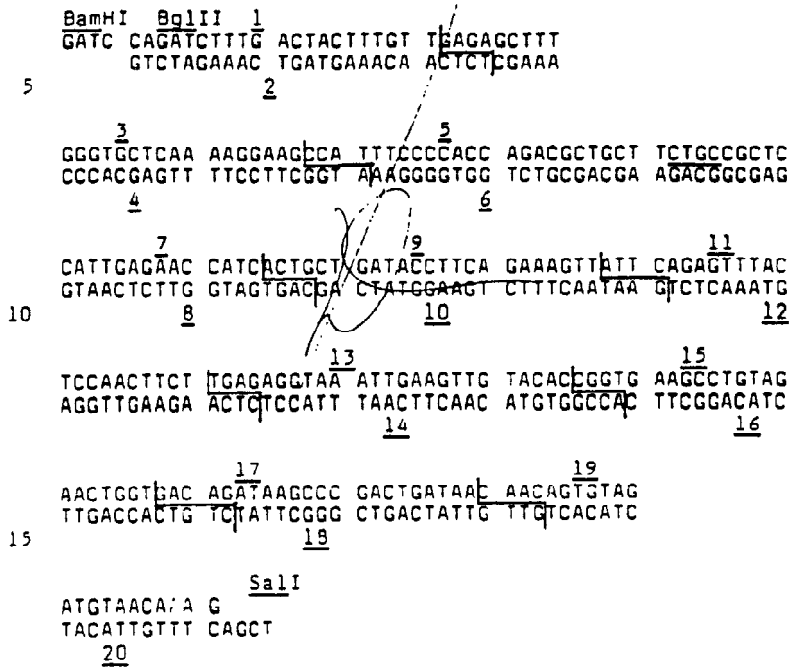

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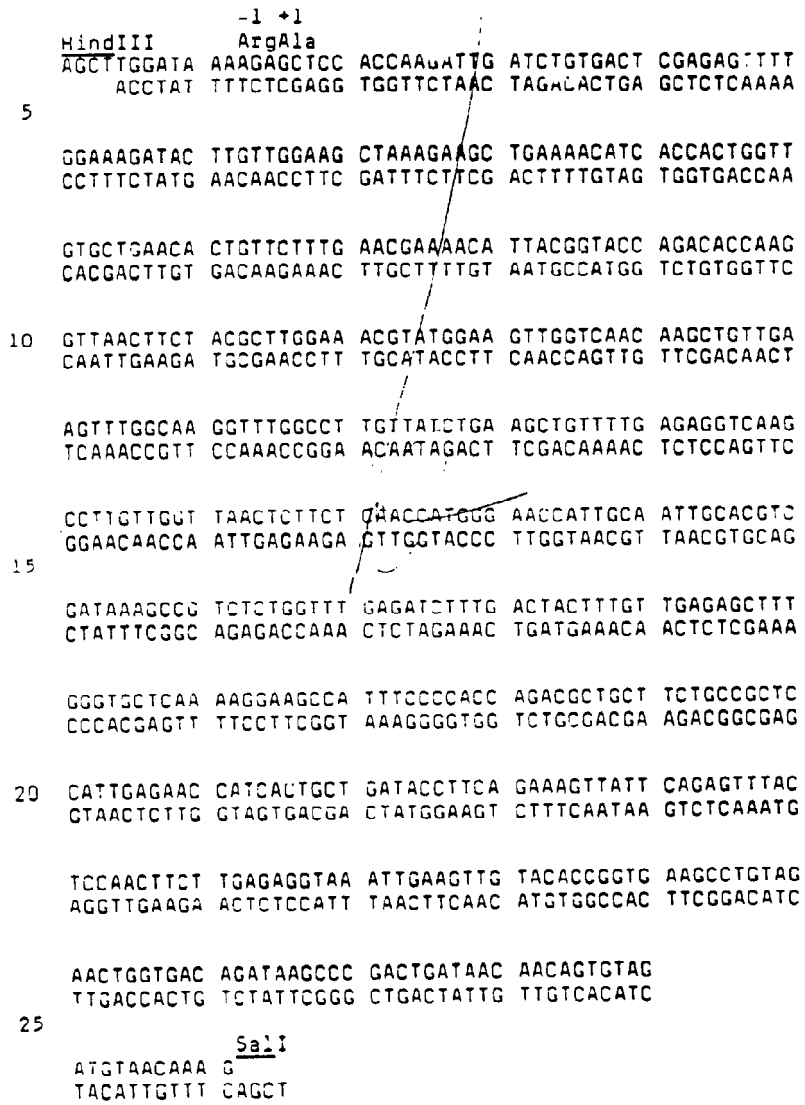
TABLE XXI

SCEPO GENE



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

The presently preferred expression system for SCEPO gene products is a secretion system based on S.cerevisiae α-factor secretion, as described in co-pending U.S. Patent Application Serial No. 487,753, filed April 22, 1983, by Grant A. Bitter, published October 31, 1984 as European Patent Application 0 123,294. Briefly put, the system involves constructions wherein DNA encoding the leader sequence of the yeast α-factor gene product is positioned immediately 5' to the coding region of the exogenous gene to be expressed. As a result, the gene product translated includes a leader or signal sequence which is "processed off" by an endogenous yeast enzyme in the course of secretion of the remainder of the product. Because the construction makes use of the α-factor translation initiation (ATG) codon, there was no need to provide such a codon at the -1 position of the SCEPO gene. As may be noted from ~~Table XXI~~ Figure 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 EPO standard.

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The EPO analog plasmids formed in parts A and B of Example 11 were each transformed into pMW1-transformed AM7 E.coli cells and the cells were cultured as above. Purified isolates were tested in both RIA and in vitro assays. RIA and in vitro assay values for [Asn^2, des-Pro^2 through Ile^6]hEPO expression products were approximately 11,000 U/mg and 6,000 U/mg protein, respectively, while the assay values for [His^7]hEPO were about 41,000 U/mg and 14,000 U/mg protein, respectively, indicating that the analog products were from one-fourth to one-tenth as "active" as the "parent" expression product in the assays.

In the expression system designed for use of S.cerevisiae host cells, plasmid pYE/SCEPO was transformed into two different strains, YSDP4 (genotype a pep4-3 trp1) and RK81 (genotype αα pep4-3 trp1). Transformed YSDP4 hosts were grown in SD medium (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 62 (1983) supplemented with casamino acids at 0.5%, pH 6.5 at 30°C. Media harvested when the cells had been grown to 36 O.D. contained EPO products at levels of about 244 U/ml (97 ug/OD liter by RIA). Transformed RK81 cells grown to either 6.5 O.D. or 60 O.D. provided media with EPO concentrations of about 80-90 U/ml (34 ug/OD liter by RIA). Preliminary analyses reveal significant heterogeneity in products produced by the expression system, likely to be due to variations in glycosylation of proteins expressed, and relatively high mannose content of the associated carbohydrate.

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

cells were likewise deposited on November 21, 1984 as A.T.C.C. 39932, 39934, and 39933, respectively. Saccharomyces cerevisiae strains YSPD4 and RK81 were deposited on November 21, 1984 as A.T.C.C. 20734 and 20733, respectively.

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

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

As previously indicated, recombinant-produced and synthetic products of the invention share, to varying degrees, the in vitro biological activity of EPO isolates from natural sources and consequently are projected to have utility as substitutes for EPO isolates in culture media employed for growth of erythropoietic cells in culture. Similarly, to the extent that polypeptide products of the invention share the in vivo activity of natural EPO isolates they are conspicuously suitable for use in erythropoietin therapy procedures practiced on mammals, including humans, to develop any or all of the effects herefore attributed in vivo to EPO, e.g., stimulation of reticulocyte response, development of ferrokinetic effects (such as plasma iron turnover effects and marrow transit time effects), erythrocyte mass charges, stimulation of hemoglobin C synthesis (see, Eschbach, et al., supra) and, as indicated in Example 10, increasing hematocrit levels in mammals. Included within the class of humans treatable with products of the invention are patients generally requiring blood transfusions and including trauma victims, surgical patients, renal disease patients including dialysis patients, and

patients with a variety of blood composition affecting disorders, such as hemophilia, sickle cell disease, physiologic anemias, and the like. The minimization of the need for transfusion therapy through use of EPO therapy can be expected to result in reduced transmission of infectious agents. Products of the invention, by virtue of their production by recombinant methods, are expected to be free of pyrogens, natural inhibitory substances, and the like, and are thus likely to provide enhanced overall effectiveness in therapeutic processes vis-a-vis naturally derived products. Erythropoietin therapy with products of the present invention is also expected to be useful in the enhancement of oxygen carrying capacity of individuals encountering hypoxic environmental conditions and possibly in providing beneficial cardiovascular effects.

A preferred method for administration of polypeptide products of the invention is by parenteral (e.g., IV, IM, SC, or IP) routes and the compositions administered would ordinarily include therapeutically effective amounts of product in combination with acceptable diluents, carriers and/or adjuvants. Preliminary pharmacokinetic studies indicate a longer half-life in vivo for monkey EPO products when administered IM rather than IV. Effective dosages are expected to vary substantially depending upon the condition treated but therapeutic doses are presently expected to be in the range of 0.1 (~7U) to 100 (~7000U) ug/kg body weight of the active material. Standard diluents such as human serum albumin are contemplated for pharmaceutical compositions of the invention, as are standard carriers such as saline.

Adjuvant materials suitable for use in compositions of the invention include compounds independently noted for erythropoietic stimulatory effects, such as testosterones, progenitor cell stimulators, insulin-like growth factor, prostaglandins, serotonin,

cyclic AMP, prolactin and triiodothyronine, as well as agents generally employed in treatment of aplastic anemia, such as methenolene, stanozolol and nandrolone [see, e.g., Resegotti, et al., Panminerva Medica, 23, 243-248 (1981); McGonigle, et al., Kidney Int., 25(2), 437-444 (1984); Pavlovic-Kantera, et al., Expt.Hematol., 8(Supp. 8), 283-291 (1980); and Kurtz, FEBS Letters, 14a(1), 105-108 (1982)]. Also contemplated as adjuvants are substances reported to enhance the effects of, or synergize, erythropoietin or asialo-EPO, such as the adrenergic agonists, thyroid hormones, androgens and BPA [see, Dunn, "Current Concepts in Erythropoiesis", John Wiley and Sons (Chichester, England, 1983); Weiland, et al., Blut, 44(3), 173-175 (1982); Kalmanti, Kidney Int., 22, 383-391 (1982); Shahidi, New.Eng.J.Med., 289, 72-80 (1973); Fisher, et al., Steroids, 30(6), 833-845 (1977); Urabe, et al., J.Exp.Med., 149, 1314-1325 (1979); and Billat, et al., Expt.Hematol., 10(1), 133-140 (1982)] as well as the classes of compounds designated "hepatic erythropoietic factors" [see, Naughton, et al., Acta.Haemat., 69, 171-179 (1983)] and "erythrotropins" [as described by Congote, et al. in Abstract 364, Proceedings 7th International Congress of Endocrinology (Quebec City, Quebec, July 1-7, 1984); Congote, Biochem.Biophys.Res.Comm., 115(2), 447-483 (1983) and Congote, Anal.Biochem., 140, 428-433 (1984)] and "erythrogenins" [as described in Rothman, et al., J.Surg.Oncol., 20, 105-108 (1982)]. Preliminary screenings designed to measure erythropoietic responses of ex-hypoxic polycythemic mice pre-treated with either 5-α-dihydrotestosterone or nandrolone and then given erythropoietin of the present invention have generated equivocal results.

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

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