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having part or all of the primary structural conformation (i.e., continuous sequence of amino acid residues) and one or more of the biological properties (e.g., immunological properties and in vivo and in vitro biological activity) of naturally-occurring erythropoietin, including allelic variants thereof. These polypeptides are also uniquely characterized by being the product of procaryotic or eucaryotic host expression (e.g., by bacterial, yeast and mammalian cells in culture) of exogenous DNA sequences obtained by genomic or cDNA cloning or by gene synthesis. Products of microbial expression in vertebrate (e.g., mammalian and avian) cells may be further characterized by freedom from association with human proteins or other contaminants which may be associated with erythropoietin in its natural mammalian cellular environment or in extracellular fluids such as plasma or urine. The products of typical yeast (e.g., Saccaromyces cerevisiae) or procaryote (e.g., E.coli) host cells are free of association with any mammalian proteins. Depending upon the host employed, polypeptides of the invention may be glycosylated with mammalian or other eucaryotic carbohydrates or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue (at position -1).

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

Vertebrate (e.g., COS-1 and CHO) cells provided by the present invention comprise the first cells ever available which can be propagated in vitro continuously and which upon growth in culture are capable of producing

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in the medium of their growth in excess of 100U (preferably in excess of 500U and most preferably in excess of 1,000 to 5,000U) of erythropoietin per  $10^6$  cells in 48 hours as determined by radioimmunoassay.

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

Illustrating the present invention are cloned DNA sequences of monkey and human species origins and polypeptide sequences suitably deduced therefrom which represent, respectively, the primary structural confor-  
25 mation of erythropoietins of monkey and human species origins.

Also provided by the present invention are novel biologically functional viral and circular plasmid DNA vectors incorporating DNA sequences of the invention and  
30 microbial (e.g., bacterial, yeast and mammalian cell) host organisms stably transformed or transfected with such vectors. Correspondingly provided by the invention are novel methods for the production of useful polypeptides comprising cultured growth of such transformed or  
35 transfected microbial hosts under conditions facilitative of large scale expression of the exogenous, vector-borne

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DNA sequence; and isolation of the desired polypeptides from the growth medium, cellular lysates or cellular membrane fractions.

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

Having herein elucidated the sequence of amino acid residues of erythropoietin, the present invention provides for the total and/or partial manufacture of DNA sequences coding for erythropoietin and including such advantageous characteristics as incorporation of codons "preferred" for expression by selected non-mammalian hosts, provision of sites for cleavage by restriction endonuclease enzymes and provision of additional initial, terminal or intermediate DNA sequences which facilitate construction of readily expressed vectors. Correspondingly, the present invention provides for manufacture (and development by site specific mutagenesis of cDNA and genomic DNA) of DNA sequences coding for microbial expression of polypeptide analogs or derivatives of erythropoietin which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (i.e., deletion analogs containing less than all of the residues specified for EPO and/or substitution analogs wherein one or more residues specified are replaced by other residues and/or addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptide); and which share some or all the properties of naturally-occurring forms.

Novel DNA sequences of the invention include all sequences useful in securing expression in procaryotic or eucaryotic host cells of polypeptide products having at

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least a part of the primary structural conformation and one or more of the biological properties of erythropoietin which are comprehended by: (a) the DNA sequences set out in Tables V and VI herein or their complementary strands; (b) DNA sequences which hybridize (under hybridization conditions such as illustrated herein or more stringent conditions) to DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to DNA sequences defined in (a) and (b) above. Specifically comprehended in part (b) are genomic DNA sequences encoding allelic variant forms of monkey and human erythropoietin and/or encoding other mammalian species of erythropoietin. Specifically comprehended by part (c) are manufactured DNA sequences encoding EPO, EPO fragments and EPO analogs which DNA sequences may incorporate codons facilitating translation of messenger RNA in non-vertebrate hosts.

Comprehended by the present invention is that class of polypeptides encoded for by portions of the DNA complement to the top strand human genomic DNA sequence of Table VI herein, i.e., "complementary inverted proteins" as described by Tramontano, et al., Nucleic Acids Research, 12, pp. 5049-5059 (1984).

Also comprehended by the invention are pharmaceutical compositions comprising effective amounts of polypeptide products of the invention together with suitable diluents, adjuvants and/or carriers which allow for provision of erythropoietin therapy, especially in the treatment of anemic disease states and most especially such anemic states as attend chronic renal failure.

Polypeptide products of the invention may be "labelled" by covalent association with a detectable marker substance (e.g., radiolabelled with  $^{125}\text{I}$ ) to provide reagents useful in detection and quantification of

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erythropoietin in solid tissue and fluid samples such as blood or urine. DNA products of the invention may also be labelled with detectable markers (such as radiolabels and non-isotopic labels such as biotin) and employed in DNA hybridization processes to locate the erythropoietin gene position and/or the position of any related gene family in the human, monkey and other mammalian species chromosomal map. They can also be used for identifying the erythropoietin gene disorders at the DNA level and used as gene markers for identifying neighboring genes and their disorders.

As hereinafter described in detail, the present invention further provides significant improvements in methods for detection of a specific single stranded polynucleotide of unknown sequence in a heterogeneous cellular or viral sample including multiple single-stranded polynucleotides where

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

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

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

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

(e) the specific polynucleotide is detected by monitoring for the presence of a hybridization reaction between it and a totally complementary probe within said

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mixture of labelled probes, as evidenced by the presence of a higher density of labelled material on the substrate at the locus of the specific polynucleotide in comparison to a background density of labelled material resulting from non-specific binding of labelled probes to the substrate.

The procedures are especially effective in situations dictating use of 64, 128, 256, 512, 1024 or more mixed polynucleotide probes having a length of 17 to 20 bases in DNA/DNA or RNA/RNA or DNA/RNA hybridizations.

As described infra, the above-noted improved procedures have illustratively allowed for the identification of cDNA clones coding for erythropoietin of monkey species origins within a library prepared from anemic monkey kidney cell mRNA. More specifically, a mixture of 128 uniformly varying 20-mer probes based on amino acid sequence information derived from sequencing fractions of human erythropoietin was employed in colony hybridization procedures to identify seven "positive" erythropoietin cDNA clones within a total of 200,000 colonies. Even more remarkably, practice of the improved procedures of the invention have allowed for the rapid isolation of three positive clones from within a screening of 1,500,000 phage plaques constituting a human genomic library. This was accomplished through use of the above-noted mixture of 128 20-mer probes together with a second set of 128 17-mer probes based on amino acid analysis of a different continuous sequence of human erythropoietin.

The above-noted illustrative procedures constitute the first known instance of the use of multiple mixed oligonucleotide probes in DNA/DNA hybridization processes directed toward isolation of mammalian genomic clones and the first known instance of the use of a mixture of more than 32 oligonucleotide probes in the isolation of cDNA clones.

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

*Handwritten initials: "dms", "BT", "pl"*

DETAILED DESCRIPTION

10 According to the present invention, DNA sequences encoding part or all of the polypeptide sequence of human and monkey species erythropoietin (hereafter, at times, "EPO") have been isolated and characterized. Further, the monkey and human origin DNA has been made the subject of eucaryotic and procaryotic  
15 expression providing isolatable quantities of polypeptides displaying biological (e.g., immunological) properties of naturally-occurring EPO as well as both in vivo and in vitro biological activities of EPO.

20 The DNA of monkey species origins was isolated from a cDNA library constructed with mRNA derived from kidney tissue of a monkey in a chemically induced anemic state and whose serum was immunologically determined to include high levels of EPO compared to normal monkey serum. The isolation of the desired cDNA clones containing EPO encoding DNA was accomplished through use of  
25 DNA/DNA colony hybridization employing a pool of 128 mixed, radiolabelled, 20-mer oligonucleotide probes and involved the rapid screening of 200,000 colonies. Design of the oligonucleotide probes was based on amino acid  
30 sequence information provided by enzymatic fragmentation and sequencing a small sample of human EPO.

The DNA of human species origins was isolated from a human genomic DNA library. The isolation of clones containing EPO-encoding DNA was accomplished  
35 through DNA/DNA plaque hybridization employing the above-noted pool of 128 mixed 20-mer oligonucleotide probes and

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a second pool of 128 radiolabelled 17-mer probes whose sequences were based on amino acids sequence information obtained from a different enzymatic human EPO fragment.

Positive colonies and plaques were verified by means of dideoxy sequencing of clonal DNA using a subset of 16 sequences within the pool of 20-mer probes and selected clones were subjected to nucleotide sequence analysis resulting in deduction of primary structural conformation of the EPO polypeptides encoded thereby. The deduced polypeptide sequences displayed a high degree of homology to each other and to a partial sequence generated by amino acid analysis of human EPO fragments.

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

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

More particularly, Example 1 is directed to amino acid sequencing of human EPO fragments and construction of mixtures of radiolabelled probes based on the results of this sequencing. Example 2 is generally directed to procedures involved in the identification of positive monkey cDNA clones and thus provides information concerning animal treatment and preliminary radioimmunoassay (RIA) analysis of animal sera. Example 3 is directed to the preparation of the cDNA library, colony

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hybridization screening and verification of positive clones, DNA sequencing of a positive cDNA clone and the generation of monkey EPO polypeptide primary structural conformation (amino acid sequence) information. Example 4 is directed to procedures involved in the identification of positive human genomic clones and thus provides information concerning the source of the genomic library, plaque hybridization procedures and verification of positive clones. Example 5 is directed to DNA sequencing of a positive genomic clone and the generation of human EPO polypeptide amino acid sequence information including a comparison thereof to the monkey EPO sequence information. Example 6 is directed to procedures for construction of a vector incorporating EPO-encoding DNA derived from a positive monkey cDNA clone, the use of the vector for transfection of COS-1 cells and cultured growth of the transfected cells. Example 7 is directed to procedures for construction of a vector incorporating EPO-encoding DNA derived from a positive human genomic clone, the use of the vector for transfection of COS-1 cells and the cultured growth of the transfected cells. Example 8 is directed to immunoassay procedures performed on media supernatants obtained from the cultured growth of transfected cells according to Example 6 and 7. Example 9 is directed to in vitro and in vivo biological activity of microbially expressed EPO of Examples 6 and 7.

Example 10 is directed to a development of mammalian host expression systems for monkey species EPO cDNA and human species genomic DNA involving Chinese hamster ovary ("CHO") cells and to the immunological and biological activities of products of these expression systems as well as characterization of such products. Example 11 is directed to the preparation of manufactured genes encoding human species EPO and EPO analogs, which genes include a number of preference codons for

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expression in E.coli and yeast host cells, and to expression systems based thereon. Example 12 relates to the immunological and biological activity profiles of expression products of the systems of Example 11.

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EXAMPLE 1

A. Human EPO Fragment Amino Acid Sequencing

Human EPO was isolated from urine and subjected  
10 to tryptic digestion resulting in the development and  
isolation of 17 discrete fragments in quantities approxi-  
17 mating 100-150 picomoles.

Fragments were arbitrarily assigned numbers and  
were analyzed for amino acid sequence by microsequence  
15 analysis using a gas phase sequencer (Applied Biosystems)  
to provide the sequence information set out in Table I,  
below, wherein single letter codes are employed and "X"  
designates a residue which was not unambiguously deter-  
mined.

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