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low levels of mRNA in initially targeted parotid gland and lung tissue sources and then address the prospects of success in probing a genomic library using a mixture of labelled probes, stating: "More generally, mixed
5 sequence oligodeoxynucleotide probes have been used to isolate protein genes of unknown sequence from cDNA libraries. Such probes are typically mixtures of 8-32
oligonucleotides, 14-17 nucleotides in length, representing every possible codon combination for a small
10 stretch (5-6 residues) of amino acid sequence. Under stringent hybridization conditions that discriminate against incorrectly base-paired probes, these mixtures are capable of locating specific gene sequences in clone
libraries of low-to-moderate complexity. Nevertheless,
15 because of their short length and heterogeneity, mixed probes often lack the specificity required for probing sequences as complex as a mammalian genome. This makes such a method impractical for the isolation of mammalian protein genes when the corresponding mRNAs are
20 unavailable." (Citations omitted).

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

B. Erythropoietin As A Polypeptide Of Interest

Erythropoiesis, the production of red blood
35 cells, occurs continuously throughout the human life span to offset cell destruction. Erythropoiesis is a very

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precisely controlled physiological mechanism enabling sufficient numbers of red blood cells to be available in the blood for proper tissue oxygenation, but not so many that the cells would impede circulation. The formation of red blood cells occurs in the bone marrow and is under the control of the hormone, erythropoietin.

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

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

See generally, Testa, et al., Exp.Hematol., 8(Supp. 8), 144-152 (1980); Tong, et al., J.Biol.Chem.,

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256(24), 12666-12672 (1981); Goldwasser, J.Cell.Physiol.,
 110(Supp. 1), 133-135 (1982); Finch, Blood, 60(6),
 1241-1246 (1982); Sytowski, et al., Expt.Hematol., 8(Supp.
 8), 52-64 (1980; Naughton, Ann.Clin.Lab.Sci., 13(5),
 432-438 (1983); Weiss, et al., Am.J.Vet.Res.,
 44(10), 1832-1835 (1983); Lappin, et al., Exp.Hematol.,
 11(7), 661-666 (1983); Baciu, et al., Ann.N.Y.Acad.Sci.,
 414, 66-72 (1983); Murphy, et al., Acta.Haematologica
Japonica, 46(7), 1380-1396 (1983); Dessypris, et al.,
 10 Brit.J.Haematol., 56, 295-306 (1984); and, Emmanouel, et
 al., Am.J.Physiol., 247 (1 Pt 2), F168-76 (1984).

Because erythropoietin is essential in the pro-
 cess of red blood cell formation, the hormone has poten-
 tial useful application in both the diagnosis and the
 15 treatment of blood disorders characterized by low or
 defective red blood cell production. See, generally,
 Pennathur-Das, et al., Blood, 63(5), 1168-71 (1984) and
 Haddy, Am.Jour.Ped.Hematol./Oncol., 4, 191-196, (1982)
 relating to erythropoietin in possible therapies for
 20 sickle cell disease, and Eschbach, et al. J.Clin.Invest.,
 74(2), pp. 434-441, (1984), describing a therapeutic
 regimen for uremic sheep based on in vivo response to
 erythropoietin-rich plasma infusions and proposing a
 dosage of 10 U EPO/kg per day for 15-40 days as correc-
 25 tive of anemia of the type associated with chronic renal
 failure. See also, Krane, Henry Ford Hosp.Med.J., 31(3),
 177-181 (1983).

It has recently been estimated that the availa-
 bility of erythropoietin in quantity would allow for
 30 treatment each year of anemias of 1,600,000 persons in
 the United States alone. See, e.g., Morrison,
 "Bioprocessing in Space -- an Overview", pp. 557-571 in
 The World Biotech Report 1984, Volume 2:USA, (Online
 Publications, New York, N.Y. 1984). Recent studies have
 35 provided a basis for projection of efficacy of erythro-

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poietin therapy in a variety of disease states, disorders and states of hematologic irregularity: Vedovato, et al., Acta.Haematol, 71, 211-213 (1984) (beta-thalassemia); Vichinsky, et al., J.Pediatr., 105(1), 15-21 (1984) (cystic fibrosis); Cotes, et al., Brit.J.Obstet.Gyneacol., 90(4), 304-311 (1983) (pregnancy, menstrual disorders); Haga, et al., Acta.Pediatr.Scand., 72, 827-831 (1983) (early anemia of prematurity); Claus-Walker, et al., Arch.Phys.Med.Rehabil., 65, 370-374 (1984) (spinal cord injury); Dunn, et al., Eur.J.Appl.Physiol., 52, 178-182 (1984) (space flight); Miller, et al., Brit.J.Haematol., 52, 545-590 (1982) (acute blood loss); Udupa, et al., J.Lab.Clin.Med., 103(4), 574-580 and 581-588 (1984); and Lipschitz, et al., Blood, 63(3), 502-509 (1983) (aging); and Dainiak, et al., Cancer, 51(6), 1101-1106 (1983) and Schwartz, et al., Otolaryngol., 109, 269-272 (1983) (various neoplastic disease states accompanied by abnormal erythropoiesis).

20 Prior attempts to obtain erythropoietin in good yield from plasma or urine have proven relatively unsuccessful. Complicated and sophisticated laboratory techniques are necessary and generally result in the collection of very small amounts of impure and unstable
25 extracts containing erythropoietin.

U.S. Letters Patent No. 3,033,753 describes a method for partially purifying erythropoietin from sheep blood plasma which provides low yields of a crude solid extract containing erythropoietin.

30 Initial attempts to isolate erythropoietin from urine yielded unstable, biologically inactive preparations of the hormone. U.S. Letters Patent No. 3,865,801 describes a method of stabilizing the biological activity of a crude substance containing erythropoietin recovered
35 from urine. The resulting crude preparation containing erythropoietin purportedly retains 90% of erythropoietin activity, and is stable.

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Another method of purifying human erythropoietin from urine of patients with aplastic anemia is described in Miike, et al., J.Biol.Chem., Vol. 252, No. 15 (August 10, 1977), pp. 5558-5564. This seven-step procedure includes ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography, and yields a pure erythropoietin preparation with a potency of 70,400 units/mg of protein in 21% yield.

U.S. Letters Patent No. 4,397,840 to Takezawa, et al. describes methods for preparing "an erythropoietin product" from healthy human urine specimens with weakly basic ion exchangers and proposes that the low molecular weight products obtained "have no inhibitory effects against erythropoietin."

U.K. Patent Application No. 2,085,887 by Sugimoto, et al., published May 6, 1982, describes a process for the production of hybrid human lymphoblastoid cells, reporting production levels ranging from 3 to 420 units of erythropoietin per ml of suspension of cells (distributed into the cultures after mammalian host propagation containing up to 10^7 cells per ml. At the highest production levels asserted to have been obtained, the rate of erythropoietin production could be calculated to be from 40 to about 4,000 units/ 10^6 cells/48 hours in in vitro culture following transfer of cells from in vivo propagation systems. (See also the equivalent U.S. Letters Patent No. 4,377,513.) Numerous proposals have been made for isolation of erythropoietin from tissue sources, including neoplastic cells, but the yields have been quite low. See, e.g., Jelkman, et al., Expt.Hematol., 11(7), 581-588 (1983); Tambourin, et al., P.N.A.S. (U.S.A.), 80, 6269-6273 (1983); Katsuoka, et al., Gann, 74, 534-541 (1983); Magiwaru, et al., Blood, 63(4), 828-835 (1984); and Choppin, et al., Blood, 64(2), 341-347 (1984).

Other isolation techniques utilized to obtain purified erythropoietin involve immunological procedures.

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A polyclonal, serum-derived antibody directed against erythropoietin is developed by injecting an animal, preferably a rat or rabbit, with human erythropoietin. The injected human erythropoietin is recognized as a foreign antigenic substance by the immune system of the animal and elicits production of antibodies against the antigen. Differing cells responding to stimulation by the antigenic substance produce and release into circulation antibodies slightly different from those produced by other responding cells. The antibody activity remains in the serum of the animal when its blood is extracted. While unpurified serum or antibody preparations purified as a serum immunoglobulin G fraction may then be used in assays to detect and complex with human erythropoietin, the materials suffer from a major disadvantage. This serum antibody, composed of all the different antibodies produced by individual cells, is polyclonal in nature and will complex with components in crude extracts other than erythropoietin alone.

Of interest to the background of the present invention are recent advances in the art of developing continuous cultures of cells capable of producing a single species of antibody which is specifically immunologically reactive with a single antigenic determinant of a selected antigen. See, generally, Chisholm, High Technology, Vol. 3, No. 1, 57-63 (1983). Attempts have been made to employ cell fusion and hybridization techniques to develop "monoclonal" antibodies to erythropoietin and to employ these antibodies in the isolation and quantitative detection of human erythropoietin. As one example, a report of the successful development of mouse-mouse hybridoma cell lines secreting monoclonal antibodies to human erythropoietin appeared in abstract form in Lee-Huang, Abstract No. 1463 of Fed.Proc., 41, 520 (1982). As another example, a detailed description

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of the preparation and use of a monoclonal, anti-erythropoietin antibody appears in Weiss, et al., P.N.A.S. (U.S.A.), 79, 5465-5469 (1982). See also, Sasaki, Biomed.Biochim.Acta., 42(11/12), S202-S206 (1983); Yanagawa, et al., Blood, 64(2), 357-364 (1984); Yanagawa, et al., J.Biol.Chem., 259(5), 2707-2710 (1984); and U.S. Letters Patent No. 4,465,624.

Also of interest to the background of the invention are reports of the immunological activity of synthetic peptides which substantially duplicate the amino acid sequence extant in naturally-occurring proteins, glycoproteins and nucleoproteins. More specifically, relatively low molecular weight polypeptides have been shown to participate in immune reactions which are similar in duration and extent to the immune reactions of physiologically significant proteins such as viral antigens, polypeptide hormones, and the like. Included among the immune reactions of such polypeptides is the provocation of the formation of specific antibodies in immunologically active animals. See, e.g., Lerner, et al., Cell, 23, 309-310 (1981); Ross, et al., Nature, 294, 654-656 (1981); Walter, et al., P.N.A.S. (U.S.A.), 77, 5197-5200 (1980); Lerner, et al., P.N.A.S. (U.S.A.), 78, 3403-3407 (1981); Walter, et al., P.N.A.S. (U.S.A.), 78, 4882-4886 (1981); Wong, et al., P.N.A.S. (U.S.A.), 78, 7412-7416 (1981); Green, et al., Cell, 28, 477-487 (1982); Nigg, et al., P.N.A.S. (U.S.A.), 79, 5322-5326 (1982); Baron, et al., Cell, 28, 395-404 (1982); Dreesman, et al., Nature, 295, 158-160 (1982); and Lerner, Scientific American, 248, No. 2, 66-74 (1983). See, also, Kaiser, et al., Science, 223, pp. 249-255 (1984) relating to biological and immunological activities of synthetic peptides which approximately share secondary structures of peptide hormones but may not share their primary structural conformation. The above studies relate, of course, to amino acid sequences of proteins other than erythro-

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poietin, a substance for which no substantial amino acid sequence information has been published. In co-owned, co-pending U.S. Patent Application Serial No. 463,724, filed February 4, 1983, by J. Egrie, published August 22, 1984 as European Patent Application No. 0 116 446, there is described a mouse-mouse hybridoma cell line (A.T.C.C. No. HB8209) which produces a highly specific monoclonal, anti-erythropoietin antibody which is also specifically immunoreactive with a polypeptide comprising the following sequence of amino acids:

NH₂-Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-COOH.

The polypeptide sequence is one assigned to the first twenty amino acid residues of mature human erythropoietin isolated according to the method of Miyake, et al., J.Biol.Chem., 252, 5558-5564 (1977) and upon which amino acid analysis was performed by the gas phase sequencer (Applied Biosystems, Inc.) according to the procedure of Hewick, M., et al., J.Biol.Chem., 256, 7990-7997 (1981). See, also, Sue, et al., Proc. Nat. Acad. Sci. (USA), 80, pp. 3651-3655 (1983) relating to development of polyclonal antibodies against a synthetic 26-mer based on a differing amino acid sequence, and Sytowski, et al., J.Immunol. Methods, 69, pp.181-186 (1984).

While polyclonal and monoclonal antibodies as described above provide highly useful materials for use in immunoassays for detection and quantification of erythropoietin and can be useful in the affinity purification of erythropoietin, it appears unlikely that these materials can readily provide for the large scale isolation of quantities of erythropoietin from mammalian sources sufficient for further analysis, clinical testing and potential wide-ranging therapeutic use of the substance in treatment of, e.g., chronic kidney disease wherein diseased tissues fail to sustain production of erythropoietin. It is consequently projected in the art that

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the best prospects for fully characterizing mammalian erythropoietin and providing large quantities of it for potential diagnostic and clinical use involve successful application of recombinant procedures to effect large scale microbial synthesis of the compound.

While substantial efforts appear to have been made in attempted isolation of DNA sequences coding for human and other mammalian species erythropoietin, none appear to have been successful. This is due principally to the scarcity of tissue sources, especially human tissue sources, enriched in mRNA such as would allow for construction of a cDNA library from which a DNA sequence coding for erythropoietin might be isolated by conventional techniques. Further, so little is known of the continuous sequence of amino acid residues of erythropoietin that it is not possible to construct, e.g., long polynucleotide probes readily capable of reliable use in DNA/DNA hybridization screening of cDNA and especially genomic DNA libraries. Illustratively, the twenty amino acid sequence employed to generate the above-named monoclonal antibody produced by A.T.C.C. No. HB8209 does not admit to the construction of an unambiguous, 60 base oligonucleotide probe in the manner described by Anderson, et al., *supra*. It is estimated that the human gene for erythropoietin may appear as a "single copy gene" within the human genome and, in any event, the genetic material coding for human erythropoietin is likely to constitute less than 0.00005% of total human genomic DNA which would be present in a genomic library.

To date, the most successful of known reported attempts at recombinant-related methods to provide DNA sequences suitable for use in microbial expression of isolatable quantities of mammalian erythropoietin have fallen far short of the goal. As an example, Farber, et al. *Exp. Hematol.*, 11, Supp. 14, Abstract 101 (1983) report the extraction of mRNA from kidney tissues of

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phenylhydrazine-treated baboons and the injection of the mRNA into Xenopus laevis oocytes with the rather transitory result of in vitro production of a mixture of "translation products" which included among them

5 displaying biological properties of erythropoietin. More recently, Farber, et al., Blood, 62, No. 5, Supp. No. 1, Abstract 392, at page 122a (1983) reported the in vitro translation of human kidney mRNA by frog oocytes. The resultant translation product mixture was estimated to

10 include on the order of 220 mU of a translation product having the activity of erythropoietin per microgram of injected mRNA. while such levels of in vitro translation of exogenous mRNA coding for erythropoietin were

15 acknowledged to be quite low (compared even to the prior reported levels of baboon mRNA translation into the sought-for product) it was held that the results confirm the human kidney as a site of erythropoietin expression, allowing for the construction of an enriched human kidney

20 cDNA library from which the desired gene might be isolated. [See also, Farber, Clin.Res., 31(4), 769A (1983).]

Since the filing of U.S. Patent Application Serial Nos. 561,024 and 582,185, there has appeared a

25 single report of the cloning and expression of what is asserted to have been human erythropoietin cDNA in E.coli. Briefly put, a number of cDNA clones were inserted into E.coli plasmids and β -lactamase fusion products were noted to be immunoreactive with a monoclonal

30 antibody to an unspecified "epitope" of human erythropoietin. See, Lee-Huang, Proc. Nat. Acad. Sci. (USA), 81, pp. 2708-2712 (1984).

BRIEF SUMMARY

35 The present invention provides, for the first time, novel purified and isolated polypeptide products

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