

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

AMGEN INC. and AMGEN)	
MANUFACTURING, LIMITED,)	
)	
Plaintiffs,)	
)	
v.)	C.A. No. 15-839 (RGA)
)	
HOSPIRA, INC.,)	DEMAND FOR JURY TRIAL
)	
Defendant.)	

AMENDED COMPLAINT

Amgen Inc. and Amgen Manufacturing, Limited (collectively “Amgen”), by and through their undersigned attorneys, for their Amended Complaint against Hospira, Inc. (“Hospira”) pursuant to Federal Rule of Civil Procedure 15(a)(1)(B), hereby allege:

THE PARTIES

1. Amgen Inc. is a corporation existing under the laws of the State of Delaware, with its principal place of business at One Amgen Center Drive, Thousand Oaks, California, 91320.

2. Amgen Manufacturing, Limited (“AML”) is a corporation existing under the laws of Bermuda with its principal place of business in Juncos, Puerto Rico.

3. Amgen discovers, develops, manufactures, and sells innovative therapeutic products based on advances in molecular biology, recombinant DNA technology, and chemistry. Founded in 1980, Amgen is a pioneer in the development of biological human therapeutics. Today, Amgen is the largest biotechnology company in the world, fueled in part by the success of its first therapeutic product EPOGEN[®] (epoetin alfa).

4. On information and belief, defendant Hospira is a corporation existing under the laws of the state of Delaware, with its principal place of business at 275 North Field Drive, Lake Forest, Illinois 60045.

5. On information and belief, Hospira, founded in 2004, is in the business of manufacturing and selling generic injectable products, and is seeking to expand its U.S. business into the manufacture and sale of biosimilar biologic therapeutics.

NATURE OF THE ACTION

6. This is one of the first actions for patent infringement under 35 U.S.C. § 271(e)(2)(C), which was enacted in 2010 as part of the Biologics Price Competition and Innovation Act (“the BPCIA”), and is the first such action in this District.

7. This is also one of the first actions seeking to give meaning and force to the disclosure and notice provisions of the BPCIA.

8. By amendment to the Public Health Service Act (“the PHSA”), the BPCIA created a new, abbreviated pathway for the approval of biological products that are highly similar to previously-licensed innovative biological products. The abbreviated pathway (42 U.S.C. § 262(k), often referred to as “the subsection (k) pathway”) allows a biosimilar applicant to secure a license from the Food and Drug Administration (“the FDA”) by relying on the prior license granted to the innovator company (“the Reference Product Sponsor” or “RPS”) for its innovative biological product (“the reference product”), provided that the reference product had been licensed by the FDA under the innovator pathway (42 U.S.C. § 262(a), often referred to as “the subsection (a) pathway”), which has traditionally required proof of safety and efficacy through a series of phased clinical trials.

9. In addition to creating an abbreviated pathway for approval, the BPCIA amended the PHSA to create an intricate and carefully orchestrated set of procedures for the biosimilar applicant and the Reference Product Sponsor to engage in a private and confidential disclosure of information, exchange of contentions, conduct of negotiations, and notice of commercial marketing to identify patents in dispute, resolve or narrow those disputes, and, if court

intervention is necessary, to facilitate an informed and orderly preliminary injunction practice after FDA licensure of the biosimilar product but before the status quo in the marketplace is disturbed. These procedures are set forth in 42 U.S.C. § 262(*l*) (“the patent provisions” of the BPCIA).

10. Seeking the benefits of the subsection (k) pathway, Hospira submitted its Biologic License Application (“BLA”) No. 125545 (“the Hospira BLA”) to the FDA, requesting that its biological product (“the Hospira Epoetin Biosimilar Product”) be licensed by relying on Amgen’s demonstration of the safety and efficacy of EPOGEN[®] (epoetin alfa).

11. Despite seeking the benefits of the subsection (k) pathway by relying on Amgen’s EPOGEN[®] license, Hospira has repeatedly refused to comply with its obligations under the patent provisions of the BPCIA.

12. In part, this lawsuit is necessary because Hospira has chosen to withhold the manufacturing information that 42 U.S.C. § 262(*l*)(2)(A) required Hospira to provide to Amgen within 20 days of the FDA having accepted Hospira’s biosimilar application for review. Hospira has thereby limited Amgen’s ability to identify patents that could reasonably be asserted against Hospira, forcing Amgen to initiate this lawsuit to get the withheld information through discovery.

13. In part, this lawsuit is necessary because Hospira has refused to engage in the good-faith negotiations with Amgen required by 42 U.S.C. § 262(*l*)(4), thereby necessitating this Court’s intervention to resolve the patent disputes identified so far.

14. In part, this lawsuit is necessary because Hospira has declared that it will not comply with 42 U.S.C. § 262(*l*)(8)(A), which requires Hospira to provide Amgen with 180 days’

notice of its first commercial marketing, on or after FDA licensure of the Hospira Epoetin Biosimilar Product.

15. Further, this lawsuit is necessary because Hospira has infringed patents that Amgen has identified under 42 U.S.C. § 262(l)(3)(A) and, upon information and belief, Hospira will infringe one or more claims of these patents should it commercially manufacture, use, offer for sale, sell, or import into the United States the Hospira Epoetin Biosimilar Product.

JURISDICTION AND VENUE

16. This is an action to declare the rights and obligations of the parties under Section 262 of the PHSA, Title 42, United States Code, and for patent infringement under the patent laws of the United States, Title 35, United States Code. This Court has subject-matter jurisdiction pursuant to 28 U.S.C. §§ 1331, 1338(a), 2201(a), and 2202.

17. Venue is proper in this Court pursuant to 28 U.S.C. §§ 1391 (b) and (c), and 28 U.S.C. § 1400(b). On information and belief, Hospira manufactures, seeks regulatory approval to market, distribute, and sell pharmaceutical products, and markets, distributes, and sells pharmaceutical products for use throughout the United States, including in this District.

18. This Court has personal jurisdiction over Hospira by virtue of, among other things, Hospira being a Delaware corporation, having conducted business in this District, having availed itself of the rights and benefits of Delaware law, and having engaged in substantial and continuing contacts with Delaware.

BACKGROUND

A. Amgen's innovative biological product, EPOGEN[®] (epoetin alfa)

19. The active ingredient in Amgen's innovative drug EPOGEN[®] (epoetin alfa) is recombinant human erythropoietin, a 165-amino-acid glycoprotein that is produced by genetically modified animal cells grown in culture vessels. By binding to specific receptors on

the surface of certain types of cells in the bone marrow, EPOGEN[®] (epoetin alfa) stimulates the production of red blood cells, known as erythrocytes. EPOGEN[®] (epoetin alfa) is used to treat anemia. Patients with anemia have a lower-than-normal level of red blood cells. EPOGEN[®] (epoetin alfa) is used to reduce or avoid the need for red blood cell transfusions in patients, for example, with chronic kidney disease.

20. Amgen is the recognized pioneer for developing therapeutically effective biological products to treat, ameliorate, or prevent disease. The availability of EPOGEN[®] represented a major advance in the treatment of anemia.

21. Biological products for human therapeutic use are regulated by the FDA under the PHSA. (In contrast, chemical pharmaceuticals are regulated by the FDA under the Food, Drug and Cosmetic Act.) A company seeking to market a biological product for human therapeutic use in the United States must first submit a BLA to obtain a license from the FDA. Developers of innovative biological products typically go through three clinical development phases to develop evidence of the safety and efficacy of the biological product for use in defined disease states before seeking FDA approval: Phase I, which typically tests safety, tolerability, and pharmacologic properties on healthy human volunteers, and Phases II and III, which typically test safety and efficacy on, respectively, a small and then a larger group of afflicted patients. If testing in each phase succeeds, the innovator may be in a position to submit a BLA under 42 U.S.C. § 262(a) seeking FDA approval. The BLA includes, among other things, technical data on the characterization and composition of the biological product, toxicology studies of the product in animals, the means for manufacturing the product, clinical trial results to establish the safety, efficacy, and dosing of the product for specific patient populations and disease states, and labeling for use of the product for which approval is requested. 21 C.F.R. §§ 601 *et seq.*

22. After submission of the BLA, innovators must pass demanding stages of clearance. For example, innovators are required to demonstrate to the FDA that “the biological product that is the subject of the application is safe, pure, and potent” (42 U.S.C. § 262(a)(2)(C)(i)(I)); and “the facility in which the biological product is manufactured, processed, packed, or held meets standards designed to assure that the biological product continues to be safe, pure, and potent.” 42 U.S.C. § 262(a)(2)(C)(i)(II). If the FDA determines that the biological product or the facility does not meet the requirements, the BLA will be denied.

23. Not surprisingly, the development of innovative pharmaceutical products requires the investment of enormous amounts of time and money. For example, it typically takes ten years to develop a drug, and the average cost to develop a drug (including the cost of failures) has been estimated at \$2.6 billion. *See* PHARMACEUTICAL RESEARCH AND MANUFACTURERS OF AMERICA, 2015 PROFILE: BIOPHARMACEUTICAL RESEARCH INDUSTRY at 35.¹

24. Amgen went through each of the requirements of the subsection (a) pathway to obtain a license from the FDA for its innovative biological product EPOGEN[®] (epoetin alfa). As a result, in 1989, the FDA approved EPOGEN[®] (epoetin alfa) pursuant to BLA No. 103234, for the treatment of anemia associated with chronic renal failure (“CRF”) (including end-stage renal disease). The initial approval of EPOGEN[®] (epoetin alfa) for use in treating anemia due to chronic renal failure was followed by approvals for additional indications: for use in patients with certain cancers suffering from anemia due to concomitant chemotherapy, in patients with HIV-infection with anemia due to anti-viral drugs, and to decrease the need for transfusion in patients scheduled for certain types of surgery. Since being granted approval, Amgen has

¹ Available at http://www.phrma.org/sites/default/files/pdf/2015_phrma_profile.pdf.

manufactured and sold EPOGEN[®] (epoetin alfa) in the U.S. for the treatment of anemia associated with chronic kidney disease in patients on dialysis. Amgen also manufactures and supplies epoetin alfa to Ortho Biotech, a division of Johnson & Johnson, for sale in the United States under the tradename PROCRIT[®] for the treatment of anemia in chronic kidney disease patients who are not receiving dialysis, as well as for other FDA-approved therapeutic indications.

B. Hospira seeks approval to market a biosimilar version of EPOGEN[®] (epoetin alfa) by taking advantage of the abbreviated subsection (k) pathway of the BPCIA

25. Hospira is seeking approval from the FDA to sell a “biosimilar” version of EPOGEN[®] (epoetin alfa) by taking advantage of a new, abbreviated approval pathway under the BPCIA.

26. But Hospira has chosen to ignore certain statutory requirements of the BPCIA that Congress put in place to protect innovators such as Amgen. Rather than follow the requirements of the BPCIA, Hospira has selectively decided to comply with certain provisions while refusing to comply with others.

C. The BPCIA reflects Congress’s balancing of the interests of innovators and biosimilar applicants

27. Congress enacted the BPCIA on March 23, 2010. The purpose of the BPCIA is to establish “a biosimilars pathway balancing innovation and consumer interests.” Biologics Price Competition and Innovation Act of 2009, Pub. L. No. 111-148, § 7001(b), 124 Stat. 119, 804 (2010) (amending 42 U.S.C. § 262). The statutory requirements of the BPCIA reflect Congress’s intent to achieve this balance.

28. On one side of the balance, the BPCIA created an abbreviated approval pathway, 42 U.S.C. § 262(k), for FDA licensure of biological products upon a determination that the

biological product is “biosimilar” to a previously-licensed “reference product.” The BPCIA defines a “biosimilar” to be a biological product that: (1) is “highly similar to the reference product notwithstanding minor differences in clinically inactive components,” and (2) has “no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product.” 42 U.S.C. §§ 262(i)(2)(A) and (B). The BPCIA defines a “reference product” to be “a single biological product licensed under subsection (a) against which the biological product is evaluated in an application submitted under subsection (k).” 42 U.S.C. § 262(i)(4).

29. As opposed to applicants following the § 262(a) pathway, biosimilar applicants following the § 262(k) pathway have the advantage of referencing the innovator’s license—the FDA evaluates the safety and efficacy of the applicant’s biological product by relying on the innovator’s prior demonstration of safety, purity, and potency of the reference product. Specifically, the § 262(k) pathway may only be used where the prior applicant for the reference product (“the Reference Product Sponsor,” or “RPS”) has submitted an application under 42 U.S.C. § 262(a) for approval of a reference product, and the FDA has determined that the RPS has demonstrated that “the biological product that is the subject of the application is safe, pure, and potent.” 42 U.S.C. § 262(a)(2)(C)(i)(I).

30. Before the BPCIA, reference to another’s biological license could be made only with the permission of the innovator RPS. An innovator RPS enjoyed permanent and exclusive rights to its clinical trial data and FDA license. The BPCIA advanced the public’s interest in price competition in part by diminishing these rights, allowing a biosimilar applicant to “reference” the innovator RPS’s license rather than incurring the delay and costs of generating its own clinical data.

31. Consequently, the § 262(k) pathway allows the biosimilar applicant to avoid the time and expense incurred by the RPS for development and clinical testing, and to gain licensure to commercialize its biological product in the market sooner as a biosimilar than it could have done through an independent demonstration of safety, purity, and potency under the § 262(a) pathway. The § 262(k) pathway is thus referred to as an “abbreviated” approval pathway.

32. In addition to providing these benefits, approval under the § 262(k) pathway offers another benefit to the biosimilar applicant: a product that is approved as a biosimilar can take advantage of the existing market for the reference product created by the RPS.

33. On the other side of the balance, Congress implemented a detailed procedure to protect the interests of the RPS, tying this procedure to the biosimilar applicant’s choice to submit a BLA under, and gain the benefit of, the abbreviated § 262(k) pathway. 42 U.S.C. § 262(l)(1)(B)(i). This procedure compels biosimilar applicants that choose the abbreviated § 262(k) pathway to provide the RPS with a defined set of information shortly after the FDA accepts the biosimilar applicant’s BLA for review.

34. Of particular relevance here, in 42 U.S.C. § 262(l), the BPCIA sets forth requirements that the biosimilar applicant must follow to obtain the benefits of filing its BLA under the § 262(k) pathway. Specifically, 42 U.S.C. § 262(l) provides the following series of steps for the disclosure of information, the exchange of contentions, the resolution or narrowing of patent disputes, and, if necessary, the commencement of litigation, all within specified times triggered initially by the biosimilar applicant’s submission and the FDA’s acceptance of a BLA under the § 262(k) pathway:

- a. Within 20 days* after the FDA has accepted its abbreviated application, the biosimilar applicant must provide the Reference Product Sponsor: (i) a copy of the biosimilar application and (ii) other information describing the

process(es) for manufacturing the biosimilar product. 42 U.S.C. § 262(l)(2).

- b.** *Within 60 days* after receiving the BLA and manufacturing information, the Reference Product Sponsor must provide the biosimilar applicant with a list of all patents that the Reference Product Sponsor believes a claim for patent infringement could reasonably be asserted by either the Reference Product Sponsor or a patent owner that has granted exclusive rights to the Reference Product Sponsor. 42 U.S.C. § 262(l)(3)(A). The Reference Product Sponsor must also identify which, if any, of these patents it would be prepared to license to the biosimilar applicant. 42 U.S.C. § 262(l)(3)(A)(ii).
- c.** *Within 60 days* after receiving the foregoing list from the Reference Product Sponsor, the biosimilar applicant may provide to the Reference Product Sponsor a list of patents that the biosimilar applicant believes could be subject to a claim of patent infringement, 42 U.S.C. § 262(l)(3)(B)(i), and with regard to any patents listed by the Reference Product Sponsor or the biosimilar applicant, the biosimilar applicant must also provide: (I) a statement describing, on a claim-by-claim basis, a factual and legal basis for an opinion that a patent is invalid, unenforceable, or not infringed; or (II) a statement that the biosimilar applicant does not intend to market until the patent expires. 42 U.S.C. § 262(l)(3)(B)(ii). The biosimilar applicant must also provide a response to the Reference Product Sponsor's identification of any patents it would be prepared to license. 42 U.S.C. § 262(l)(3)(B)(iii).
- d.** *Within 60 days* after receiving the information described immediately above, the Reference Product Sponsor must provide, regarding each patent discussed in (I) above, a reciprocal statement describing, on a claim by claim basis, a factual and legal basis for an opinion that a patent will be infringed as well as a response to any statement regarding validity and enforceability. 42 U.S.C. § 262(l)(3)(C).
- e.** After this exchange of information, both parties must engage in good-faith negotiations to identify which patents, if any, should be subject to patent infringement litigation. 42 U.S.C. § 262(l)(4)(A). If the parties reach agreement *within 15 days* of starting negotiations, the Reference Product Sponsor must bring a patent-infringement action against the biosimilar applicant on the negotiated list of patents *within 30 days* of such agreement. 42 U.S.C. § 262(l)(6)(A). If the parties do not reach agreement *within 15 days* of starting negotiations, the biosimilar applicant must notify the Reference Product Sponsor of the number of patents it will provide in a second list, and the parties then simultaneously exchange within five days of this notice a list of patents that each party believes should be the subject of infringement litigation. 42 U.S.C. § 262(l)(5). *Within 30 days* after exchanging these lists, the Reference Product

Sponsor must bring an “immediate” patent infringement action against the biosimilar applicant on all patents on these simultaneously exchanged lists. 42 U.S.C. § 262(l)(6)(B).

- f.* Even after the litigation contemplated by 42 U.S.C. § 262(l)(6)(B) has commenced, the Reference Product Sponsor must identify additional patents that are newly issued or licensed after the Reference Product Sponsor provided its patent list under 42 U.S.C. § 262(l)(3)(A). Specifically, the Reference Product Sponsor must, not later than 30 days after the issuance or licensing, supplement that list with the newly issued or licensed patent(s). 42 U.S.C. § 262(l)(7).

35. Section 262(l) also requires the biosimilar applicant to provide the RPS with at least 180 days’ notice before the biosimilar applicant’s first commercial marketing of the biosimilar product. 42 U.S.C. § 262(l)(8)(A) (the “subsection (k) applicant shall provide notice to the Reference Product Sponsor not later than 180 days before the date of first commercial marketing of the biological product licensed under subsection (k)”). The notice of commercial marketing can only be provided *on or after* the biosimilar applicant has received FDA approval to market its product. *Amgen Inc. v. Sandoz, Inc.*, 794 F.3d 1347, 1357 (Fed. Cir. 2015) (“[U]nder paragraph (l)(8)(A), a subsection (k) applicant may only give effective notice of commercial marketing after the FDA has licensed its product.”). The biosimilar applicant’s obligation to provide this advance notice of commercial marketing is mandatory; it is not conditioned on performance of any act by the RPS, nor exempted if the biosimilar applicant fails to make the initial disclosures under 42 U.S.C. § 262(l)(2)(A). *Amgen*, 794 F.3d at 1359 (“A question exists, however, concerning whether the ‘shall’ provision in paragraph (l)(8)(A) is mandatory. We conclude that it is.”); *id.* at *1359-60 (“Paragraph (l)(8)(A) is a standalone notice provision in subsection (l). . . . Unlike the actions described in paragraphs (l)(3) through (l)(7), which all depend on, or are triggered by, the disclosure under paragraph (l)(2)(A), nothing in paragraph (l)(8)(A) conditions the notice requirement on paragraph (l)(2)(A) or other provisions of subsection (l).”).

36. The 180-days' notice of commercial marketing enables the RPS to seek a preliminary injunction before the biosimilar applicant commences commercial marketing of the biosimilar product, enjoining the biosimilar applicant from commercially manufacturing or selling the biosimilar product until the court decides any disputed patent issues. Accordingly, this provision gives the courts an opportunity to consider the RPS's motion for preliminary injunction when the issues are fully crystallized and before the status quo has changed.

D. Hospira seeks the benefits of the BPCIA pathway under 42 U.S.C. § 262(k) but refuses to comply with all of its obligations under § 262(l)

1. The Hospira BLA

37. In December 2014, Hospira submitted the Hospira BLA to the FDA under the abbreviated § 262(k) pathway to obtain approval to commercially manufacture, market, and sell the Hospira Epoetin Biosimilar Product, a biosimilar version of EPOGEN[®] (epoetin alfa) (which Hospira refers to as "Hospira Epoetin") for treating particular diseases in the United States.

38. The Hospira Epoetin Biosimilar Product is designed to copy and compete with Amgen's EPOGEN[®] (epoetin alfa). Hospira will instruct or direct others to administer the Hospira Epoetin Biosimilar Product to certain patients in the U.S. to treat particular diseases in the same way that Amgen's EPOGEN[®] (epoetin alfa) is administered. Hospira is seeking FDA approval for one or more indications for which EPOGEN[®] (epoetin alfa) is already approved.

39. Hospira does not seek to independently demonstrate to the FDA that its biological product is "safe, pure, and potent" pursuant to 42 U.S.C. § 262(a), as Amgen did in its BLA for its innovative biological product EPOGEN[®] (epoetin alfa). Rather, Hospira has requested that the FDA evaluate the suitability of its biological product for licensure by expressly referencing EPOGEN[®] (epoetin alfa) and thereby relying on the data supporting Amgen's FDA license for EPOGEN[®] (epoetin alfa) 42 U.S.C. § 262(k)(2)(A)(iii)(I).

40. On February 23, 2015, Hospira notified Amgen that the Hospira BLA had “recently been accepted for filing by FDA.” On information and belief, the FDA has not yet approved the Hospira BLA or given any indication whether it will be approved, when it will be approved, or what the scope of any approval will be. Under the Biosimilar Biological Product Authorization Performance Goal and Procedures, which sets forth FDA goals for fiscal years 2013-2017, the FDA is committed to reviewing and acting “on 70 percent of original biosimilar biological product application submissions within 10 months of receipt” for biosimilar biological product applications filed in 2014.² Therefore, the FDA may complete its final review of the Hospira Epoetin Biosimilar Product before November, 2015.

41. Hospira’s choice to submit its BLA using the abbreviated subsection (k) pathway triggered its mandatory obligation to also comply with the disclosure obligations at the outset of FDA review. 42 U.S.C. § 262(l)(1)(B)(i).

42. Hospira’s receipt of FDA notification that its BLA had been accepted for review triggered the 20-day deadline to provide its BLA and manufacturing information to Amgen as required by 42 U.S.C. § 262(l)(2)(A).

43. Purporting to comply with § 262(l)(2)(A), on March 3, 2015, Hospira provided a copy of its BLA for the Hospira Epoetin Biosimilar Product to Amgen.

²

FDA, Biosimilar Biological Product Authorization Performance Goals and Procedures Fiscal Years 2013 through 2017, *available at* <http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/Biosimilars/UCM281991.pdf>.

2. Hospira violated § 262(l)(2)(A)

44. Although Hospira provided a copy of the Hospira BLA to Amgen, it did not provide Amgen with the other information describing the processes used to manufacture the Hospira Epoetin Biosimilar Product as required by § 262(l)(2)(A).

45. In correspondence dated March 31, April 17, April 27, and May 1, 2015, Amgen specifically identified for Hospira the manufacturing information that was missing from the Hospira BLA. Amgen repeatedly requested that Hospira comply with § 262(l)(2)(A) and provide that information.

46. In correspondence dated March 5, April 21, April 30, August 19, and September 15, 2015, Hospira repeatedly refused to provide Amgen with the other information describing the processes used to manufacture the Hospira Epoetin Biosimilar Product as required by § 262(l)(2)(A).

47. Hospira deliberately decided not to provide Amgen with the information necessary to describe the processes for manufacturing the Hospira Epoetin Biosimilar Product within 20 days of receiving notification of FDA acceptance of its application for review.

48. To date, Amgen still has not received this manufacturing information, while Hospira continues to enjoy the benefit of FDA review of its application in reliance on Amgen's prior biological product license for EPOGEN[®] (epoetin alfa).

49. In *Amgen Inc. v. Sandoz, Inc.*, 794 F.3d 1347 (Fed. Cir. 2015), the Federal Circuit held that Sandoz's failure to provide its BLA and other manufacturing information to Amgen as required by 42 U.S.C. § 262(l)(2)(A), did not violate the BPCIA. The panel majority held that because the BPCIA provides consequences for a biosimilar applicant's failure to comply with § 262(l)(2)(A), the word "'shall' in paragraph § 262(l)(2)(A) does not mean 'must.'" *Id.* at 1355. The majority held instead that if the applicant fails to provide the required information, the RPS

may bring a declaratory-judgment action under 42 U.S.C. § 262(l)(9)(C) or a patent-infringement action under 35 U.S.C. § 271(e)(2)(C)(ii), and obtain the required information through discovery. *Id.* at 1356. Judge Newman dissented, because the BPCIA “leaves no uncertainty as to which of its provisions are mandatory and which are permissive,” with § 262(l)(2)(A) being mandatory, and because § 262(l)(2)(A) is central to the entire BPCIA: “Subsection (k) and subsection (l) are components of an integrated framework; to enjoy the benefits of subsection (k), the biosimilar applicant is obligated to comply with subsection (l),” and an applicant that fails to provide the required information violates the “explicit balance of obligations and benefits” of the BPCIA. *Id.* at 1365-66.

50. Receipt of the required manufacturing information would have given Amgen the opportunity to evaluate the manufacturing processes used by Hospira to determine whether those processes would infringe any patents held by Amgen, including under 35 U.S.C. § 271(a), (b), (c), (e), or (g). The purpose of the statutory requirements of 42 U.S.C. § 262(l)(2) is, among other things, to permit such an evaluation. In the absence of such a disclosure, the Reference Product Sponsor has no access to the manufacturing information.

51. Had Hospira provided Amgen with the required manufacturing information, Amgen would have been in a position: (1) to provide to Hospira a list of all patents for which Amgen believes a claim of patent infringement could reasonably be asserted as to the Hospira Epoetin Biosimilar Product, and (2) to identify to Hospira whether Amgen would be prepared to grant a license to Hospira under each of the patents included on such a list. 42 U.S.C. § 262(l)(3)(A). Amgen has an extensive portfolio of patents relating to various aspects of the manufacture of biological products. Because Hospira’s manufacturing process for the Hospira Epoetin Biosimilar Product is still secret, however, without the disclosure required by 42 U.S.C.

§ 262(l)(2), Amgen cannot conduct a full and complete evaluation of its patent portfolio as to Hospira's specific processes of manufacture. By unlawfully withholding the information required by 42 U.S.C. § 262, Hospira has thereby frustrated the statutory purpose and deprived Amgen of the opportunity to seek redress for potential infringement.

52. Amgen may therefore seek to assert additional patents following eventual receipt of Hospira's manufacturing information to be produced in discovery in this action under the Federal Rules.

53. Hospira's actions also create the substantial and continuing risk that Amgen cannot obtain manufacturing information regarding the Hospira Epoetin Biosimilar Product that would permit Amgen to assert its process patents before commercialization of that product. Forcing Amgen to assert one or more of its patents (including process patents) after Hospira's commercial entry into the market harms Amgen by, *e.g.*, diminishing the value of such patents.

3. Amgen has complied with the BPCIA procedures

54. Amgen complied (to the extent possible, given Hospira's non-compliance) with its obligations under the BPCIA.

55. Within 60 days after receiving a copy of the Hospira BLA, Amgen provided Hospira with a list of patents that Amgen believed could reasonably be asserted by Amgen if a person not licensed under the patents engaged in the manufacture, use, sale, offer for sale, or import into the United States of the Hospira Epoetin Biosimilar Product, thus satisfying 42 U.S.C. § 262(l)(3)(A)(i). Amgen also provided the required statement as to which, if any, of these patents it would be prepared to license to Hospira, thus satisfying 42 U.S.C. § 262(l)(3)(A)(ii).

56. Within 60 days after receiving Hospira's statement pursuant to 42 U.S.C. § 262(l)(3)(B) (which did not satisfy the statute's requirement that Hospira address each patent

on a claim-by-claim basis), Amgen provided its reciprocal statement under 42 U.S.C. § 262(l)(3)(C), describing, on a claim-by-claim basis, the factual and legal bases for Amgen's opinion that each patent will be infringed, as well as a response to Hospira's statement regarding validity and enforceability (to the extent Hospira provided such a statement).

4. Hospira violated § 262(l)(4)

57. Beginning on August 18, 2015, Amgen sought to comply with the requirements of 42 U.S.C. § 262(l)(4)(A) to engage in "good-faith negotiations" with Hospira to "agree on which, if any, patents . . . shall be the subject of an action for patent infringement under [42 U.S.C. § 262(l)(6)]."

58. Hospira refused to engage in any of the negotiations required by 42 U.S.C. § 262(l)(4)(A). Instead, in correspondence dated August 19, August 24, and September 15, 2015, Hospira purported to bypass its obligations by merely declaring that it was "accepting" the patents that Amgen had initially listed in accordance with § 262(l)(3)(A).

59. In correspondence dated August 21 and September 14, 2015, respectively, Amgen thereafter sought to gain Hospira's cooperation to commence good-faith negotiations with the goal of resolving or narrowing the issues to be put before the Court. Hospira has steadfastly refused to engage in *any* negotiation with Amgen, in violation of the statute.

5. Hospira violated § 262(l)(8)(A)

60. Under 42 U.S.C. § 262(l)(8)(A), Hospira is also required to provide Amgen with at least 180 days' notice of the date of first commercial marketing of the licensed Hospira Epoetin Biosimilar Product. At product licensure, when the issues are fully crystalized and the threat of injury is imminent, this provision will permit Amgen to assess its patent rights and seek injunctive relief before the status quo in the marketplace has changed, *i.e.*, before Hospira first

markets commercially or launches the Hospira Epoetin Biosimilar Product. It avoids the need for emergency motions and the attendant disruption to the Court's administration of its docket.

61. Hospira's obligation to provide this notice of commercial marketing is not conditioned on performance of any act by Amgen, and Hospira must provide the notice *on or after* the date that the FDA approves its biosimilar application. *Amgen Inc. v. Sandoz, Inc.*, 794 F.3d 1347, 1357 (Fed. Cir. 2015) ("[U]nder paragraph (l)(8)(A), a subsection (k) applicant may only give effective notice of commercial marketing after the FDA has licensed its product"); *id.* at 1359-60 ("Paragraph (l)(8)(A) is a standalone notice provision in subsection (l). . . . Unlike the actions described in paragraphs (l)(3) through (l)(7), which all depend on, or are triggered by, the disclosure under paragraph (l)(2)(A), nothing in paragraph (l)(8)(A) conditions the notice requirement on paragraph (l)(2)(A) or other provisions of subsection (l).").

62. Despite its obligation under § 262(l)(8)(A), Hospira provided Amgen with a purported (8)(A) notice on April 8, 2015, *before* Amgen had provided its initial disclosure of patents under (3)(A) and *before* Hospira received FDA approval for its Hospira Epoetin Biosimilar Product. On May 8, 2015, Amgen objected to this premature attempt to provide notice, but Hospira has repeatedly refused to withdraw it.

63. On August 18, 2015, after the Federal Circuit issued its decision in *Amgen v. Sandoz* holding that the notice must be provided on or after FDA approval, Amgen renewed its objection and requested that Hospira confirm that it would follow the law.

64. But Hospira has refused to acknowledge the import of the holding in *Amgen v. Sandoz*. Instead, in correspondence dated August 19 and September 15, 2015, Hospira has taken the position that it is under no obligation to, and will not, provide *any* notice under § 262(l)(8)(A).

65. If Hospira is allowed to proceed based on its invalid notice of commercial marketing (or no notice at all), the 180-day period that the statute requires before commercial marketing may begin would run when the precise nature of the dispute between the parties, and even the need for litigation on certain patents, has not yet crystallized.

66. Hospira has indicated that it intends to violate the statute by categorically refusing to provide Amgen with a legally operative notice of commercial marketing under 42 U.S.C. § 262(l)(8)(A). In serving a purported “notice of commercial marketing” before its biosimilar product is licensed, Hospira intends to deprive Amgen of the statutory time period for considering the need for and, if appropriate, seeking adjudication of, a potential preliminary injunction motion. Therefore, Hospira intends to continue violating this provision of the BPCIA absent an order of the Court compelling Hospira to comply.

67. Hospira’s scheme to follow only those parts of the BPCIA it considers helpful to it, and to evade the parts it considers unhelpful to it, is unlawful and inequitable.

THE PATENTS-IN-SUIT

A. U.S. Patent No. 5,856,298

68. Amgen Inc. is the owner of all rights, title, and interest in U.S. Patent No. 5,856,298 (“the ’298 Patent”).

69. The ’298 Patent is titled “Erythropoietin Isoforms.” The ’298 Patent was duly and legally issued on January 5, 1999 by the United States Patent and Trademark Office (“USPTO”). The inventor of the ’298 Patent is Dr. Thomas Strickland, a former Amgen scientist. A true and correct copy of the ’298 Patent is attached to this Complaint as Exhibit A.

70. AML is an exclusive licensee under the ’298 Patent.

71. The '298 Patent is directed to erythropoietin isoforms and erythropoietin compositions having specific numbers of attached sialic acid moieties, and methods for preparing the same.

B. U.S. Patent No. 5,756,349

72. Amgen Inc. is the owner of all rights, title, and interest in U.S. Patent No. 5,756,349 ("the '349 Patent").

73. The '349 Patent is titled "Production of Erythropoietin." The '349 Patent was duly and legally issued on May 26, 1998 by the USPTO. The inventor of the '349 Patent is Dr. Fu-Kuen Lin, a former Amgen scientist. A true and correct copy of the '349 Patent is attached to this Complaint as Exhibit B.

74. AML is an exclusive licensee under the '349 Patent.

75. The '349 Patent is directed to vertebrate cells which are capable of producing recombinant human erythropoietin, and processes for producing recombinant erythropoietin using such cells.

CAUSES OF ACTION

FIRST COUNT

**(DECLARATORY JUDGMENT THAT HOSPIRA'S
REFUSAL TO GIVE LEGALLY EFFECTIVE NOTICE OF
COMMERCIAL MARKETING VIOLATES 42 U.S.C. § 262(l)(8)(A))**

76. Amgen incorporates by reference paragraphs 1-75 as if fully set forth herein.

77. This Count arises under 42 U.S.C. § 262 and the Declaratory Judgment Act, 28 U.S.C. §§ 2201(a) & 2202.

78. The BPCIA, 42 U.S.C. § 262(l), requires Hospira to follow mandatory procedures related to the filing of a BLA under 42 U.S.C. § 262(k).

79. Hospira has failed to comply with the mandatory requirements of the BPCIA. Hospira's violations of the BPCIA have injured Amgen, for example, by depriving Amgen of the procedural protections of the statute, by diminishing the value of Amgen's patents, and by subjecting Amgen to the burden of potentially unnecessary litigation.

80. To comply with 42 U.S.C. § 262(l)(8)(A), Hospira must provide notice to Amgen "not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)."

81. Amgen received a letter from Hospira dated April 8, 2015, in which Hospira purported to provide notice of commercial marketing of the Hospira Epoetin Biosimilar Product, which the FDA has not yet approved for licensure. This purported notice is ineffective because, among other things, a biosimilar applicant may only give effective notice of commercial marketing after the FDA has licensed its product.

82. In later letters, Hospira has indicated that it does not intend to rely upon its April 8, 2015 notice.

83. Hospira has categorically represented to Amgen that it does not intend to provide Amgen with notice of commercial marketing after the FDA licenses the Hospira Epoetin Biosimilar Product and 180 days before commercial marketing of the Hospira Epoetin Biosimilar Product is to begin.

84. Hospira's refusal to provide Amgen with commercial notice after the FDA licenses the Hospira Epoetin Biosimilar Product and 180 days before commercial marketing of the Hospira Epoetin Biosimilar Product is to commence, is a violation of 42 U.S.C. § 262(l)(8)(A).

85. Amgen is entitled to a declaration of its rights under the statute and injunctive relief requiring Hospira to provide Amgen with legally effective notice of commercial marketing and for such further relief as may be appropriate in equity.

SECOND COUNT
(INFRINGEMENT OF U.S. PATENT NO. 5,856,298
UNDER 35 U.S.C. § 271(e)(2)(C))

86. Amgen incorporates by reference paragraphs 1-85 as if fully set forth herein.

87. Amgen is the owner of all rights, title, and interest in U.S. Patent No. 5,856,298 (“the ’298 Patent”).

88. Hospira seeks FDA approval under 42 U.S.C. § 262(k) to manufacture, use, offer to sell, or sell within the United States the Hospira Epoetin Biosimilar Product, a biosimilar version of Amgen’s EPOGEN[®] (epoetin alfa) product.

89. Amgen included the ’298 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A).

90. On information and belief (including Hospira’s failure to state otherwise in its disclosures required by the BPCIA), Hospira intends to, and will, manufacture, use, offer to sell, or sell within the United States the Hospira Epoetin Biosimilar Product before the expiration of the ’298 Patent.

91. On information and belief, Hospira has, intends to, and will immediately and imminently upon FDA licensure of the Hospira BLA, manufacture, use, offer to sell, or sell within the United States, or import into the United States, the Hospira Epoetin Biosimilar Product.

92. The submission of the Hospira BLA to the FDA to obtain approval to engage in the commercial manufacture, use, offer for sale, or sale of the Hospira Epoetin Biosimilar

Product before the expiration of the '298 Patent is an act of infringement of one or more claims of the '298 Patent under 35 U.S.C. § 271(e)(2)(C).

93. Amgen will be irreparably harmed if Hospira is not enjoined from infringing one or more claims of the '298 Patent. Amgen is entitled to injunctive relief under 35 U.S.C. § 271(e)(4)(B) preventing Hospira from any further infringement. Amgen does not have an adequate remedy at law.

94. Hospira's commercial manufacture, use, offer for sale, or sale within the United States, or importation into the United States, upon FDA approval of the Hospira Epoetin Biosimilar Product and before the expiration of the '298 Patent will cause Amgen injury, entitling Amgen to damages or other monetary relief under 35 U.S.C. § 271(e)(4).

THIRD COUNT
(INFRINGEMENT OF THE '298 PATENT
UNDER 35 U.S.C. § 271(a))

95. Amgen incorporates by reference paragraphs 1-94 as if fully set forth herein.

96. Hospira seeks FDA approval under 42 U.S.C. § 262(k) to manufacture and sell the Hospira Epoetin Biosimilar Product, a biosimilar version of Amgen's EPOGEN[®] (epoetin alfa) product.

97. On information and belief, Hospira has, intends to, and will immediately and imminently upon FDA licensure of the Hospira BLA, manufacture, use, offer to sell, or sell within the United States, or import into the United States, the Hospira Epoetin Biosimilar Product.

98. Hospira's manufacture, use, offer to sell, or sale within the United States, or importation into the United States, of the Hospira Epoetin Biosimilar Product before the expiration of the '298 Patent, will infringe one or more claims of the '298 Patent under 35 U.S.C. § 271(a).

99. An actual controversy has arisen and now exists between the parties concerning whether the Hospira Epoetin Biosimilar Product has or will infringe one or more claims of the '298 Patent.

100. Amgen is entitled to a judgment that Hospira has or will infringe one or more claims of the '298 Patent by making, using, offering to sell, or selling within the United States, or importing into the United States, the Hospira Epoetin Biosimilar Product before the expiration of the '298 Patent.

101. Amgen is entitled to injunctive relief prohibiting Hospira from making, using, offering to sell, or selling within the United States, or importing into the United States, the Hospira Epoetin Biosimilar Product before the expiration of the '298 Patent. Amgen does not have an adequate remedy at law.

102. Hospira's manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of the Hospira Epoetin Biosimilar Product before the expiration of the '298 Patent will cause Amgen injury, entitling Amgen to damages under 35 U.S.C. § 284.

FOURTH COUNT
(INFRINGEMENT OF U.S. PATENT NO. 5,756,349
UNDER 35 U.S.C. § 271(a))

103. Amgen incorporates by reference paragraphs 1-102 as if fully set forth herein.

104. On information and belief, Hospira infringed one or more claims of the '349 Patent under 35 U.S.C. § 271(a) by engaging in the manufacture or use of the vertebrate cells claimed in the '349 patent before the expiration of the '349 Patent.

105. Hospira's infringement of one or more claims of the '349 Patent before the expiration of the '349 Patent entitles Amgen to damages under 35 U.S.C. § 284.

106. Hospira's infringement of one or more claims of the '349 Patent before the expiration of the '349 Patent entitles Amgen to an injunction prohibiting Hospira from exporting, using, offering for sale, or selling any infringing vertebrate cells produced or used before the expiration of the '349 patent, and from exporting, using, offering for sale, or selling any Hospira Epoetin Biosimilar Product manufactured by an infringing process prior to the expiry of the '349 patent. Amgen does not have an adequate remedy at law.

PRAYER FOR RELIEF

WHEREFORE, Amgen respectfully requests that this Court enter judgment in its favor against Hospira and grant the following relief:

A. An order enjoining Hospira from commercially marketing the Hospira Epoetin Biosimilar Product until Amgen is restored to the position it would have been in had Hospira met its obligations under the BPCIA;

B. An order enjoining Hospira from continuing to seek FDA review of its § 262(k) application and/or compelling Hospira to suspend FDA review of its § 262(k) application until Hospira has obtained permission from Amgen to use the EPOGEN[®] (epoetin alfa) license or Hospira has restored to Amgen the benefits afforded to Reference Product Sponsors in the BPCIA;

C. A declaration that the notice of commercial marketing that Hospira provided on April 9, 2015 is ineffective under 42 U.S.C. § 262(l)(8)(A);

D. A declaration of Amgen's rights under 42 U.S.C. § 262(l)(8)(A);

E. An injunction requiring Hospira to provide Amgen, on or after FDA licensure of the Hospira Epoetin Biosimilar Product, notice of the date of the first commercial marketing of the Hospira Epoetin Biosimilar Product thereby complying with 42 U.S.C. § 262(l)(8)(A) and prohibiting Hospira from commencing first commercial marketing of the licensed Hospira

Epoetin Biosimilar Product until a date that is 180 days after Hospira provides this notice to Amgen;

F. A judgment that Hospira has infringed one or more claims of the '298 Patent under 35 U.S.C. § 271(e)(2)(C)(i), by submitting to the FDA BLA No. 125545 to obtain approval of the Hospira Epoetin Biological Product under the Public Health Service Act to engage in the commercial manufacture, use, or sale of the Hospira Epoetin Biosimilar Product before the expiration of a patent that claims the product or use of the product;

G. A judgment that Hospira has or will infringe one or more claims of the '298 Patent by engaging in the manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of the Hospira Epoetin Biosimilar Product before the expiration of the '298 Patent;

H. A judgment that Hospira has infringed one or more claims of the '349 Patent by engaging in the manufacture or use of the vertebrate cells claimed in the '349 patent before the expiration of the '349 Patent and by engaging in a process claimed in the '349 patent to produce Hospira Epoetin Biosimilar Product before the expiration of the '349 patent;

I. An order enjoining Hospira, its officers, partners, agents, servants, employees, attorneys, affiliates, divisions, subsidiaries, other related business entities, and those persons in active concert or participation with any of them from infringing the '298 Patent, or contributing to or inducing anyone to do the same, including the manufacture, use, offer to sell, or sale within the United States, or importation into the United States, of any current or future versions of the Hospira Epoetin Biosimilar Product;

J. An order enjoining Hospira, its officers, partners, agents, servants, employees, attorneys, affiliates, divisions, subsidiaries, other related business entities, and those persons in

active concert or participation with any of them from exporting, using, offering for sale, or selling any infringing vertebrate cells produced or used before the expiration of the '349 patent, and from exporting, using, offering for sale, or selling any Hospira Epoetin Biosimilar Product manufactured by an infringing process before the expiration of the '349 patent;

K. A judgment compelling Hospira to pay to Amgen damages or other monetary relief adequate to compensate for Hospira's infringement, in accordance with 35 U.S.C. § 271(e)(4)(C) and § 284;

L. A declaration that this is an exceptional case and awarding to Amgen its attorneys' fees and costs pursuant to 35 U.S.C. § 285; and

M. Such other relief as this Court may deem just and proper.

DEMAND FOR A JURY TRIAL

Amgen hereby demands a jury trial on all issues so triable.

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November 6, 2015

CERTIFICATE OF SERVICE

I hereby certify that on November 6, 2015, I caused the foregoing to be electronically filed with the Clerk of the Court using CM/ECF, which will send notification of such filing to all registered participants.

I further certify that I caused copies of the foregoing document to be served on November 6, 2015, upon the following in the manner indicated:

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EXHIBIT A

United States Patent [19]
Strickland

[11] Patent Number: 5,856,298
[45] Date of Patent: Jan. 5, 1999

[54] ERYTHROPOIETIN ISOFORMS

[75] Inventor: Thomas Wayne Strickland, Moorpark, Calif.

[73] Assignee: Amgen Inc., Thousand Oaks, Calif.

[21] Appl. No.: 334,882

[22] Filed: Nov. 3, 1994

Related U.S. Application Data

[63] Continuation of Ser. No. 942,126, Sep. 8, 1992, abandoned, which is a continuation of Ser. No. 594,448, Oct. 12, 1990, abandoned, which is a continuation-in-part of Ser. No. 421,444, Oct. 13, 1989, abandoned.

[51] Int. Cl.⁶ A61K 38/18; C07K 14/505; C07K 1/18; C07K 1/28

[52] U.S. Cl. 514/8; 530/395; 530/397; 530/412; 530/416; 435/69.4; 204/182.9

[58] Field of Search 530/395, 397, 530/416, 412; 514/8; 435/69.4; 204/182.9

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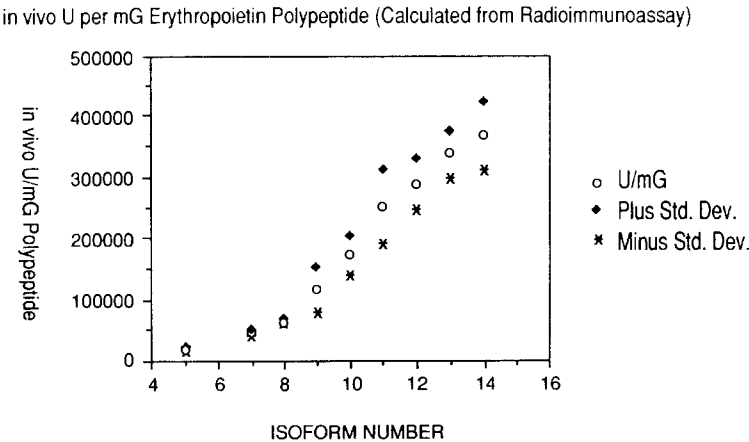
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Primary Examiner—Stephen G. Walsh
Attorney, Agent, or Firm—Robert B. Winter; Steven M. Odre; Ron K. Levy

[57] ABSTRACT

Erythropoietin isoforms having a specific number of sialic acids per erythropoietin molecule are disclosed. Also disclosed are mixtures of such isoforms, pharmaceutical compositions containing such isoforms or mixtures thereof and methods of obtaining the erythropoietin isoforms.

31 Claims, 13 Drawing Sheets



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FIG. 1

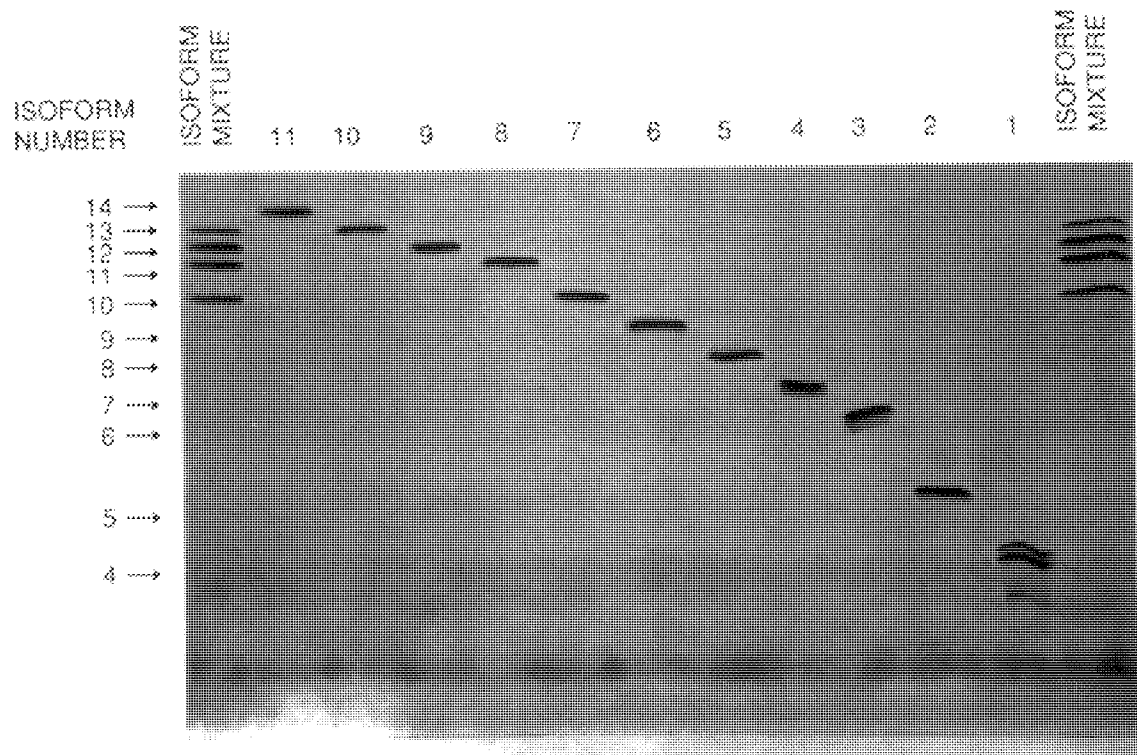


FIG. 2A

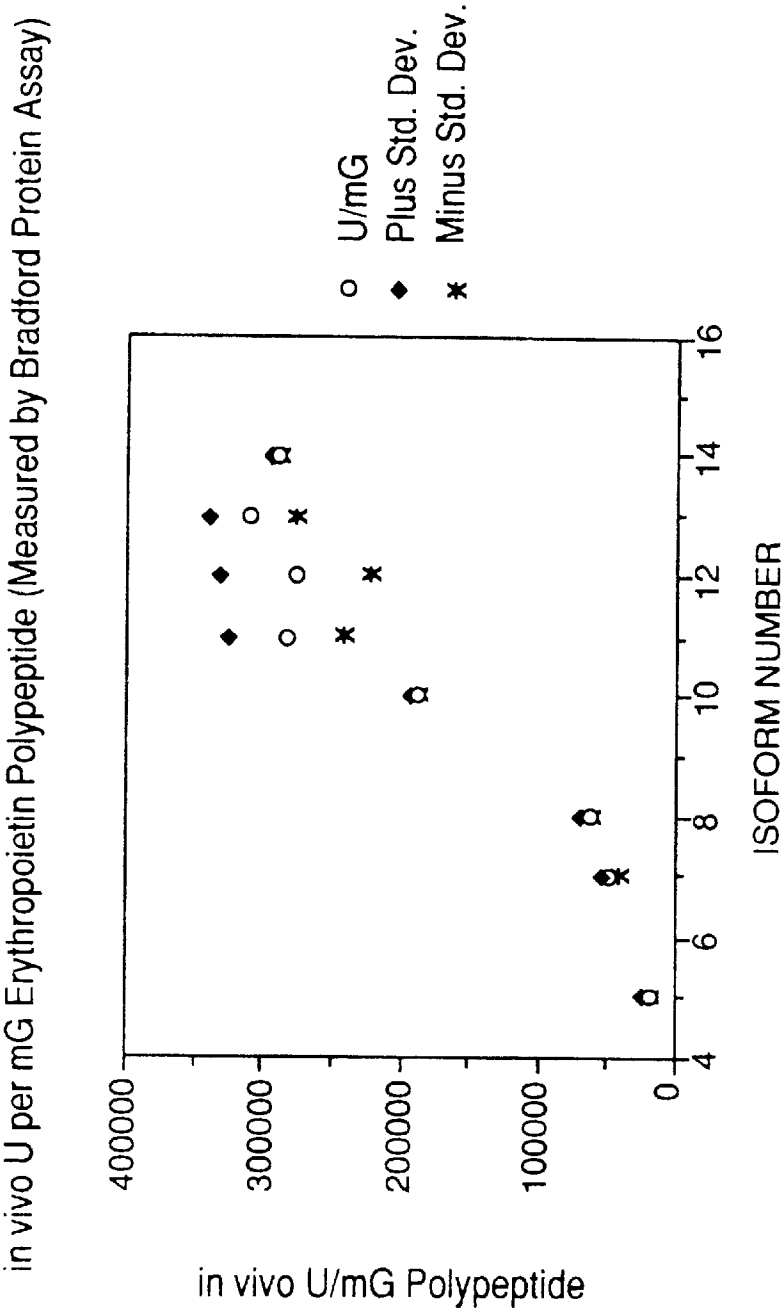
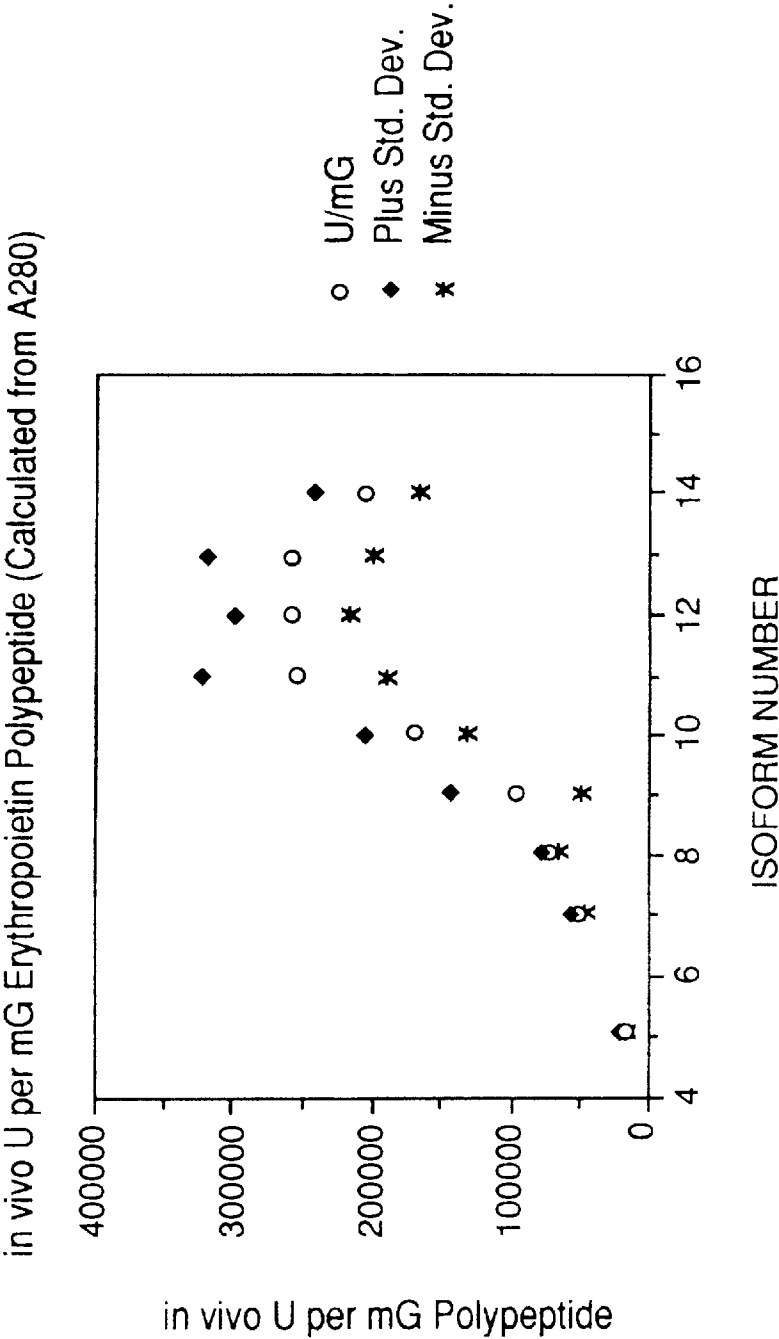


FIG. 2B



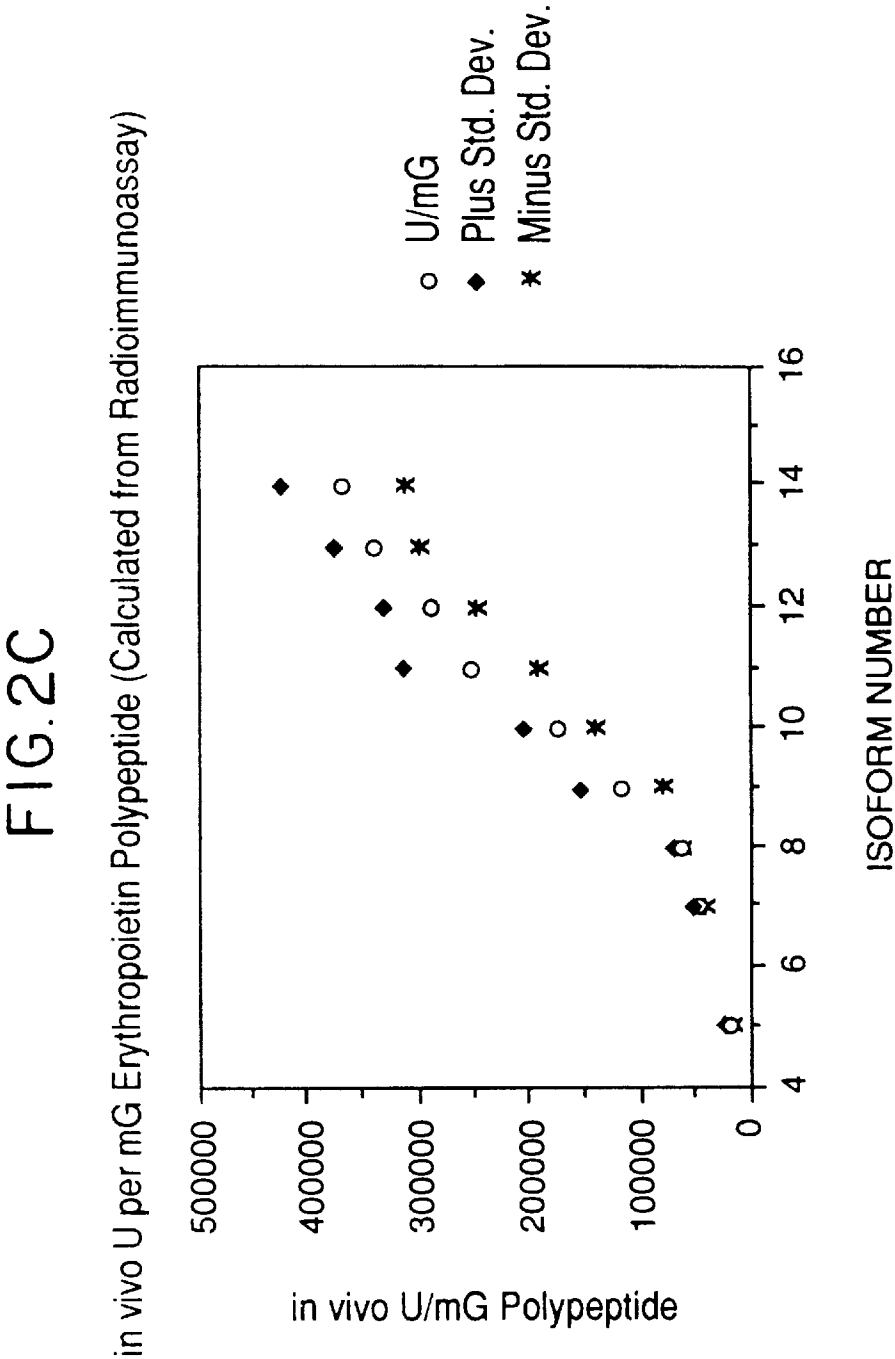


FIG. 3

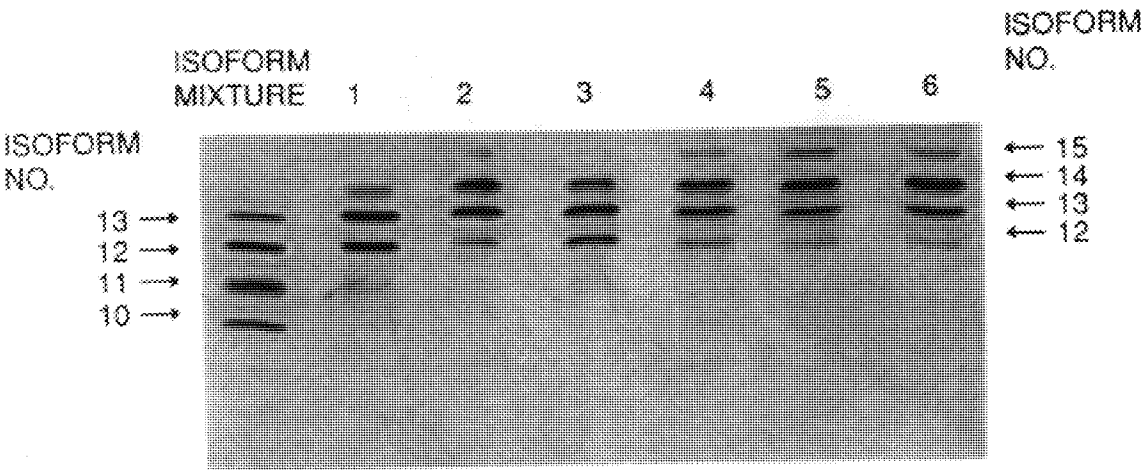


FIG. 4

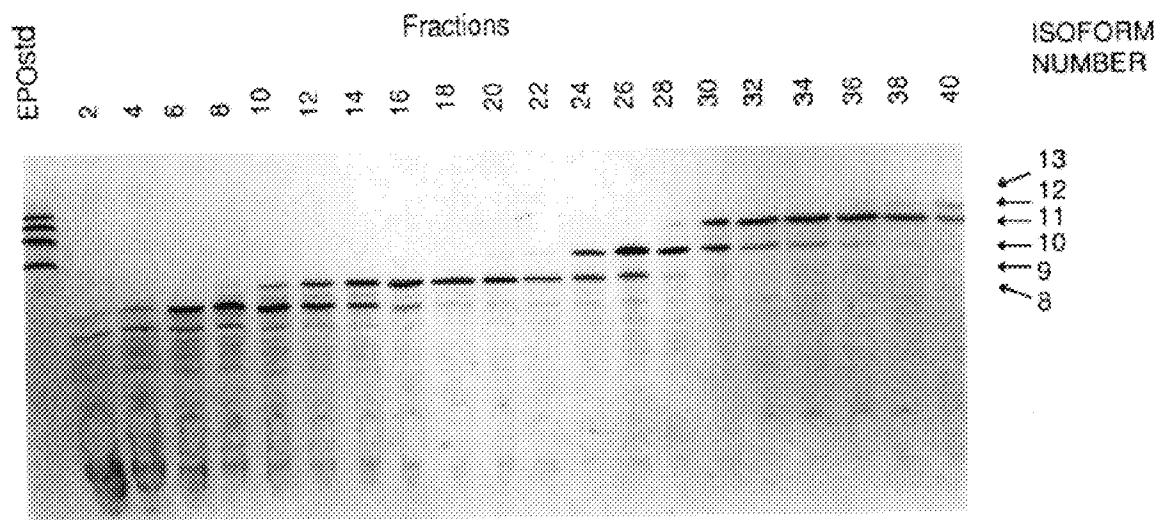


FIG. 5



FIG. 6A

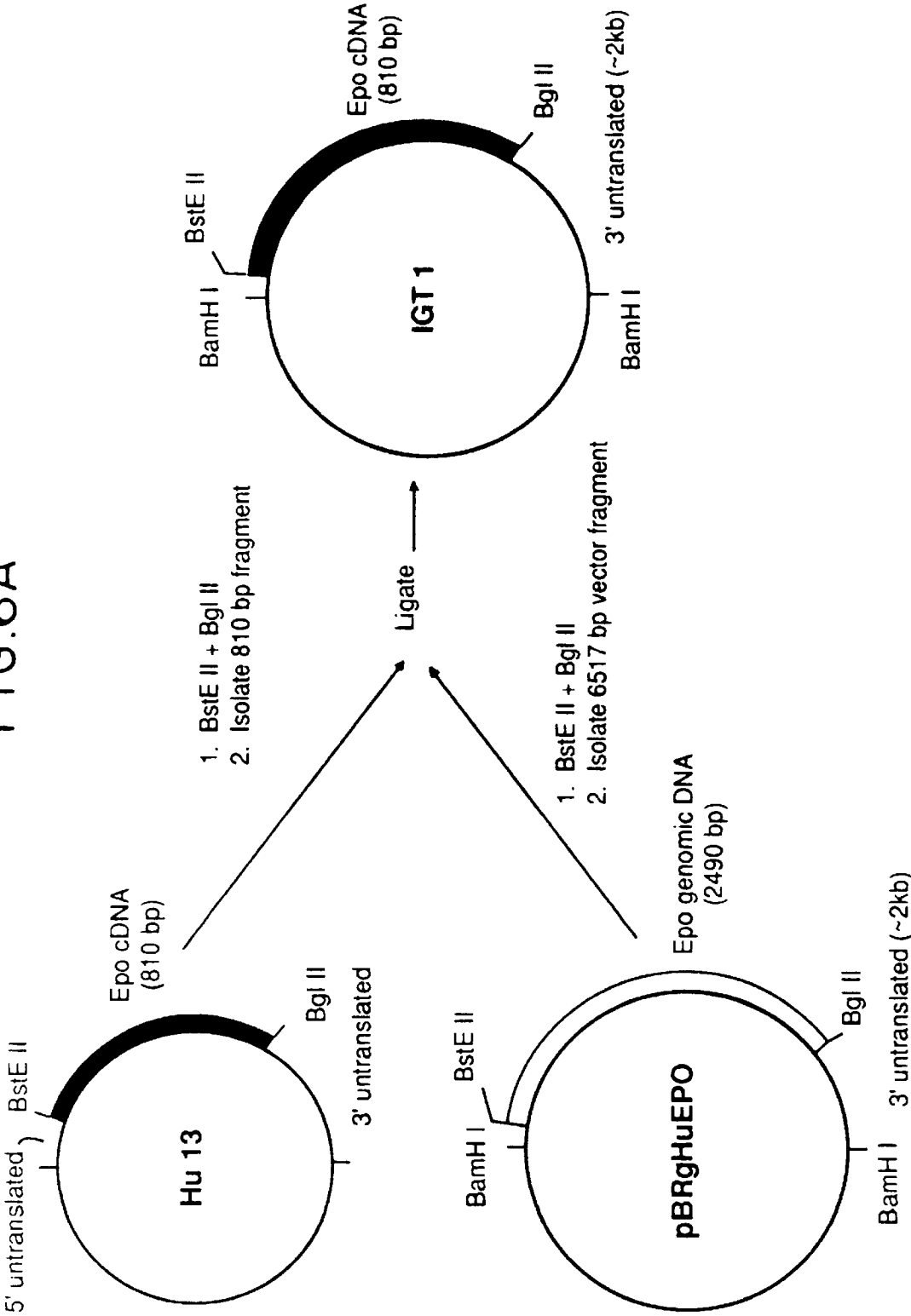


FIG. 6B

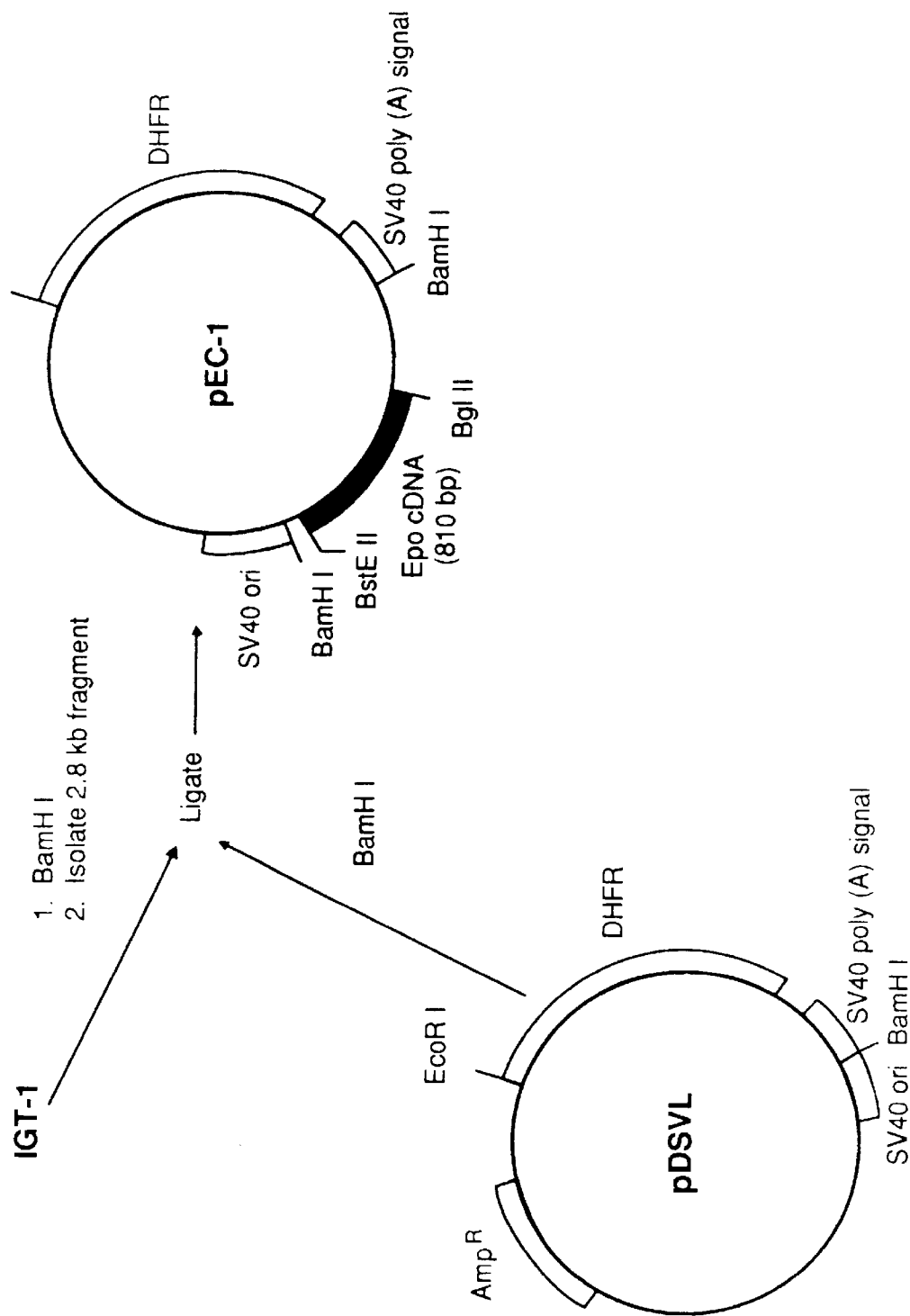


FIG. 6C

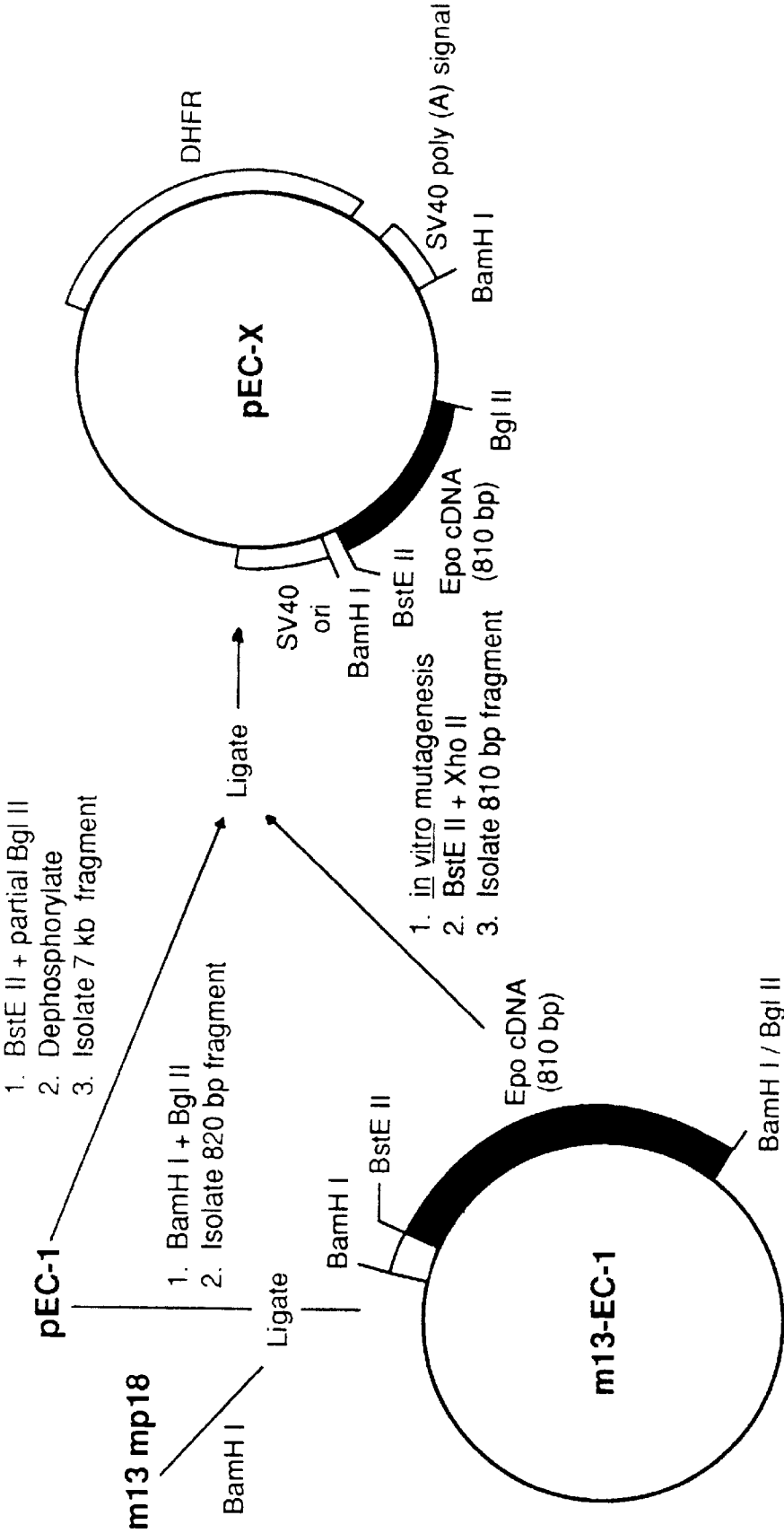


FIG. 7

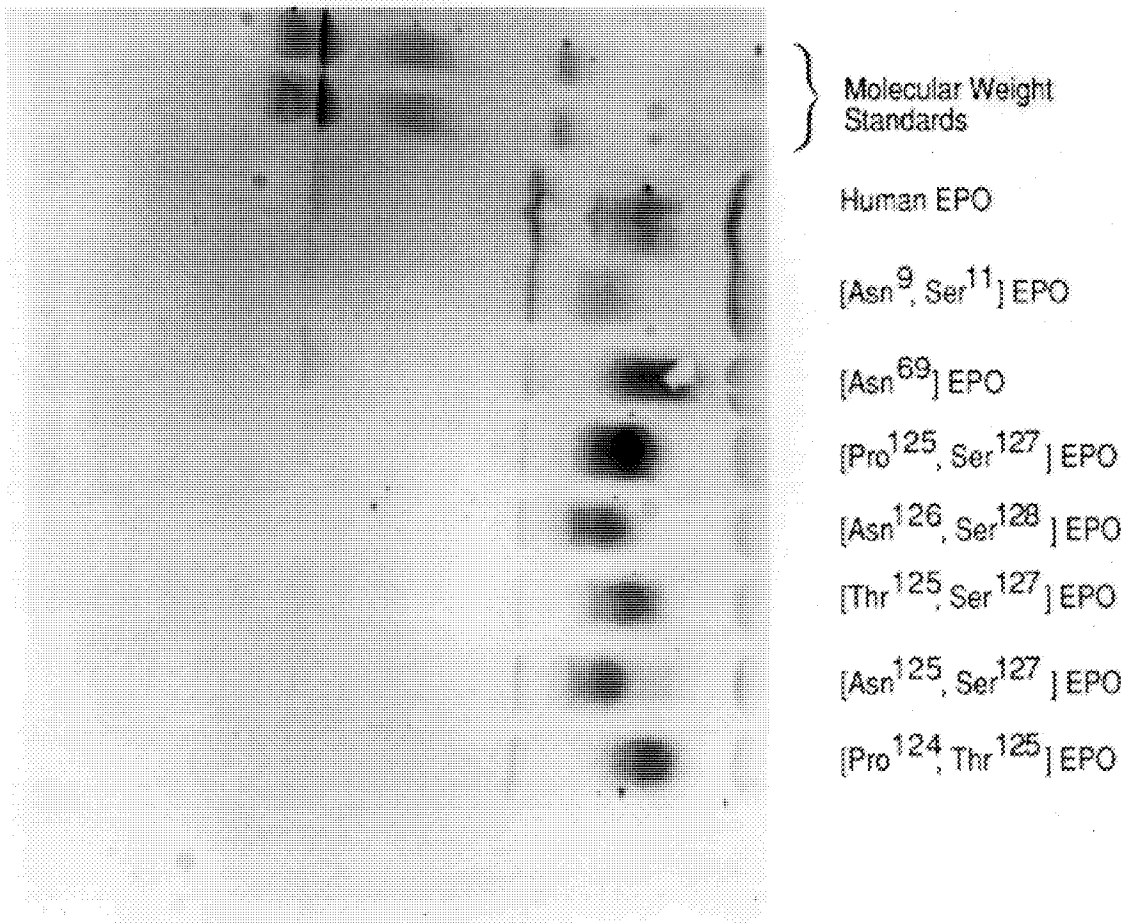
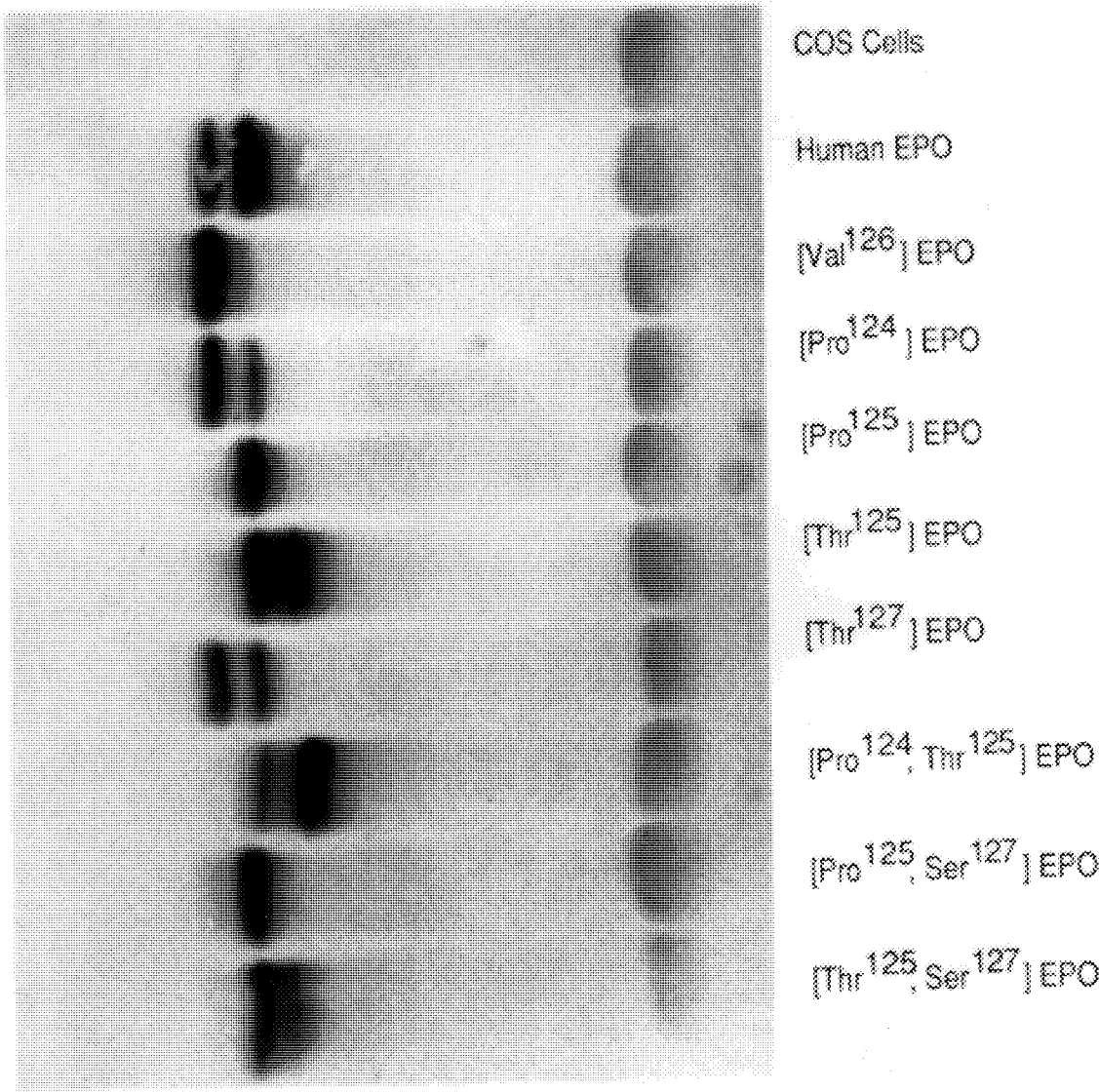
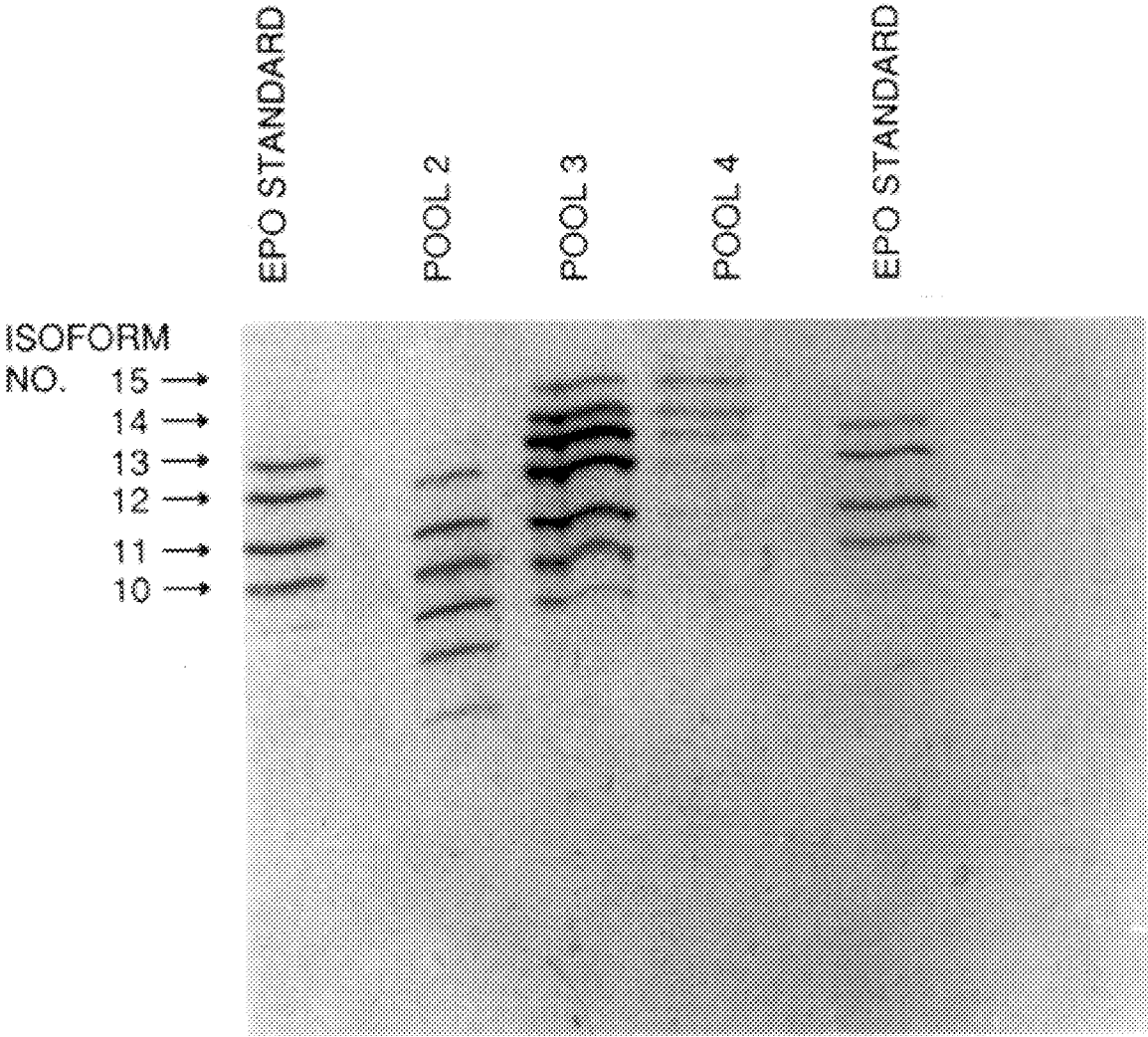


FIG. 8



↑ additional O-linked chains
↑ one O-linked chain
↑ unglycosylated

FIG. 9



ERYTHROPOIETIN ISOFORMS

This application is a continuation, of application Ser. No. 07/942,126, filed Sep. 8, 1992, now abandoned, which is hereby incorporated by reference, which is a continuation of application Ser. No. 07/594,448 filed on Oct. 12, 1990, now abandoned, which is a continuation-in-part application of U.S. application Ser. No. 421,444, filed Oct. 13, 1989, now abandoned, which is incorporated by reference. The present invention relates to erythropoietin isoforms or mixtures thereof, to the methods for the preparation of specific isoforms or mixtures thereof, to pharmaceutical compositions comprising such isoforms or mixtures thereof, and to methods of treatment utilizing such isoforms and compositions.

BACKGROUND OF THE INVENTION

Erythropoietin is a glycoprotein hormone involved in the maturation of erythroid progenitor cells into erythrocytes. It is essential in regulating levels of red blood cells in circulation. Naturally occurring erythropoietin is produced by the liver during fetal life and by the kidney of adults and circulates in the blood and stimulates the production of red blood cells in bone marrow. Anemia is almost invariably a consequence of renal failure due to decreased production of erythropoietin from the kidney. Recombinant erythropoietin produced by genetic engineering techniques involving the expression of a protein product from a host cell transformed with the gene encoding erythropoietin has been found to be effective when used in the treatment of anemia resulting from chronic renal failure.

Until recently, the availability of erythropoietin has been very limited. Although the protein is present in human urine, excreted levels are too low to make this a practical source of erythropoietin for therapeutic use. Patients suffering from aplastic anemia exhibit elevated levels of urinary erythropoietin relative to healthy individuals, but limited supplies of this urine also make such a source impractical. The purification of human urinary erythropoietin by Miyake et al. in J. Biol. Chem., 252, 5558 (1977), used, as starting material, urine from aplastic anemic individuals.

The identification, cloning, and expression of genes encoding erythropoietin are described in U.S. Pat. No. 4,703,008 to Lin. A description of the purification of recombinant erythropoietin from cell medium that supported the growth of mammalian cells containing recombinant erythropoietin plasmids for example, is included in U.S. Pat. No. 4,667,016 to Lai et al. The expression and recovery of biologically active recombinant erythropoietin from mammalian cell hosts containing the erythropoietin gene on recombinant plasmids has, for the first time, made available quantities of erythropoietin suitable for therapeutic applications. In addition, knowledge of the gene sequence and the availability of larger quantities of purified protein has led to a better understanding of the mode of action of this protein.

The biological activity of a protein is dependent upon its structure. In particular, the primary structure of a protein (i.e., its amino acid sequence) provides information that allows the formation of secondary (e.g., α helix or β -sheet) and tertiary (overall three-dimensional folding) structures by a polypeptide during and after its synthesis. The disruption of proper secondary and tertiary structures by the introduction of mutations or by chemical or enzymatic treatments can result in a reduction in biological activity.

In procaryotic organisms, the biological activities of proteins are largely governed by the structures described above.

Unlike proteins from procaryotic cells, many cell surface and secretory proteins produced by eucaryotic cells are modified with one or more oligosaccharide groups. This modification, referred to as glycosylation, can dramatically affect the physical properties of proteins and can also be important in protein stability, secretion, and subcellular localization. Proper glycosylation can be essential for biological activity. In fact, some genes from eucaryotic organisms, when expressed in bacteria (e.g., *E. coli*) which lack cellular processes for glycosylating proteins, yield proteins that are recovered with little or no activity by virtue of their lack of glycosylation.

Glycosylation occurs at specific locations along the polypeptide backbone and is usually of two types: O-linked oligosaccharides are attached to serine or threonine residues while N-linked oligosaccharides are attached to asparagine residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (hereafter referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycoprotein.

Both human urinary derived erythropoietin and recombinant erythropoietin (expressed in mammalian cells) having the amino acid sequence 1-165 of human erythropoietin contain three N-linked and one O-linked oligosaccharide chains which together comprise about 40% of the total molecular weight of the glycoprotein. N-linked glycosylation occurs at asparagine residues located at positions 24, 38 and 83 while O-linked glycosylation occurs at a serine residue located at position 126 (Lai et al. J. Biol. Chem. 261, 3116 (1986); Broudy et al. Arch. Biochem. Biophys. 265, 329 (1988)). The oligosaccharide chains have been shown to be modified with terminal sialic acid residues. Enzymatic treatment of glycosylated erythropoietin to remove all sialic acid residues results in a loss of in vivo activity but does not affect in vitro activity (Lowy et al. Nature 185, 102 (1960); Goldwasser et al. J. Biol. Chem. 249, 4202 (1974)). This behavior has been explained by rapid clearance of asialo-erythropoietin from circulation upon interaction with the hepatic asialoglycoprotein binding protein (Morrell et al. J. Biol. Chem. 243, 155 (1968); Briggs, et al. Am. J. Physiol. 227, 1385 (1974); Ashwell et al. Methods Enzymol. 50, 287 (1978)). Thus, erythropoietin possesses in vivo biological activity only when it is sialylated to avoid its binding by the hepatic binding protein.

The role of the other components in the oligosaccharide chains of erythropoietin is not well defined. It has been shown that non-glycosylated erythropoietin has greatly reduced in vivo activity compared to the glycosylated form but does retain in vitro activity (Dordal et al. Endocrinology 116, 2293 (1985); Lin patent, supra). In another study, however, the removal of N-linked or O-linked oligosaccharide chains singly or together by mutagenesis of asparagine or serine residues that are glycosylation sites sharply reduces in vitro activity of the altered erythropoietin that is produced in mammalian cells (Dube et al. J. Biol. Chem. 263, 17516 (1988)).

Glycoproteins such as erythropoietin can be separated into different charged forms using techniques such as isoelectric focusing (IEF). Several parties have reported IEF studies of crude and partially purified erythropoietin preparations (Lukowsky et al., J. Biochem 50, 909 (1972); Shelton et al. Biochem. Med. 12, 45 (1975); Fuhr et al. Biochem.

Biophys. Res. Comm. 98, 930 (1981)). At most, three or four fractions having erythropoietin activity were distinguished by IEF in these studies and none were characterized with respect to carbohydrate content. In addition, no correlation between the isoelectric points of the fractions and their biological activity was made.

During the purification of urinary erythropoietin from human urine discussed in Miyake et. al. supra, two erythropoietin fractions from hydroxylapatite chromatography designated II and IIIA were reported to have the same specific activity. A subsequent carbohydrate analysis of fractions II and IIIA revealed that fraction II had a greater average sialic acid content than fraction IIIA (Dordal et. al. supra).

It is an object of the present invention to provide separated and isolated isoforms of erythropoietin having a defined sialic acid content and biological activity. Pharmaceutical compositions containing such molecules would have therapeutic benefit.

SUMMARY OF THE INVENTION

The subject invention relates to erythropoietin isoforms. Also provided is a method of preparing an erythropoietin isoform comprising the steps of subjecting purified erythropoietin to preparative isoelectric focusing, and eluting a single isoform from the gel. Pharmaceutically acceptable compositions comprising erythropoietin isoforms are also provided. This invention also relates to methods of increasing hematocrit levels in mammals comprising administering a therapeutically acceptable amount of these compositions to increase production of reticulocytes and red blood cells.

The subject invention relates to a method of preparing a mixture of erythropoietin molecules having greater than or alternatively less than a predetermined number of sialic acids per molecule comprising subjecting material containing erythropoietin to ion exchange chromatography. Also comprised by the subject invention is a method of preparing a mixture of erythropoietin molecules having greater than or alternatively less than a predetermined number of sialic acids per molecule comprising subjecting a material containing erythropoietin to chromatofocusing.

The invention also comprises analogs of human erythropoietin having a greater number of sites for carbohydrate chain attachment than human erythropoietin, such as [Asn⁶⁹] EPO; [Asn¹²⁵, Ser¹²⁷] EPO; [Thr¹²⁵] EPO; and [Pro¹²⁴, Thr¹²⁵] EPO.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows an analytical isoelectric focusing gel of the separate recombinant erythropoietin isoforms. Gel lanes 1–11 show isoforms ranging from less acidic (higher pI) in lane 1 to more acidic (lower pI), in lane 11. Purified recombinant erythropoietin containing a mixture of isoforms 9–14 is also shown in the far left and right lanes of the gel.

FIG. 2A, FIG. 2B and FIG. 2C show the relationship between the number of sialic acids per erythropoietin isoform and the in vivo specific activity of each isoform expressed as units per mg of erythropoietin polypeptide. In FIG. 2A, the concentration of each erythropoietin isoform was determined by the Bradford protein assay; in 2B, the concentration was determined by absorbance at 280 nm, in 2C, the concentration was determined by RIA.

FIG. 3 shows an analytical isoelectric focusing gel of defined mixtures of recombinant erythropoietin isoforms prepared by anion exchange chromatography under different

conditions. Gel lanes 1–6 represent, respectively, erythropoietin isoforms eluted in a high salt wash after washing the Q-Sepharose fast flow column with 150 mM acetic acid, pH 4.7, 150 mM acetic acid (unbuffered), 200 mM acetic acid, pH 4.7, 250 mM acetic acid, pH 4.7, 300 mM acetic acid, pH 4.7 or 300 mM acetic acid (unbuffered). Purified recombinant erythropoietin containing a mixture of isoforms as obtained using procedures described in Example 2 of Lai et al., supra, except that DEAE-Agarose chromatography is replaced by Q-Sepharose chromatography, is also shown in the far left lane of the gel.

FIG. 4 shows the separation of erythropoietin isoforms 8 to 12 achieved by subjecting cell conditioned medium applied to a column of Q-Sepharose to a gradient of decreasing pH and increasing ionic strength. Aliquots of even numbered fractions from Fraction 2 to Fraction 40 were subjected to analytical isoelectric focusing. Purified recombinant erythropoietin containing a mixture of isoforms obtained using procedures described in Example 2 of Lai et al. supra, except that DEAE-Agarose chromatography is replaced by Q-Sepharose chromatography, is also shown in the far left lane of the gel.

FIG. 5 shows the amino acid sequence of human erythropoietin. Squares indicate asparagine residues to which carbohydrate chains are attached and asterisks indicate threonine and serine residues modified with carbohydrate. Additional glycosylation sites provided in the analogs of Example 6 are indicated by mutations to asparagine serine, and threonine.

FIG. 6A, FIG. 6B, and FIG. 6C show the series of cloning steps used in generating plasmids for the construction and analysis of analogs of human erythropoietin. These analogs have amino acids altered as shown in FIG. 5 which provide additional glycosylation sites.

FIG. 7 shows a Western blot analysis of COS cell supernatants of human sequence erythropoietin and indicated erythropoietin analogs. The analogs [Asn⁹, Ser¹¹] EPO, [Asn⁶⁹] EPO, [Asn¹²⁵, Ser¹²⁷] EPO, and [Pro¹²⁴, Thr¹²⁵] EPO are constructed as described in Example 6. The analogs [Pro¹²⁵, Thr¹²⁷] EPO, [Asn¹²⁶, Ser¹²⁸] EPO and [Thr¹²⁵, Ser¹²⁷] EPO which do not contain additional carbohydrate chains are shown for comparison.

FIG. 8 shows a Western blot analysis of COS cell supernatants of human sequences erythropoietin and indicated erythropoietin analogs after treatment with N-glycanase. The analogs [Thr¹²⁵] EPO and [Pro¹²⁴, Thr¹²⁵] EPO are constructed as described in Example 6. The analogs [Val¹²⁶] EPO, [Pro¹²⁴] EPO, [Pro¹²⁵] EPO, [Thr¹²⁷] EPO, [Pro¹²⁵, Ser¹²⁷] EPO and [Thr¹²⁵, Ser¹²⁷] EPO are shown for comparison.

FIG. 9 shows an isoelectric focusing gel of pools 2, 3 and 4 obtained by Q-Sepharose and C4 reverse phase chromatography of cell medium that supported the growth of CHO cells transfected with erythropoietin cDNA containing the [Thr¹²⁵] mutation. Purified recombinant erythropoietin containing a mixture of isoforms are obtained using procedures described in Example 2 of Lai et al., supra, except that DEAE-Agarose chromatography is replaced by Q-Sepharose chromatography, is also shown in the left and right lanes of the gel.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, erythropoietin isoforms are provided. Isoelectric focusing (IEF) separates proteins on the basis of charge. When placed in a pH

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gradient and subjected to an electric field, proteins will migrate to the point at which they have no net charge and remain there. This is the isoelectric point (pI) of the protein. Each distinct band observed on IEF represents molecules that have a particular pI and therefore the same overall charge, and is termed an isoform. The term "erythropoietin isoform" as used herein refers to erythropoietin preparations having a single pI, and having the same amino acid sequence.

In a preferred embodiment the erythropoietin is the product of the expression of an exogenous DNA sequence that has been transfected into a non-human eucaryotic host cell, that is, in a preferred embodiment the erythropoietin is "recombinant erythropoietin". Recombinant erythropoietin is advantageously produced according to the procedures described in commonly owned Lin U.S. Pat. No. 4,703,008 hereby incorporated by reference. Recombinant erythropoietin is advantageously purified according to the general procedures described in Example 2 of commonly owned Lai et al. U.S. Pat. No. 4,667,016 hereby incorporated by reference, or alternatively the procedure described in Example 2 wherein DEAE-Agarose chromatography is replaced by Q-Sepharose chromatography. In the Q-Sepharose column modification, 55 mM NaCl replaces 25 mM NaCl in the buffer solution used to bring the column to neutral pH, and 140 mM NaCl replaces 75 mM NaCl in the buffer solution used to elute erythropoietin from the column. This material, when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, migrates as a single species (i.e. band). When purified erythropoietin is subjected to IEF, multiple bands in the gel are apparent, indicating that different charged forms of the glycoprotein are present.

It has been found that discrete isoforms of recombinant erythropoietin having the amino acid sequence of urinary derived human erythropoietin correspond to erythropoietin molecules having from 1–14 sialic acids, and each isoform present in purified recombinant erythropoietin has an in vivo activity which is related to the number of sialic acids the isoform possesses. The term "erythropoietin", as used herein, includes naturally occurring erythropoietin, urinary derived human erythropoietin as well as non-naturally occurring polypeptides having an amino acid sequence and glycosylation sufficiently duplicative of that of naturally occurring erythropoietin to allow possession of in vivo biological properties of causing bone marrow cells to increase production of reticulocytes and red blood cells.

Crude preparations of erythropoietin have many isoforms but material purified for example, as in the Lai et al. patent supra Example 2, contains predominantly six isoforms when analyzed by IEF. In addition, at least one additional isoform of greater acidity has been detected using the chromatographic procedures described in Example 4. (This more acidic form, migrating at >14 sialic acids on an IEF gel may contain nonsialic acid negative charges as shown by the resistance of some of the charge to sialidase digestion). These isoforms differ from each other by sialic acid content. As shown in the Examples, this is demonstrated by isolating 10 of these isoforms by preparative IEF and determining the sialic acid content of five of them. Of the isoforms assayed for sialic acid content, it is found that the five isoforms contained either 9, 10, 11, 12 or 13 sialic acid residues.

There is a relationship between the relative in vivo specific activity of erythropoietin and number of sialic acid residues per erythropoietin molecule from the isoforms 5 through 11 (each isoform is designated herein by the number of sialic acids per erythropoietin molecule). Isoforms 11 through 14 have approximately the same relative in vivo

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specific activity. Isoforms 5–14 are assayed for in vivo activity by the exhypoxic polycythemic mouse bioassay and the amount of each isoform present is determined by Bradford protein assay, absorbance at 280 nm or by radioimmunoassay (RIA) for erythropoietin. RIA determinations (Egrie et al. Immunobiology 172, 213, (1986)), expressed as units/ml, are divided by 212,770 units/mg erythropoietin polypeptide, the average specific activity of purified erythropoietin as determined by RIA, to give protein concentrations of isolated isoforms or isoform mixtures expressed as mg erythropoietin polypeptide/ml. As shown in the Examples, the relative in vivo specific activities increase step-wise from isoform 5 to isoform 11 (see Table 2).

The in vivo specific activities referred to herein are measurements of relative in vivo specific activities and are not measurements of absolute in vivo specific activities. For the purposes of this application, the specific activities are used only to compare relative activities of isoforms that have been assayed using the same assay, using the same conditions including the same internal standard, the same type of animals, having the same analysis of the data used to calculate specific activity, the same assay for determining protein content. It is not intended that any in vivo specific activity value reported for any isoform represents an inherent or absolute value for that isoform.

The subject invention provides erythropoietin isoforms. The specific isoforms of erythropoietin obtained in accordance with the present invention, and their properties, may vary depending upon the source of the starting material. For example, the isoforms of urinary derived human erythropoietin are different than the isoforms of recombinant erythropoietin. In a preferred embodiment, the invention relates to an erythropoietin isoform having a specific number (i.e. a fixed number greater than 0) of sialic acids per erythropoietin molecule, said number selected from the group consisting of 1–14. Advantageously said number is 9, 10, 11, 12, 13, or 14. In another embodiment, said number is greater than 14, advantageously 16–23.

This invention also provides compositions comprising two or more erythropoietin isoforms. In one embodiment the compositions comprise a mixture of isoforms having greater than a predetermined number of sialic acids per erythropoietin molecule, e.g. greater than 11 sialic acids per erythropoietin molecule, or greater than 12 sialic acids per molecule, e.g. a mixture of isoforms 12, 13 and 14. In another embodiment the compositions comprise mixtures of isoforms having a predetermined number of sialic acids per erythropoietin molecule, e.g. less than 12, but greater than 8 sialic acids per molecule as in, for example, a mixture of isoforms 9, 10, and 11. The invention also provides for compositions of erythropoietin isoforms wherein the relative amounts of the isoforms are the same or different. For example, a mixture of isoforms 9, 10 and 11 could have the isoforms present in a variety of ratios such as 1:1:1, 2:3:1 or 20:20:1.

Advantageously, the compositions comprise mixtures of less than four isoforms, for example a mixture of isoforms 11, 12, and 13, or a mixture of 12 and 14, or a mixture of 7 and 13.

In order to produce mixtures of erythropoietin isoforms, this invention also provides methods of isolating selected erythropoietin isoforms simultaneously. These methods include isolation of individual isoforms by techniques such as preparative isoelectric focusing or preparation of mixtures of isoforms having a predetermined number of sialic acids per molecule (for example, greater than 11) by techniques

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such as ion exchange chromatography or chromatofocusing. All of these techniques have as their basis the separation of proteins according to charge.

In general, ion exchange chromatography and chromatofocusing involve application of either crude human erythropoietin (cell conditioned media) or purified material to a column resin under conditions that permit binding of some or all of the erythropoietin isoforms to the resin. For crude erythropoietin preparations, it is preferable to apply the protein to the column at about pH 7 while for purified preparations the protein can be applied to the column at pH 7 down to about pH 4. After washing the column with buffer at about pH 4, those erythropoietin isoforms that remain bound on the ion exchange column are eluted by increasing the pH and the salt concentration of the buffer or by applying a gradient of decreasing pH and increasing ionic strength at about pH 4. For chromatofocusing, the isoforms are eluted from the column by a gradient of decreasing pH or by washing the column with a high concentration of salt.

One embodiment the invention relates to mammalian (e.g., Chinese Hamster Ovary, CHO) host cells which preferentially synthesize erythropoietin isoforms having greater than a specific number, e.g. greater than 10 sialic acids per molecule. Erythropoietin molecules have N-linked or O-linked oligosaccharides structures which can limit the sialic acid content of the molecule. For example, tetraantennary (four-branched) N-linked oligosaccharides most commonly provide four possible sites for sialic acid attachment while bi- and triantennary oligosaccharide chains, which can substitute for the tetraantennary form at asparagine-linked sites, commonly have at most only two or three sialic acids attached. O-linked oligosaccharides commonly provide two sites for sialic acid attachment. Thus, erythropoietin molecules can accommodate a total of 14 sialic acid residues provided all three N-linked oligosaccharides are tetraantennary. Mammalian cell cultures are screened for those cells that preferentially add tetraantennary chains to recombinant erythropoietin, thereby maximizing the number of sites for sialic acid attachment.

The N-linked oligosaccharides of urinary erythropoietin contain sialic acid in both an α 2,3 and an α 2,6 linkage to galactose (Takeuchi et al. J. Biol. Chem. 263, 3657(1988)). Typically the sialic acid in the α 2,3 linkage is added to galactose on the mannose α 1,6 branch and the sialic acid in the α 2,6 linkage is added to the galactose on the mannose α 1,3 branch. The enzymes that add these sialic acids (β -galactoside α 2,3 sialyltransferase and β -galactoside α 2,6 sialyltransferase) are most efficient at adding sialic acid to the mannose α 1,6 and mannose α 1,3 branches respectively.

Dihydrofolate reductase (DHFR) deficient Chinese Hamster Ovary (CHO) cells are a commonly used host cell for the production of recombinant glycoproteins including recombinant erythropoietin. These cells do not express the enzyme β -galactoside α 2,6 sialyltransferase and therefore do not add sialic acid in the α 2,6 linkage to N-linked oligosaccharides of glycoproteins produced in these cells. (Mutsaers et al. Eur. J. Biochem. 156, 651 (1986); Takeuchi et al. J. Chromatogr. 400, 207 (1987)). Consequently, recombinant erythropoietin produced in CHO cells lacks sialic acid in the 2,6 linkage to galactose (Sasaki et al. (1987), supra; Takeuchi et al. (1987), supra). In another embodiment of the subject invention, the erythropoietin used to produce the isoforms is made in CHO cells that are transfected with a functional β -galactoside α 2,6 sialyltransferase gene to give incorporation of sialic acid in α 2,6 linkage to galactose. See Lee et al. J. Biol. Chem. 264, 13848 (1989), hereby

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incorporated by reference, for a disclosure of techniques for creating modified CHO cells or other mammalian host cells.

Also encompassed by the invention are certain analogs of human erythropoietin. As used herein the phrase "analog of human erythropoietin" refers to erythropoietin with one or more changes in the amino acid sequence of human erythropoietin which result in an increase in the number of sites for sialic acid attachment. Analogs are generated by site-directed mutagenesis having additions, deletions, or substitutions of amino acid residues that alter sites that are available for glycosylation. Such analogs have a greater number of carbohydrate chains than human erythropoietin.

Analogs that result in increased biological activity are constructed by increasing the sialic acid content of the erythropoietin molecule. Analogs having levels of sialic acid greater than that found in human erythropoietin are generated by adding glycosylation sites which do not perturb the secondary or tertiary conformation required for biological activity. Advantageously, the analog of human erythropoietin has 1, 2 or 3 additional sites for N-glycosylation or O-glycosylation. For example, a leucine at position 69 is replaced by an asparagine to give the sequence Asn-Leu-Ser, which serves as a fourth site for N-glycosylation. Such a change can commonly provide up to four additional sialic acids per molecule. Examples of other changes that generate additional N- or O-glycosylation sites are alanines at positions 125 and 127 to asparagine and serine, respectively, alanine at position 125 to threonine and alanines at positions 124 and 125 to proline and threonine, respectively. As will be appreciated by those skilled in the art, the subject invention includes many other analogs of human erythropoietin having additional sites for glycosylation.

Also comprehended by the invention are pharmaceutical compositions comprising a therapeutically effective amount of a specific isoform or mixture of isoforms together with a suitable diluent, adjuvant and/or carrier useful in erythropoietin therapy. A "therapeutically effective amount" as used herein refers to that amount which provides therapeutic effect for a given condition and administration regimen. The administration of erythropoietin isoforms is preferably by parental routes. The specific route chosen will depend upon the condition being treated. The administration of erythropoietin isoforms is preferably done as part of a formulation containing a suitable carrier, such as human serum albumin, a suitable diluent, such as a buffered saline solution, and/or a suitable adjuvant. The required dosage will be in amounts sufficient to raise the hematocrit of patients and will vary depending upon the severity of the condition being treated, the method of administration used and the like.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof. The erythropoietin standard used in the in vivo bioassays employed in the Examples is a recombinant erythropoietin standard that was standardized against a partially purified urinary erythropoietin standard. Thus, only relative in vivo specific activities are being measured. Also the in vivo specific activities are expressed in "units/ml", "units/mg" and units/ A_{280} and not as "IU/ml", "IU/mg" and IU/ A_{280} , because the erythropoietin standard employed has not been directly correlated to any existing international standard.

EXAMPLE 1

Isolation of Recombinant Erythropoietin Isoforms

Recombinant erythropoietin is produced as described in Lin, supra. Recombinant erythropoietin used as starting

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material for the first and third isoform isolations is purified according to the procedure described in Example 2 of commonly owned Lai et al., supra. Starting material for the second and fifth isoform isolation is purified according to Lai et al. supra using the modification of Q-Sepharose chromatography. These preparations contain a mixture of isoforms of recombinant erythropoietin having the same amino acid sequence as urinary derived human erythropoietin and contain predominantly isoforms 9 to 14. Starting material for the fourth isoform preparation is the material which elutes during the 5 mM acetic acid/1 mM glycine/6M urea wash of the anion exchange column in Example 2 of Lai et al. This fraction contains isoforms with less than or equal to 9 sialic acids and was further purified by gel filtration chromatography as described in Example 2 of Lai et al. prior to use in the preparative isoelectric focusing procedure. The sixth isoform preparation used as its starting material a purified preparation of recombinant erythropoietin having from 4 to 13 sialic residues. This material was purified as per Example 2 of Lai et al. except for a modification to the ion exchange column (elution of the recombinant erythropoietin with a sodium chloride gradient at pH 8.4 and omission of the acetic acid/urea wash) which results in retention of most of the isoforms present in the starting material.

Six different preparations of individual isoforms are carried out by preparative isoelectric focusing in a granulated gel bed (Ultradex, LKB) essentially as per LKB Application Note 198. Pharmalyte (Pharmacia) 2.5–5 ampholytes (Pharmacia) are used and the gel bed contains 5M urea.

In the first preparation, approximately 20 mg of recombinant erythropoietin in 6.8 ml of 20 mM sodium citrate/100 mM sodium chloride, pH 7.0 are applied to the gel and focused at 8 watts for approximately 16 hours. After isoelectric focusing, the isoform bands in the gel are visualized by a paper contact print of the gel bed. The print is made and then fixed by soaking in 3 changes (approximately 10 minutes each, room temperature) of fixing solution (40% methanol/10% acetic acid/10% TCA/3.5% sulfosalicylic acid), subjected to one change (approximately 10 minutes) of 40% methanol/10% acetic acid (30°–60° C.), stained for 15 minutes at 60° C. in 0.125% Coomassie Blue R-250/40% methanol/10% acetic acid, and then destained in 7.5% methanol/10% acetic acid in order to visualize the separated isoforms. The region of the granulated gel bed containing the isoforms (~50% of the resin) is removed, water is added (~16 ml), and the slurry is poured into a 5.5x24.5 inch tray and evaporated to ~40 g net weight. This preparation is focused for a second time and a contact print of the gel bed is made as before. The portion of gel containing each of the six discernible isoforms is removed from the gel bed.

In order to elute the isoforms from the gel, a solution containing 10 mM Tris-HCl, pH 7.0/5 mM Chaps is added to each isoform to generate a slurry. The slurries are placed in small columns and washed with the Tris-Chaps buffer. The flow throughs are collected and applied separately to small columns (open column configuration) containing Vydac C4 reversed phase resin equilibrated in 20% ethanol/10 mM Tris-HCl, pH 7.0. The columns are developed stepwise with 20% ethanol/10 mM Tris-HCl, pH 7.0, 35% ethanol/10 mM Tris-HCl, pH 7.0, and 65% ethanol/10 mM Tris-HCl, pH 7.0. The fraction eluting at 65% ethanol/10 mM Tris is diluted 1:1 with 10 mM Tris-HCl, pH 7.0 and subjected to concentration and then buffer exchanged to 10 mM Tris-HCl, pH 7.0 using a Centricon-10 (Amicon) micro-concentrator. Analytical isoelectric focusing of this preparation is performed essentially as described in LKB techni-

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cal note 250 using Servalyte 3–5 ampholines (Serva) in a polyacrylamide gel containing 5M urea.

In a second preparation, approximately 26 mg of recombinant erythropoietin in 6.5 ml of deionized water are applied to the gel and focused at 2.5 watts for 35 minutes and 10 watts for approximately 17 hours. The bands of focused protein, which are visible in the gel bed, are removed as 11 different pools. Each pool is brought to about 7.5 ml with deionized water and 20 ml of each of the resulting pool supernatants is subjected to analytical isoelectric focusing as described above. To each of the pools is added 5 ml of 1.5M Tris-HCl, pH 8.8 and the slurries are each placed in small columns and the liquid phase allowed to flow through. The resin is washed with approximately three volumes of 0.5M Tris-HCl, pH 7 and the rinse solution is combined with the flow through. The eluants are concentrated and buffer exchanged to 20 mM sodium citrate/100 mM sodium chloride, pH 7.0 using Amicon disposable ultrafiltration devices having a 10,000 dalton molecular weight cutoff. The concentrated solutions (approximately 0.5 ml) are then passed through a 0.22 micron cutoff cellulose acetate filter. Based upon analytical isoelectric focusing, five pools are found to contain predominantly the single isoforms 10, 11, 12, 13 and 14.

In a third preparation, approximately 30 mg of recombinant erythropoietin in 21.8 ml of distilled water is applied to the gel and focused at 2 watts for 25 minutes, 10 watts for 20 hours and 15 watts for 15 minutes. Protein bands corresponding to the individual isoforms are observed visually and removed from the gel bed. Distilled water is added to gel-isolated isoforms to generate a slurry and the resulting supernatants are analyzed by analytical isoelectric focusing. An equal volume of 1M Tris-HCl, pH 7.2 is added to each slurry, the suspensions are placed into separate small columns, and the liquid phase is allowed to flow through the column to elute the isoforms. Each flow through is concentrated and buffer exchanged to 20 mM sodium citrate/100 mM sodium chloride, pH 7.0 using Amicon disposable ultrafiltration devices having a 10,000 dalton molecular weight cutoff. An analytical isoelectric focusing gel revealed that pools containing predominantly the single isoforms 9, 10, 11, 12, 13 and 14 were obtained.

A fourth isoform preparation used as its starting material erythropoietin containing isoforms 3–9 (prepared as described above). Prior to preparative isoelectric focusing carried out essentially as described for preparations 1–3 above, the ampholytes (Pharmalyte 2.5–5) were pre-fractionated in a Rotofor (Bio-Rad, Richmond, Calif.) liquid phase isoelectric focusing cell to yield an ampholyte range more suitable for the lower isoelectric points of the starting material. The pre-fractionation was carried out by mixing 6.7 mL of Pharmalyte 2.5–5 with 15 g of urea and adding purified water to bring the volume to 50 mL. This mixture was fractionated in the Rotofor at 10 Watts, 1° C., for 5 ½ hours using 0.1M phosphoric acid and 0.1M sodium hydroxide as the anolyte and catholyte, respectively. The ampholyte fractions having measured pHs of between 4.5 and approximately 6 were used in the flat-bed isoelectric focusing.

Ampholytes were removed from the isoforms using a Centriuluter (Amicon, Danvers, Mass.) and a 10,000 MW cutoff Centricon (Amicon) using the following parameters: 0.18 Tris buffer pH 8.8, 100 Volts, 25–30 mA, for 3 hours. The isoforms were then buffer exchanged into 0.1M sodium chloride by gel filtration using Sephadex G-25 (Pharmacia). Analytical isoelectric focusing of the five resulting pools showed them to contain isoforms 4,5,6,7, and 8. Isoform 4 ran as several bands, indicating that it may have undergone some degradation.

The fifth isoform preparation was modified by the addition of a pre-focusing step to the flat bed isoelectric focusing procedure. In this modification, the protein was not added to the ampholyte/urea/gel mixture prior to electrophoresis but was added to the isoelectric focusing apparatus following generation of the pH gradient in the gel bed. Following prefocusing for 75 minutes (1500 volt-hrs) the section of gel bed from 2.25–4.25 cm from the cathode was removed, mixed with the erythropoietin solution, and added back to the gel bed. Following isoelectric focusing, isoforms 10,11, 12,13, and 14 were eluted from the gel bed and separated from the ampholytes by ultrafiltration using Centricon-10 (Amicon) devices.

The pre-focusing modification was undertaken to make the ultraviolet absorbance characteristics of the isoform preparations more similar to that of the starting recombinant erythropoietin. This improvement in spectral characteristics can be seen in the ratio of absorbance at 280 and 260 nm for the isolated isoforms. The average ratio of absorbance at 280 nm to that at 260 nm (A280/A260) for isoforms from preparations 2 and 3 (non-prefocused) is 1.36±0.11 while the average A280/A260 ratio for preparations 5 and 6 (pre-focused) is 1.68±0.20. When isoform #14 is excluded from the calculation, the average A280/A260 ratios are 1.39±0.11 and 1.74±0.09 for preparations 2 & 3 and 5 & 6, respectively. (Isoform 14 may have the most atypical spectrum because it is present in the smallest amounts and is thus more subject to interferences by trace contamination by ampholyte components or because it is nearest to the electrode during the flat bed isoelectric focusing procedure). The average A280/A260 ratio for recombinant erythropoietin prepared according to Example 2 of Lai et al. (modified as described earlier by using Q-Sepharose as the anion exchange resin) is 1.91±0.04.

As described above, the starting material for isoform preparation #6 was a recombinant erythropoietin preparation containing isoforms 4–13. The ampholytes were pre-focused in the Rotofor apparatus as per the fourth preparation. Ampholyte fractions having measured pHs of between 3.7 and 4.8 were used for the flat bed isoelectric focusing. The flat bed was pre-focused as in run #5 and isoforms 9,10,11, 12 and 13 were obtained after ultrafiltration (Centricon-10) to remove carrier ampholytes.

EXAMPLE 2

Sialic Acid Content of Recombinant Erythropoietin Isoforms

The isoforms isolated as described in Example 1 and erythropoietin purified according to procedures described in Lai et al., supra (mixture of isoforms 9 to 14) are buffer exchanged into 0.10–0.15M sodium chloride and analyzed for sialic acid content by a modification of the procedure of Jourdan et al. J. Biol. Chem. 246, 430 (1971). The sialic acid residues are cleaved from the glycoproteins by hydrolysis with 0.35M sulfuric acid at 80° C. for 30 minutes and the solutions are neutralized with sodium hydroxide prior to analysis. In order to estimate the amount of erythropoietin protein present, a Bradford protein assay (Bradford Anal. Biochem. 72, 248 (1976)) using recombinant erythropoietin having the amino acid sequence of human erythropoietin as standard is performed using the assay reagents and the micro-method procedure supplied by Bio-Rad. The results, expressed as moles of sialic acids per mole of erythropoietin, are shown in Table 1. Isoforms are designated according to the number of sialic acids per molecule and range from least acidic (Isoform 9) to most acidic (Isoform 13). Isoforms

9–13 are shown in gel lanes 6–10 of FIG. 1. Quantities of Isoform 14 are insufficient to accurately measure the sialic acid content. The sialic acid content of this isoform is inferred from its migration on IEF gels relative to other isoforms. The sialic acid content of isoforms 5–8 (preparation #4) has not been measured but is likewise inferred from their migration on IEF gels.

TABLE 1

ERYTHROPOIETIN ISOFORM	MOLES SIALIC ACID/MOLE ERYTHROPOIETIN
Isoform 13	12.9 ± 0.5
Isoform 12	11.8 ± 0.2
Isoform 11	11.0 ± 0.2
Isoform 10	9.8 ± 0.3
Isoform 9	8.9 ± 0.6
Isoform Mixture (9-14)	11.3 ± 0.2

EXAMPLE 3

Activity of Recombinant Erythropoietin Isoforms

The isoforms isolated as described in Example 1 are assayed by absorbance at 280 nm, by Bradford protein assay and by RIA for erythropoietin to determine the amount of recombinant erythropoietin present. The exhypoxic polycythemic mouse bioassay (Cotes et al. Nature 191, 1065 (1961)) is used to determine the relative in vivo biological activity. Quantitation of the amount of erythropoietin protein present using a radioimmunoassay for erythropoietin produced results having higher relative in vivo specific activity for certain isoforms because of an apparent decreased immunoreactivity of isoforms containing large amounts of sialic acid leading to an underestimation of the erythropoietin concentration and thus an overestimation of the relative in vivo specific activity for the most negative isoforms. Mouse bioassay determinations, expressed as units/ml, are divided by the corresponding protein concentrations to give in vivo specific activities expressed as units/mg erythropoietin polypeptide. These specific activities are shown in Table 2.

In Table 2, “n” is the number of independent isoform preparations which contribute to the specific activity value. In most cases several in vivo assays were performed on each isoform preparation. The same in vivo data contribute to the specific activity calculations for all three columns, units/mg erythropoietin polypeptide was determined by the absorbance at 280 nm, from radioimmunoassay potencies, or from Bradford protein assay results. Purified recombinant erythropoietin containing isoforms 9–14 was used as the standard in the Bradford protein assay. “n” may be less for the calculation made using the Bradford protein assay as some preparations were no longer available at the time the Bradford assays were performed.

Erythropoietin purified according to the procedures described in Lai et al., supra and containing a mixture of isoforms 9 to 14 is used as a standard for the RIAs and in vivo assays.

The relative specific activities expressed as units/mg erythropoietin polypeptide can be converted to units/A₂₈₀ by multiplying by 0.807 mg erythropoietin polypeptide/A₂₈₀. The conversion factor is derived by multiplying the extinction coefficient of erythropoietin (1.345 mg/A₂₈₀) by the protein content of the erythropoietin glycoprotein (about 60% by weight, Davis et al. Biochemistry 26, 2633 (1987)) to obtain mg erythropoietin polypeptide/A₂₈₀ (i.e., 1.345 mg erythropoietin/A₂₈₀ × 0.60 mg polypeptide/mg

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erythropoietin=0.807 mg polypeptide/A₂₈₀). In addition, specific activities expressed as units/mg erythropoietin polypeptide can be multiplied by the factor 0.60 mg polypeptide/mg erythropoietin glycoprotein to give specific activities expressed as units/mg erythropoietin glycoprotein.

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adjusted to pH 4.7 with NaOH; Column #5, 150 mM acetic acid, 1 mM glycine, 20 μM CuSO₄, 6M urea; Column #6, 300 mM acetic acid, 1 mM glycine, 20 μM CuSO₄, 6M urea. The pH of the columns is increased to approximately pH 7 by washing each one with 8 to 11 column volumes of 10 mM

TABLE 2

Isoform	U/mG Polypeptide (Bradford Protein Assay)		n	U/mG Polypeptide (From A280)		n	U/mG Polypeptide (From RIA)		n
14	289,400 ± 3,100		2	205,800 ± 37,700		2	366,700 ± 55,900		2
13	307,600 ± 30,600		4	258,700 ± 59,500		5	337,200 ± 40,200		5
12	275,200 ± 55,600		4	258,400 ± 41,700		5	287,700 ± 42,600		5
11	282,700 ± 41,100		3	255,800 ± 67,300		4	251,400 ± 62,700		4
10	188,000 ± 1,900		1	170,300 ± 34,500		3	171,900 ± 31,600		3
9	—			96,600 ± 46,700		2	113,600 ± 39,600		2
8	65,200 ± 3,800		1	70,600 ± 4,100		1	61,000 ± 3,500		1
7	46,200 ± 5,800		1	50,300 ± 6,300		1	42,800 ± 5,400		1
5	16,600 ± 1,700		1	18,300 ± 1,900		1	15,500 ± 1,600		1

The data in Table 2 are also presented graphically in FIGS. 2A, 2B and 2C. These data show that the relative in vivo activity of erythropoietin increases as a function of sialic acid content up until isoform #11. Isoforms 11–14 have essentially the same relative in vivo bioactivity. (This is most apparent when the concentration of isoform 14 is expressed using the Bradford assay value. The Bradford value may be more accurate for isoform 14 because of the generally low levels obtained and the resulting difficulty in determination by A₂₈₀ and the most apparent decreased reactivity in the RIA of very negative forms discussed previously). The greater relative in vivo specific activity of erythropoietin isoforms having more sialic acid is most likely due to a longer circulating half-life of these forms. Isoforms 9 and 13 were labeled with radioactive iodine (¹²⁵I) and their rate of clearance in rats was determined. The half-life in circulation was significantly longer for isoform 13 than for isoform 9.

EXAMPLE 4

Selection of Recombinant Erythropoietin Isoform Mixtures by O-Sepharose Chromatography

Cell conditioned media from the production of recombinant erythropoietin according to the procedures described in Lin, supra are concentrated and diafiltered against 10 mM Tris, pH 7.2. Protein concentration is determined by the Bradford microprotein assay using bovine serum albumin as a standard. 19.6 ml of the solution containing 40 mg of total protein is made 20 μM in CuSO₄, filtered through a 0.45 micron cutoff filter and loaded onto a 4 ml bed volume (1.05 cm heightx2.2 cm diameter) column packed with Q Sepharose Fast Flow (Pharmacia) which has been equilibrated with 10 mM Tris, pH 6.8 to 7.0 at 4° C. After sample application, the column is washed with two column volumes of the same buffer. The column flow rate is about 1 ml/min. Six separate columns are set up using this procedure to select defined erythropoietin isoform mixtures.

Columns are washed with 6 to 9 column volumes of a low pH buffer consisting of: Column #1, 150 mM acetic acid, 1 mM glycine, 20 μM CuSO₄, 6M urea adjusted to pH 4.7 with NaOH; Column #2, 200 mM acetic acid, 1 mM glycine, 20 μM CuSO₄, 6M urea adjusted to pH 4.7 with NaOH; Column #3, 250 mM acetic acid, 1 mM glycine, 20 μM CuSO₄, 6M urea adjusted to pH 4.7 with NaOH; Column #4, 300 mM acetic acid, 1 mM glycine, 20 μM CuSO₄, 6M urea

Tris-HCl, 55 mM NaCl, 20 μM CuSO₄, pH 7. The defined erythropoietin isoform mixtures are eluted from the columns by washing with 10 mM Tris-HCl, 140 mM NaCl, 20 μM CuSO₄, pH 7.0.

The eluted isoform pools from each column are concentrated and solvent exchanged into water using an Amicon Centricon-10 microconcentrator. The results of analytical isoelectric focusing of these concentrated pools are shown in FIG. 3. Gel lanes 1–6 represent defined erythropoietin isoform mixtures eluted from column 1–6, respectively. The “isoform mixture” shown in the far right gel lane of FIG. 3 represents cell media which is applied to a Q-Sepharose column as described above, the column is washed with 5 mM acetic acid, 1 mM glycine, 20 μM CuSO₄, 6M urea, and the erythropoietin isoform mixture is eluted from the column using the procedures described above. This eluted mixture of isoforms is further purified according to the procedures described in Lai et al., supra prior to analytical isoelectric focusing.

EXAMPLE 5

Fractionation of Recombinant Erythropoietin Isoforms Using a Low pH Gradient on O-Sepharose

In another procedure, erythropoietin isoforms are separated using a gradient of decreasing pH and increasing ionic strength. The concentrated diafiltered erythropoietin containing media is loaded to a column of Q-Sepharose at a ratio of approximately 40 mg total protein/mL gel. The column is then washed with approximately two column volumes of 10 mM Tris HCl, pH 7.0 and then approximately 10 column volumes of 2 mM acetic acid/1 mM glycine/20 μM CuSO₄/6M urea (pH approximately 4.8) to remove contaminating proteins and erythropoietin isoforms containing less than approximately 7 sialic acid residues. Isoforms containing from approximately 8 to approximately 12 sialic acids are eluted from the column using a gradient starting at approximately 2 mM acetic acid in 6M urea/1 mM glycine/20 μM CuSO₄ and running to 40 mM acetic acid/6M urea/1 mM glycine/20 μM CuSO₄ (pH approximately 4). The total volume of the gradient is approximately 40 column volumes and fractions of approximately one column volume each are collected into vessels containing a volume of Tris buffer sufficient to bring the pH into the range of 6–8.5 so as to avoid long term exposure of the collected fractions to low

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pH. Aliquots of the fractions are subjected to analytical isoelectric focusing to monitor the separation. FIG. 4 shows the separation of isoforms 8–11 which may be achieved by this procedure. Isoforms 12–14 which remain bound to the column at the end of the gradient are eluted by washing with a buffer consisting of 10 mM TrisHCl, 140 mM NaCl, 20 μM CuSO₄ (pH 7.0). The isoforms (separated during the gradient or eluted by the sodium chloride solution) are freed of contaminating proteins by reverse phase chromatography followed by gel filtration chromatography as described in Example 2 of Lai et al.

EXAMPLE 6

Analogs of Human Erythropoietin Having Additional Glycosylation Sites.

A. Construction of Human Erythropoietin Analogs.

The locations of existing and proposed carbohydrate attachment sites within the erythropoietin amino acid sequence are shown in FIG. 5 and the procedure for generating these additional glycosylation sites is summarized in FIGS. 6A–C and described below.

The following oligonucleotide primers were synthesized for use in in vitro mutagenesis:

[Asn⁴, Ser⁶] EPO: 5' CGCCACCAAACCTC
AGCTGTGACAGCCGA 3'
[Asn⁹, Ser¹¹] EPO: 5' ATCTGTACAACCGA
AGCCTGGAGAGGT 3'
[Asn⁶⁹]EPO: 5' GGGCCTGGCCAACCTGTGCGAAG 3'
[Asn¹²⁴] EPO: 5' TCCCCTCCAGAT
AATGCCTCAGCTGC 3'
[Asn¹²⁵, Ser¹²⁷] EPO: 5' CAGATGCGAACTCA
TCTGCTCCAC 3'
[Asn¹⁶³, Ser¹⁶⁵] EPO: 5' AGGCCTGCAGGAATGGG
AGCAGATGACCAGGTG 3'
[Thr¹²⁵] EPO: 5' TCCAGATGCGACCTCAGCTGCTC 3'
[Pro¹²⁴, Thr¹²⁵] EPO: 5' CCTCCAGATCCG
ACCTCAGCTGC 3'

The underlined codons show the mismatched regions where the amino acids indicated in brackets replace the wild-type amino acids.

[Asn⁴, Ser⁶] EPO was constructed to add an N-glycosylation site at Asn 4. [Asn⁹, Ser¹¹] EPO was constructed to add an N-glycosylation site at Asn 9. [Asn⁶⁹] EPO was constructed to add an N-glycosylation site at Asn 69. [Asn¹²⁵, Ser¹²⁷] EPO was constructed to add an N-glycosylation site at Asn 125. [Thr¹²⁵] EPO and [Pro¹²⁴, Thr¹²⁵] EPO were constructed to add an O-glycosylation site at Thr 125.

The following oligonucleotide primers are synthesized for use in in vitro mutagenesis:

[Asn⁶⁹, Thr⁷¹] EPO: 5' GGGCCTGGCCAACCTGAC
AGAAGCTGTGTC 3'
[Ser⁶⁸, Asn⁶⁹, Thr⁷¹] EPO: 5' CAGGGCCTG
TCCAACCTGACAGAAGCTGTGTC 3'
[Asn¹²⁵, Thr¹²⁷] EPO: 5' CAGATGCGAACTCAA
CGGCTCCAC 3'
[Asn¹²⁵, Thr¹²⁷, Thr¹³¹] EPO: 5'ATGCGAACTCAA
CGGCTCCACTCACAACAATCACT 3'
[Pro¹²⁴, Asn¹²⁵, Ser¹²⁷] EPO: 5' CCAGATCCAAATCA
TCTGCTCCACTC 3'
[Pro¹²⁴, Asn¹²⁵, Thr¹²⁷] EPO: 5' CCAGATCCAAATCA
ACAGCTCCACTC 3'
[Thr¹²⁵, Thr¹²⁶] EPO: 5' CCAGATGCG
ACAACAGCTGCTCCA 3'
[Pro¹²⁴, Thr¹²⁵, Thr¹²⁶, Thr¹³¹] EPO:

Starting from [Pro¹²⁴, Thr¹²⁵] EPO cDNA, the oligonucleotide primer 5' AGATCCGACCAC

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CGCTGCTCCAC 3' is used to generate [Pro¹²⁴,Thr¹²⁵, Thr¹²⁶] EPO. The oligonucleotide primer 5'TGCTCCACTC ACAACAATCACTG 3' is then used to generate [Pro¹²⁴, Thr¹²⁵, Thr¹²⁶, Thr¹³¹] EPO.

[Asn⁶⁹, Thr⁷¹] EPO and [Ser⁶⁸, Asn⁶⁹, Thr⁷¹] EPO are constructed to add an N-glycosylation site at Asn 69 and to enhance N-glycosylation at that site. [Asn¹²⁵, Thr¹²⁷] EPO, [Asn¹²⁵, Thr¹²⁷, Thr¹³¹] EPO, [Pro¹²⁴, Asn¹²⁵, Ser¹²⁷] EPO and [Pro¹²⁴, Asn¹²⁵, Thr¹²⁷] EPO are constructed to add an N-glycosylation site at Asn 125 and to increase glycosylation at that site. [Thr¹²⁵, Thr¹²⁶] EPO and [Pro¹²⁴, Thr¹²⁵, Thr¹²⁶, Ser¹³¹] EPO are constructed to add an O-glycosylation site at Thr 125 and to increase glycosylation at that site.

The source of erythropoietin DNA for in vitro mutagenesis was plasmid Hu13, a human erythropoietin cDNA clone in pUC 8 (Law et al. Proc Natl. Acad. Sci. 83, 6920 (1986)). Plasmid DNA derived from Hu13 was digested with BstEII and BglII restriction enzymes, the resulting DNA fragments were subjected to agarose gel electrophoresis, and the 810 base pair (bp) erythropoietin DNA fragment was isolated from the gel using a GeneClean™ kit and procedures supplied by the manufacturer (BIO 101, Inc.). Plasmid pBRgHuEPO contains the erythropoietin genomic gene as a BamHI fragment inserted into a derivative of pBR322, as described in commonly owned Lin patent, supra. pBR-gHuEPO was also digested with BstEII and BglII and the 6517 bp vector fragment was recovered. Ligation of the two fragments results in IGT1. To construct pEC-1, pDSVL (described in commonly owned Lin patent, supra, and shown in FIG. 5B) was digested with BamHI and the isolated 2.8 kilobase (kb) BamHI fragment from IGT1 containing erythropoietin cDNA was ligated into it.

In order to generate single-stranded DNA for in vitro mutagenesis, pEC-1 was digested with BamHI and BglII and the 820 bp erythropoietin cDNA fragment was isolated. It was ligated into the BamHI site of m13mp18 to give m13-EC-1. Single stranded DNA was recovered from supernatants of *E. coli* strain RZ1032 infected by m13-EC-1 as described by Kunkel et al. Methods in Enzymol. 154, 367 (1987) and Messing, Methods in Enzymol. 101, 20 (1983). For in vitro mutagenesis approximately 1 μg of single-stranded DNA and 0.2 pmole of one of the synthetic primers described above were mixed with 6 ml of buffer (250 mM Tris pH 7.8, 50 mM MgCl₂, and 50 mM dithiothreitol). For annealing of the primer to the template, the reaction volume was adjusted to 10 μl with water, the mixture was heated to 65° C. for 5 minutes and then allowed to cool to room temperature. For the elongation reaction 2.5 ml of each of dTTP, dATP, dGTP, dCTP and ATP (all at 10 μM) were added, followed by 1 μl (1 unit) of *E. coli* DNA polymerase (Klenow fragment) and 1 μl (1 unit) of T4 DNA ligase. The mixture was then incubated overnight at 14° C. and used to transform *E. coli* JM 109 (Yanisch-Perron et al. Gene 33, 103 (1985)) as described (Messing, supra).

To identify mutant clones by differential hybridization, plaques on nutrient agar were transferred to Gene Screen filters (New England Nuclear). The filters were dried under a heat lamp and then incubated for one hour in 6×SSC containing 1% SDS at 60° C. For the hybridization, the oligonucleotide primer above (8 pmoles) was end-labeled with T4 polynucleotide kinase and γ 32p-labeled ATP and incubated with the filters overnight in 6×SSC, 0.5% SDS and 100 mg/ml salmon sperm DNA at 37° C. for the [Asn¹²⁴] mutation, 55° C. for the [Asn⁴, Ser⁶] mutation, 65° C. for the [Thr¹²⁵] and the [Pro¹²⁴, Thr¹²⁵] mutations, and 70° C. for the [Asn⁹, Ser¹¹] and [Asn¹⁶³, Ser¹⁶⁵] mutations.

The next day, the filters were washed three times with 6xSSC at room temperature and subjected to autoradiography. If necessary, the filters were then washed with 6xSSC at increasing temperatures until little or no hybridization was detected to plaques having the wild-type erythropoietin CDNA sequence. Clones that gave positive hybridization signals under these conditions were identified and retransfected into JM109 to isolate a pure clone. Dideoxy chain termination sequence analysis indicated that the mutations to asparagine, serine threonine and proline residues were present.

Double stranded m13 EC-1 DNAs carrying the [Asn⁴, Ser⁶], [Asn⁹, Ser¹¹], [Asn⁶⁹], [Asn¹²⁴], [Asn¹²⁵], [Ser¹²⁷], [Asn¹⁶³, Ser¹⁶⁵][Thr¹²⁵], and [Pro¹²⁴, Thr¹²⁵] changes were recovered from JM109 transfected cells by the boiling method (Holmes et al. Anal. Biochem 117, 193 (1981)). The DNAs were digested with BstEII and XhoII and the 810 bp erythropoietin DNA fragments were isolated. pEC-1 were digested with BstEII followed by a partial digestion with BglII and the 5' termini of the resulting fragments are dephosphorylated with bacterial alkaline phosphatase in 10 mM Tris, pH 8 at 60° C. for 60 minutes. The 7 kb vector fragment lacking the 810 bp BstEII-BglII fragment was isolated and ligated to the erythropoietin fragments above. The resulting plasmids (designated pEC-X where X identifies the particular mutation) contain human erythropoietin with altered amino acid residues at the indicated positions.

cDNA clones of the human erythropoietin sequence and analogs corresponding to [Asn⁴, Ser⁶], [Asn⁹, Ser¹¹], [Asn⁶⁹], [Asn¹²⁴], [Asn¹²⁵, Ser¹²⁷], [Asn¹⁶³, Ser¹⁶⁵], [Thr¹²⁵] and [Pro¹²⁴, Thr¹²⁵] erythropoietin cDNA clones were transferred into COS-1 cells (ATCC No. CRL-1650) by electroporation. COS-1 cells were harvested from semi-confluent dishes, washed with medium (Dulbecco's modified essential medium containing 5% fetal calf serum and 1% L-glutamine/penicillin/streptomycin (Irvine Scientific)) and resuspended at 4x10⁶ cells/ml. One ml of cells was transferred to an electroporation cuvette (Bio-Rad) and electroporated with a Bio-Rad Gene Pulser™ at 25 μFarads and 1600 volts in the presence of 100 to 200 μg of carrier DNA and 2 to 20 μg of plasmid DNA encoding the erythropoietin analog. The electroporated cells were plated at 2x10⁶ cells per 60 mm tissue culture dish in 5 ml of medium. Two to four hours after plating the medium was replaced with 5 ml of fresh medium. The conditioned medium was collected 3 to 5 days after electroporation.

B. Assays for erythropoietin analog activity

RIAs were performed according to Egrie et al., supra. The in vivo biological activity of erythropoietin analogs was determined by the exhypoxic polycythemic mouse bioassay (Cotes et al., supra).

In vitro erythropoietin activity was determined by the erythroid colony forming assay as described by Iscove et al. J. Cell Physiol. 83, 309–320 (1974) with modifications. The mononucleated cells from human bone marrow cells were partially purified on a ficoll-paque cushion and washed in Iscove medium before plating to remove the adherent cells. The culture medium contained 0.9% methyl cellulose and did not include any bovine serum albumin. The erythroid colonies are scored after 8 to 10 days of culture.

The erythropoietin analogs transfected and expressed in COS cells as described in Section A were analyzed in crude COS cell supernatants by RIA and by the erythroid colony

forming assay. Human sequence erythropoietin has an in vitro activity that is comparable to the RIA activity as determined by the above-mentioned assays. The analogs [Asn⁶⁹] EPO, [Asn¹²⁵, Ser¹²⁷] EPO, [Thr¹²⁵] EPO and [Pro¹²⁴, Thr¹²⁵] EPO exhibited an in vitro activity that is comparable to the RIA activity and gave evidence of having additional carbohydrate chains (as determined in Section C). These analogs are analyzed further by transfecting a cDNA clone encoding the erythropoietin analog into CHO cells, purifying the erythropoietin analog and measuring the in vivo biological activity of the purified analog.

C. Western Blot Analysis

A volume of supernatant containing 5–20 units from COS cells transfected with erythropoietin analog cDNAs as described in Section A was immunoprecipitated overnight at room temperature with a rabbit anti-erythropoietin polyclonal antibody. 20–80 μl of 1:1 Protein A-Sepharose in phosphate buffered saline (PBS) was added to the immunoprecipitate and allowed to incubate for one hour at room temperature. The samples were centrifuged, washed with PBS and, where indicated, the pellet was treated with N-glycanase to remove N-linked carbohydrate chains. The samples were analyzed by 15% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and subjected to Western analysis as described (Burnette et al. Anal. Biochem. 112, 195–203 (1981); Elliot et al. Gene 79, 167–180 (1989)) using a mixture of mouse anti-erythropoietin monoclonal antibodies. One such antibody, 9G8A, is described in Elliot et al. (1989) Blood 74, Supp. 1, A. 1228.

Analysis of COS cell supernatants transfected with [Asn⁶⁹] EPO and [Asn¹²⁵, Ser¹²⁷] EPO cDNA revealed increased protein size compared to human sequence erythropoietin. This increased size is indicative of an additional N-linked carbohydrate chain (FIG. 7). Treatment of supernatants from COS cells transfected with [Thr¹²⁵] EPO and [Pro¹²⁴, Thr¹²⁵] EPO cDNA with N-glycanase revealed an increased protein size compared to human sequence erythropoietin. This increased size is indicative of an additional O-linked carbohydrate chain (FIG. 8).

D. Isolation of Erythropoietin Analog Isoforms

The erythropoietin analog [Thr¹²⁵] EPO was constructed as described in Section A. An 810 bp. erythropoietin cDNA fragment carrying the [Thr¹²⁵] mutation was isolated by cleaving the plasmid pEC containing the [Thr¹²⁵] mutation with BstEII and BglII and ligating the fragment to pDECA, a derivative of pDSα2. pDSα2 is generally described in commonly owned U.S. patent application Ser. No. 501,904, now abandoned, hereby incorporated by reference. pDECA was derived from pDSα2 by the following steps:

- (1) The HindIII site of pDSα2 was deleted by digesting pDSα2 DNA with HindIII, treating the HindIII cohesive ends with *E. coli* DNA Polymerase (Klenow fragment) and dNTPs, and religating the blunt-ended vector. The resulting plasmid was pDSα2ΔH.
- (2) pDSα2ΔH was digested with SalI and a synthetic oligonucleotide having an SV40 splice signal with a SalI linker attached to the 3' end of the splice signal was ligated to it. The synthetic oligonucleotide had the following sequence:
5' TCGAGGAACTGAAAAACCAGAAAGT-TAACTGGTAAGTTTAGT CTTTTTGTCCTTT-

TATTTTCAGGTCCCGGATCCGGTGGTGGT-
G C A A A T C A
AAGAAGTCTCCTCAGTGGATGTTGC-
CTTTACTTCTAGGCCTGTACGG AAGTGT-
TACTTCTGCTCTAAAAGCTGCTGCAA-
CAAGCTGGTCGACC 3'

The resulting plasmid was pDSα2ΔH splice.

3) pDSα2ΔH splice was digested with SalI and blunt-ended by treating the cohesive ends with T4 DNA polymerase and dNTPs. An 820 bp. BamHI-BglII human erythropoietin CDNA fragment was blunt-ended by the same method and ligated to the plasmid. The resulting plasmid was pDEC.

4) pDEC was digested with KpnI and PvuII and blunt-ended by treating the cohesive ends with mung bean nuclease. The plasmid was religated to delete the excised KpnI-PvuII fragment resulting in the plasmid pDECA.

Plasmid pDECA containing [Thr¹²⁵] erythropoietin cDNA was transfected into DHFR-deficient CHO cells. 770 ml of CHO cell conditioned medium was concentrated using a 10,000 dalton molecular weight cutoff membrane and diafiltered against 10 mM Tris-HCl, pH 8.6 to a final volume of 34 ml. A 17 ml. aliquot of the concentrate was loaded onto a Q-Sepharose fast flow column (5 ml bed volume) equilibrated in the same buffer and eluted in a linear gradient of 0–250 mM NaCl in 10 mM Tris-HCl, pH 8.6. Aliquots of column fractions, either untreated or digested with N-glycanase, were analyzed by SDS-PAGE or IEF and pools (designated 2, 3 and 4) were made based upon the isoform and/or carbohydrate composition of the fractions. Each pool was loaded onto a Vydac C4 column (214TPB 2030; 1 cm diameter; 1.8–2.5 ml bed volume; 0.34 ml/min) and washed with two column volumes of 20% ethanol in 10 mM Tris-HCl, pH 7.0. The columns were eluted with linear gradients of 20–94% ethanol, 10 mM Tris, pH 7.0. Pools were made, diluted into 10 mM Tris-HCl, pH 7.0, and loaded onto Q-Sepharose fast flow columns. Following a wash in 10 mM Tris-HCl, pH 7.0, the samples were eluted with 20 mM sodium citrate, 250 mM NaCl, pH 7.0. The purified [Thr¹²⁵] pools were analyzed by IEF and are shown in FIG. 9. EPO analog is analyzed for in vivo biological activity as described above (Cotes et al., supra).

While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such modifications and equivalents.

What is claimed is:

1. An isolated biologically active erythropoietin isoform having a single isoelectric point and having a specific number of sialic acids per molecule, said number selected from the group consisting of 1–14, and said isoform being the product of the expression of an exogenous DNA sequence in a non-human eucaryotic host cell.
2. An erythropoietin isoform according to claim 1 wherein said isoform comprises erythropoietin having the amino acid sequence of 1-165 or 1-166 human erythropoietin.
3. An erythropoietin isoform according to claim 1 having 14 sialic acids per erythropoietin molecule.
4. An erythropoietin isoform according to claim 1 having 13 sialic acids per erythropoietin molecule.
5. An erythropoietin isoform according to claim 1 having 10 sialic acids per erythropoietin molecule.

6. An erythropoietin isoform according to claim 1 wherein said eucaryotic host cell is a CHO cell.

7. A pharmaceutical composition comprising a therapeutically effective amount of said erythropoietin isoform of claim 1 and a pharmaceutically acceptable diluent, adjuvant or carrier.

8. A composition consisting essentially of two or three erythropoietin isoforms according to claim 1.

9. A composition according to claim 8 wherein said isoforms have from 1 to 12 sialic acids per erythropoietin molecule.

10. A composition according to claim 9 wherein said isoforms have 9, 10 and 11 sialic acids per erythropoietin molecule.

11. A composition according to claim 8 wherein said isoforms have greater than 11 sialic acids per erythropoietin molecule.

12. A composition according to claim 11 wherein said isoforms have from 13–14 sialic acids per erythropoietin molecule.

13. Erythropoietin consisting essentially of erythropoietin molecules having a single specific number of sialic acids per molecule, said number selected from the group consisting of 1–14, and said molecules being the product of the expression of an exogenous DNA sequence in a non-human eucaryotic host cell.

14. Erythropoietin according to claim 13 having 14 sialic acids per erythropoietin molecule.

15. Erythropoietin according to claim 13 having 13 sialic acids per erythropoietin molecule.

16. Erythropoietin according to claim 13 having 10 sialic acids per erythropoietin molecule.

17. Erythropoietin according to claim 13 wherein said erythropoietin has the amino acid sequence of human erythropoietin.

18. A pharmaceutical composition comprising a therapeutically effective amount of the erythropoietin according to claim 13 and a pharmaceutically acceptable diluent, adjuvant or carrier.

19. A composition consisting essentially of erythropoietin molecules according to claim 13 having two or three specific numbers of sialic acids per erythropoietin molecule.

20. A composition according to claim 19 wherein said molecules have from 1 to 12 sialic acids per erythropoietin molecule.

21. A composition according to claim 19 wherein said molecules have greater than 11 sialic acids per erythropoietin molecule.

22. A composition according to claim 21 wherein said molecules have 13 and 14 sialic acids per erythropoietin molecule.

23. A method of preparing an erythropoietin isoform according to claim 1 comprising the steps of:

subjecting a purified erythropoietin to preparative isoelectric focusing, and

eluting a single isoform.

24. A method of preparing erythropoietin molecules having a predetermined number of sialic acids per molecule said number selected from the group consisting of 1–14, comprising applying material containing erythropoietin to an ion exchange column and selectively eluting said molecules from the column.

25. A method of preparing erythropoietin molecules having a predetermined number of sialic acids per molecule said number selected from the group consisting of 1–14, comprising applying material containing erythropoietin to a chromatofocusing column and selectively eluting said molecules from the column.

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26. A method of increasing hematocrit levels in mammals comprising administering a therapeutically effective amount of the composition according to claim 19.

27. A method for obtaining an erythropoietin composition having a predetermined in vivo specific activity comprising preparing a mixture of two or more erythropoietin isoforms of claim 1.

28. The method of claim 27 wherein said mixture consists essentially of at least two isoforms having less than 12 sialic acids per molecule.

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29. The method of claim 28 wherein said mixture consists essentially of erythropoietin isoforms having 9, 10 and 11 sialic acids per molecule.

30. The method of claim 27 wherein said mixture consists essentially of at least two isoforms having greater than 11 sialic acids per molecule.

31. The method as in claim 28 wherein said mixture consists essentially of erythropoietin isoforms having 13 and 14 sialic acids per molecule.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,856,298
DATED : January 5, 1999
INVENTOR(S) : Strickland, Thomas

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 13, line 43 change "O" to - - Q - -.

Column 14, line 45 change "O" to - - Q - -.

Column 17, line 29 change "[Asn4, Ser6]" to - - [Asn⁴, Ser⁶] - -.

Column 17, line 29 change "[Asn9, Ser11]" to - - [Asn⁹, Ser¹¹] - -.

Signed and Sealed this
Eighteenth Day of May, 1999

Attest:



Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks

EXHIBIT B



US005756349A

United States Patent [19]

Lin

[11] **Patent Number:** **5,756,349**[45] **Date of Patent:** **May 26, 1998**[54] **PRODUCTION OF ERYTHROPOIETIN**[75] **Inventor:** **Fu-Kuen Lin**, Thousand Oaks, Calif.[73] **Assignee:** **Amgen Inc.**, Thousand Oaks, Calif.[21] **Appl. No.:** **468,369**[22] **Filed:** **Jun. 6, 1995****Related U.S. Application Data**

[63] Continuation of Ser. No. 113,179, Oct. 23, 1987, Pat. No. 5,441,868, which is a continuation of Ser. No. 675,298, Nov. 30, 1984, Pat. No. 4,703,008, which is a continuation-in-part of Ser. No. 561,024, Dec. 13, 1983, abandoned, which is a continuation-in-part of Ser. No. 582,185, Feb. 21, 1984, abandoned, which is a continuation-in-part of Ser. No. 655,841, Sep. 28, 1984, abandoned.

[51] **Int. Cl.⁶** **C12N 15/00**[52] **U.S. Cl.** **435/325; 435/358; 435/365**[58] **Field of Search** **435/69.1, 69.4, 435/240.2, 240.22, 325, 358, 365**[56] **References Cited****U.S. PATENT DOCUMENTS**

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Primary Examiner—James Martinell**Attorney, Agent, or Firm**—Marshall, O'Toole, Gerstein, Murray & Borun[57] **ABSTRACT**

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

7 Claims, 27 Drawing Sheets

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FIG. 1

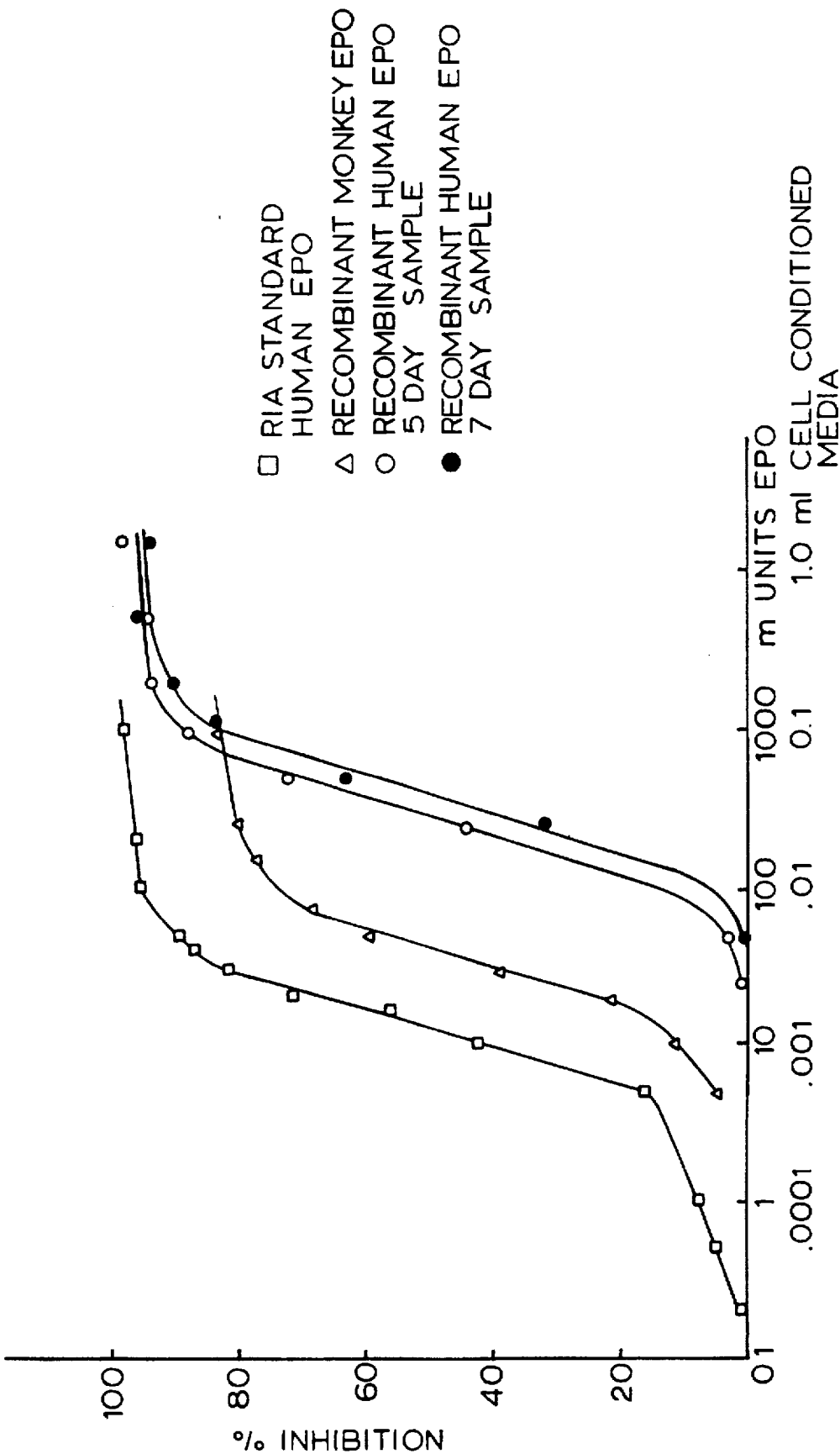


FIG. 2

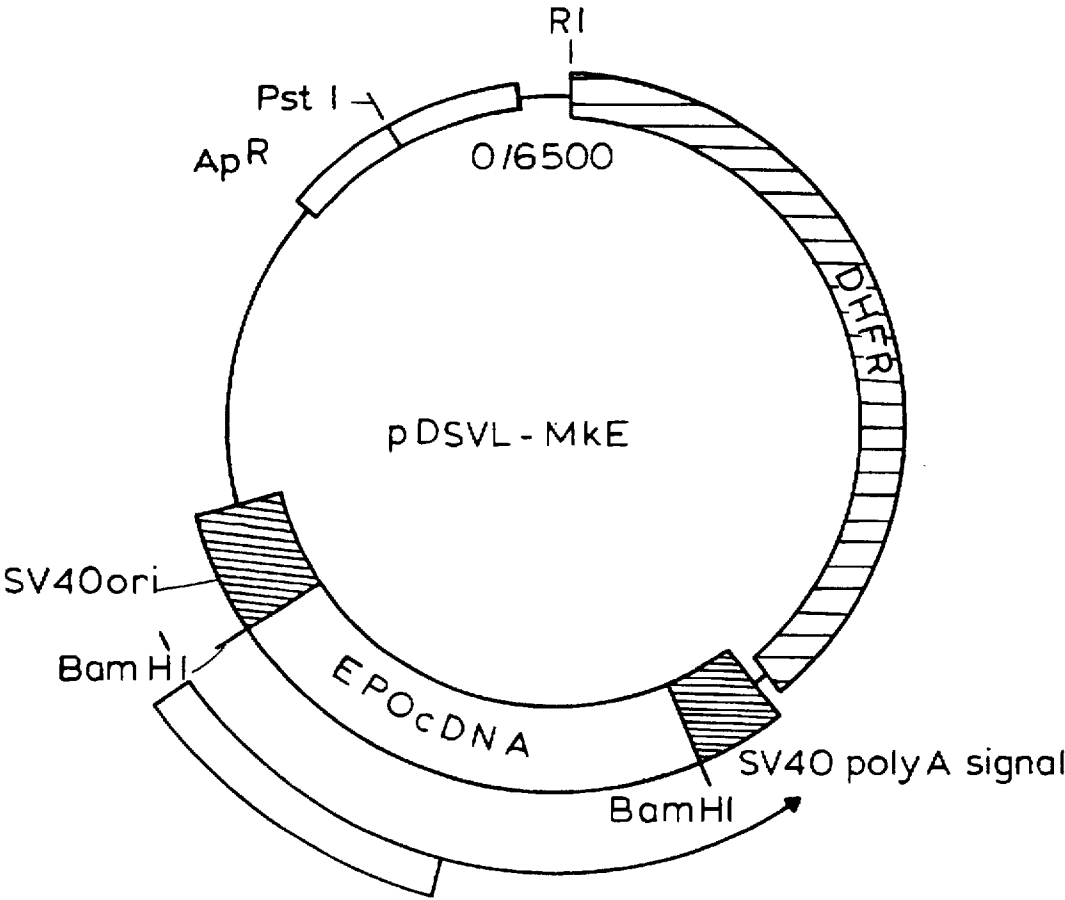


FIG. 3

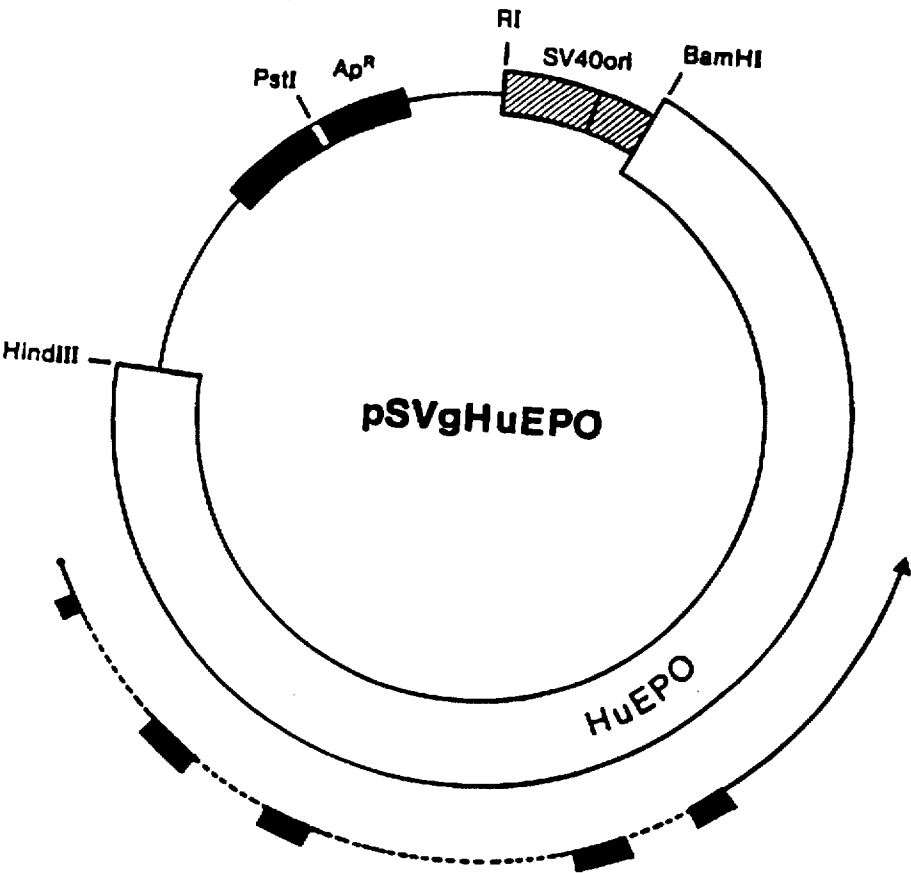


FIG. 4

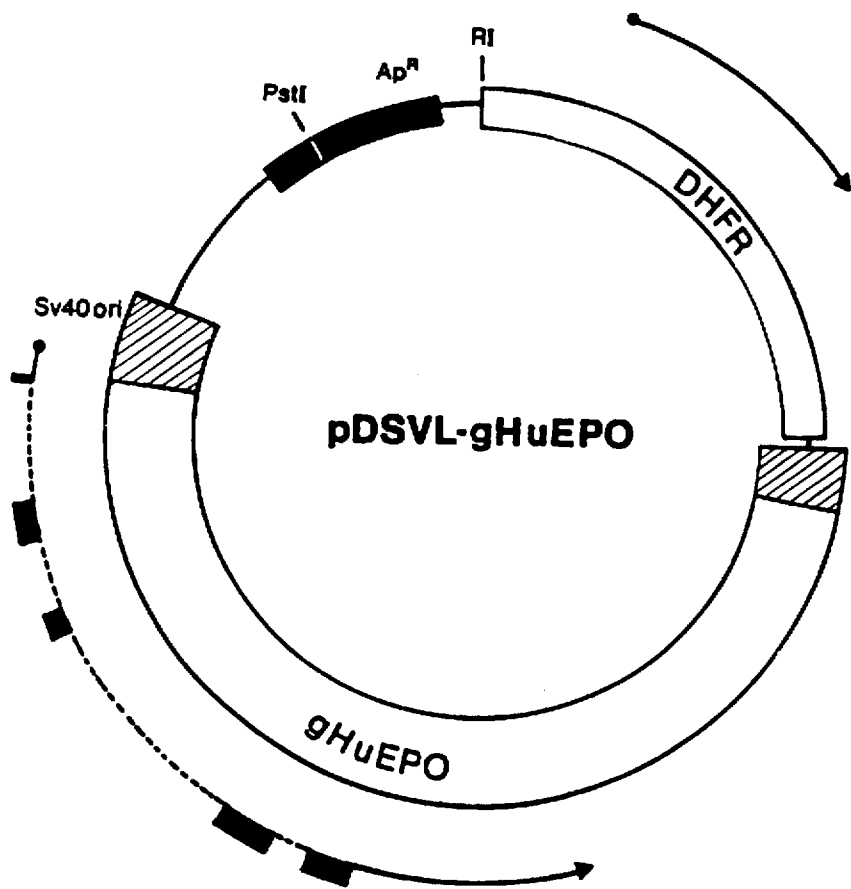


FIG. 5A

Sau3A
GATCCGGCGCCCTTGACAGCGCCCTCTCCTCCAGGCCCGTGGGGCTGGCCCTGCCCC
CGCTGAACCTTCCCGGATGAGGACTCCCGGTGTGGTCAACCGCGCCTAGGTCGCTGAG
-27
Met Gly Val His Glu Cys Pro Ala Trp
GGACCCCGCCAGCGCGGAGATG GGG GTG CAC GAA TGT CCT GCC TGG
-10
Leu Trp Leu Leu Leu Ser Leu Val Ser Leu Pro Leu Gly Leu Pro
CTG TGG CTT CTC CTG TCT TCT CTC GTG TCG CTC CCT CTG GGC CTC CCA
-1 +1
Val Pro Gly Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu
GTC CCG GGC GCC CCA CCA CGC CTC ATC TGT GAC AGC CGA GTC CTG
20
Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Val Thr Met
GAG AGG TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT GTC ACG ATG
30
Gly Cys Ser Glu Ser Cys Ser Leu Asn Glu Asn Ile Thr Val Pro
GGC TGT TCC GAA AGC TGC AGC TTG AAT GAG AAT ATC ACC GTC CCA
40
*

FIG.5B

50 Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly
GAC ACC AAA GTT AAC TTC TAT GCC TGG AAG AGG ATG GAG GTC GGG

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

70

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

*

90 Phe Glu Pro Leu Gln Leu His Met Asp Lys Ala Ile Ser Gly Leu
TTC GAG CCC CTG CAG CTG CAG CTG CAC ATG GAT AAA GCC ATC AGT GGC CTT

100

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

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

130

140 Thr Ala Asp Thr Phe Cys Lys Leu Phe Arg Val Tyr Ser Asn Phe
ACT GCT GAC ACT TTC TGC TGC AAA CTC TTC CGA GTC TAC TCC AAT TTC

FIG. 5C

150 Leu Arg Gly Lys Leu Lys Tyr Thr Gly Glu Ala Cys Arg Arg
CTC CGG GGA AAG CTG AAG CTG TAC ACG GGG GAG GCC TGC AGG AGA
160
Gly Asp Arg OP
GGG GAC AGA TGA CCAGGTGCGTCCAGCTGGGCACATCCACCACCTCCCTCACCAACA
CTGCCCTGTGCCACACCCCTCCCTCACCACTCCCGAACCCCATCGAGGGGCTCTCAGCTAAG
CGCCAGCCTGTCCCATGGACACTCCAGTGCCAGCAATGACATCTCAGGGGCCAGAGGAAC
TGTCAGAGCACAACCTCTGAGATCTAAGGATGTCCGAGGGCCAACTTGAGGGCCCCAGAGC
AGGAAGCATTCAGAGAGCAGCTTTAAACTCAGGAGCAGAGACAAATGCAGGGGAAAACACCT
GAGCTCACTCGGCCACCTGCAAAATTTGATGCAGGACACGCTTTGGAGGCAATTTACCTG
TTTTTGCACCTACCATCAGGGACAGGATGACTGGAGAACTTAGGTGGCAAGCTGTGACTT
CTCAAGGCCCTCAGGGCACTCCCTTGGTGGCAAGAGCCCCCTTGACACTGAGAGAAATATT
TTGCAATCTGCAGCAGGAAAAATTACGGACAGGTTTTGGAGGTTGGAGGGTACTTGACAG
GTGTGTGGGAAGCAGGGCGGTAGGGGTGGAGCTGGGATCGGAGTGAGAACCGTGAAGAC
AGGATGGGGCTGGCCTCTGGTTCTCGTGGGGTCCAAGCTT
HindIII

FIG.6A

AAGCTTCTGGGCTTCCAGACCCAGCTACTTTGCGGAACTCAGCAACCCAGGCATCTCTGAGTCTCCGCCCA
AGACCGGGATGCCCCCAGGGGAGGTGTCCGGGAGCCAGCCTTTCCAGATAGCAGCTCCGCCAGTCCC
AAGGGTGCGCAACCGGCTGCACTCCCCCTCCCGCGACCCAGGGCCCCGGGAGCAGCCCCCATGACCCACAGC
ACGCTGTCAGCAGCCCCGCTCACGCCCCGGCGAGCCTCAACCCAGGCGTCCTGCCCTGCTCTGACCCCCGG
GTGGCCCCCTACCCCTGGCGACCCCTCACGCACACAGCCTCTCTCCCCACCCCCACCCGGCACGCACACATG
CAGATAACAGCCCCGACCCCCGGCCAGAGCCGXAGAGTCCCTGGGGCCACCCCGGCCGCTCGCCTGCCGCTG
CGCCGCACCGCGCTGTCTCTCCCGGAGCCGACCGGGGCCACCGCGCCCGXGCTCTGCTCCGACACCGCGCCC
CTTGGACAGCCGCCCTCTCCTCTAGGCCCGTGGGGCTGGCCCTGCACCGCCGAGCTTCCCCGGGATGAGGXX
-27
-24
Met Gly Val His
ATG GGG GTG CAC G
CCCCGTGACCGGCGGCCCCCAAGTCGCTGAGGGACCCCGGCCAAGCGCGGAG
GTGAGTACTCGCGGGCTGGGCGCTCCCGGCGCCGGGTTCCTGTGTTGAGCGGGGATTTAGCGCCCCCGGCT

FIG.6B

ATTGGCCAAGAGGTGGTGGTTCAAGACCGCGGACTTGTCAAGGACCCCGAAGGGGAGGGGGTGGG

GCAGCTCCACGTGCCGGGGACTTGGGGAGTTCTTGGGGATGGCAAAACCTGGCCTGTTGAGGGGCA

CAGTTTGGGGTTGGGAGGAGGTTTGGGGTTCTGCTGTGCAGTTGTGTGCTGTCAGTGTCTCG[I.S.]

TTGCACACGACAGATCAATAAGCCAGAGGCACACCTGAGTGTGCTTGCATGGTTGGGACAGGAAGGACGAG

CTGGGGCAGAGACGTGGGGATGAAGGAAGCTGTCTTCCACAGCCACCTTCTCCCCCCCCCGCTGACTCT

-23

Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu

AA TGT CCT GCC TGG CTG TGG CTG TGG CTT CTC CTG TCC CTG

CAGCCTGGCTATCTGTTCTAG

-10

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu Ile Cys

CTG TCG CTC CCT CTG GGC CTC CCA GTC CTG GGC GCC CCA CCA CGC CTC ATC TGT

10

Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Ile

GAC AGC CGA GTC CTG GAG AGG TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT ATC

26

Thr

ACG GTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTCAGGGCTTCAGGGAACCTCCTCCAGAT

CCAGGAACCTGGCACTTGTTGGGGTGAGTTGGGAAGCTAGACACTGCCCCCTACATAAGAATAAGTC

FIG. 6D

```

AAAAAGAAAAAGAAAAAATAATGAGGGCTGTATGGAATACATTATTATTCACTCACTCACT
CACTCATTCATTTCATTTCATTCACAAAGTCTTATTGCATACCTTCTGTGTTGCTCAGCTTGGTGCTTGG
GGCTGCTGAGGGGCAGGAGGGGACATGGGTACATGGGTGAGCTCGACTCCAGAGTCCACTCCCTGTAG

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

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

80          *          90

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

100
Arg Ala Leu Gly Ala Gln
CGG GCT CTG GGA GCC CAG GTGAGTAGGAGCGGACACTTCTGCTTGCCCTTCTGTAAAGAGGGGA

GAAGGGTCTTGCTAAGGAGTACAGGAAGTGTCCGTATTCTCCCTTCTGTGGCACTGCAGCGACCTCCT

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

116          120
Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
AAG GAA GCC ATC TCC CCT CCA GAT GCG GCC TCA GCT GCT
GTTTTCTCCTTGGCAG
```

FIG. 6E

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

140

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

160

166 Asp Arg OP
GAC AGA TGA CCAGGTGTGCCACCTGGGCATATCCACCACCTCCCTCACCACATTGCTTGTGCCACA
CCCTCCCGCCACTCCTGAACCCCGTCGAGGGGCTCTCAGCTCAGCGCCAGCCTGTCCCATGGACACTCC
AGTGCCAGCAATGACATCTCAGGGGCCAGAGGAACCTGTCCAGAGAGCAACTCTGAGATCTAAGGATGTCAC
AGGGCCAACTTGAAGGGCCAGAGCAGGAAGCATTCAGAGAGCAGCTTTAACTCAGGGACAGAGCCATGC
TGGGAAGACGCCCTGAGCTCACTCGGCACCCCTGCAAAATTGTATGCCAGGACACGCTTTGGAGGCGATTAC
CTGTTTTTCGCACCTACCATCAGGGACAGGATGACCTGGAGAACTTAGTGGCAAGCTGTGACTTCTCCAGG
TCTCAGGGCATGGGCACTCCCTTGGTGGCAAGAGCCCCCTTGACACCGGGTGGTGGGAACCATGAAGAC
AXGATXGGGGCTGGCCTCTGGCTCTCATGGGGTCCAAAGTTTGTGTATTCTCAACCTATTGACAGACTGAA
ACACAATATGAC

FIG. 7

XbaI

CTAG

-11

MetAla

AAACCATGAGGGTAATAAAA

TAATGGCTCCGCCGCGTCTG

TTTGGTACTCCATTATTTT

ATTACCGAGGCGGCGCAGAC

ATCTGCGACTCGAGAGTTCTGGAACGTTAC

CTGCTGGAAGCTAAAGAAGC

TAGACGCTGAGCTCTCAAGACCTTGCAATG

GACGACCTTCGATTTCTTCG

TGAAAACATCACCCTGGTTGTGCTGAACA

CTGTTCTTTGAACGAAAACA

ACTTTTGTAGTGGTGACCAACACGACTTGT

GACAAGAACTTGCTTTTGT

TTACGGTACCAGACACCAAGGTTAACTTCT

ACGCTTGGAAACGTATGGAA

AATGCCATGCTCTGTGGTCCAATTGAAGA

TGCGAACCTTGCATACCTT

GTTGGTCAACAAGCAGTTGAGTTTGGCAG

GGTCTGGCACTGCTGAGCGA

CAACCAGTTGTTCGTCAACTCAAACCGTC

CCAGACCGTGACGACTCGCT

GGCTGTACTGCGTGGCCAGGCACTGCTGGT

AAACTCCTCTCAGCCGTGGG

CCGACATGACGCACCGGTCCGTGACGACCA

TTTGAGGAGAGTCGGGCACCC

AACCGCTGCA

GCTGCATGTTGACAAAGCAG

TATCTGGCCTGAGATCTCTG

TTGGCGACGT

CGACGTACAACTGTTTTCGTC

ATAGACCGGACTCTAGAGAC

ACTACTCTGCTGCGTGCTCTGGGTGCACAG

AAAGAGGCTATCTCTCCGCC

TGATGAGACGACGCACGAGACCCACGTGTC

TTTCTCCGATAGAGAGGCGG

GGATGCTGCA

TCTGCTGCACCGCTGCGTAC

CATCACTGCTGATACCTTCC

CCTACGACGT

AGACGACGTGGCGACGCATG

GTAGTGACGACTATGGAAGG

GCAAACCTGTT

TCGTGTATACTCTAACTTCC

TGCGTGGTAAACTGAAACTG

CGTTTGACAA

AGCACATATGAGATTGAAGG

ACGCACCATTTGACTTTGAC

SaI

TATACTGGCG

AAGCATGCCGTACTGGTGAC

CGCTAATAG

ATATGACCGCTTCGTACGGCATGACCACTG

GCGATTATCA

GCT

FIG. 8

	-1	+1		
<u>HindIII</u>	ArgAla			
AGCTTGGATA	AAAGAGCTCC	ACCAAGATTG	ATCTGTGACT	CGAGAGTTTT
ACCTAT	TTTCTCGAGG	TGGTTCTAAC	TAGACACTGA	GCTCTCAAAA
GGAAAGATAC	TTGTTGGAAG	CTAAAGAAGC	TGAAAACATC	ACCACTGGTT
CCTTTCTATG	AACAACCTTC	GATTTCTTCG	ACTTTTGTAG	TGGTGACCAA
GTGCTGAACA	CTGTTCTTTG	AACGAAAACA	TTACGGTACC	AGACACCAAG
CACGACTTGT	GACAAGAAAC	TTGCTTTTGT	AATGCCATGG	TCTGTGGTTC
GTTAACTTCT	ACGCTTGGAA	ACGTATGGAA	GTTGGTCAAC	AAGCTGTTGA
CAATTGAAGA	TGCGAACCTT	TGCATACCTT	CAACCAGTTG	TTCGACAAC
AGTTTGGCAA	GGTTTGGCCT	TGTTATCTGA	AGCTGTTTTG	AGAGGTCAAG
TCAAACCGTT	CCAAACCGGA	ACAATAGACT	TCGACAAAAC	TCTCCAGTTC
CCTTGTTGGT	TAACTCTTCT	CAACCATGGG	AACCATTGCA	ATTGCACGTC
GGAACAACCA	ATTGAGAAGA	GTTGGTACCC	TTGGTAACGT	TAACGTGCAG
GATAAAGCCG	TCTCTGGTTT	GAGATCTTTG	ACTACTTTGT	TGAGAGCTTT
CTATTTTCGGC	AGAGACCAAA	CTCTAGAAAC	TGATGAAACA	ACTCTCGAAA
GGGTGCTCAA	AAGGAAGCCA	TTTCCCCACC	AGACGCTGCT	TCTGCCGCTC
CCCACGAGTT	TTCCTTCGGT	AAAGGGGTGG	TCTGCGACGA	AGACGGCGAG
CATTGAGAAC	CATCACTGCT	GATACCTTCA	GAAAGTTATT	CAGAGTTTAC
GTAACCTTTG	GTAGTGACGA	CTATGGAAGT	CTTTCAATAA	GTCTCAAATG
TCCAACCTTCT	TGAGAGGTAA	ATTGAAGTTG	TACACCGGTG	AAGCCTGTAG
AGGTTGAAGA	ACTCTCCATT	TAACTTCAAC	ATGTGGCCAC	TTCGGACATC
AACTGGTGAC	AGATAAGCCC	GACTGATAAC	AACAGTGTAG	
TTGACCACTG	TCTATTCGGG	CTGACTATTG	TTGTCACATC	
	<u>SalI</u>			
ATGTAACAAA	G			
TACATTGTTT	CAGCT			

FIG. 9

	-20	-10	+1	10	20	30	40
Human	MGVHECPAWLWLLLSLSPLGLPVLGAPPRLICDSRVLERYLLEAKEAENITTGCAEHCSLNENITV	PD	TK				
Monkey	MGVHECPAWLWLLLSLSPLGLPVLGAPPRLICDSRVLERYLLEAKEAENITTMGCSESCSLNENITV	PD	TK				
	50	60	70	80	90	100	110
Human	VNFWKRMVEVGQQA	VEVWQGLALLSEAVLRGQALLVNSSQPWEPLQLHV	DKAVSGLRSLTTL	RALGAQKE			
Monkey	VNFWKRMVEVGQQA	VEVWQGLALLSEAVLRGQAVLANSSQPFEPQLHMDKAISGLRSITTL	RALGAQ-E				
	120	130	140	150	160		
Human	AISPPDAASAAPLRTITADTFRKLLFRVYSNFLRGKLLKLYTGEACRTGDR						
Monkey	AISLPDAASAAPLRTITADTFC	KLLFRVYSNFLRGKLLKLYTGEACRRGDR					

FIG. 10

1. AATTCTAGAAACCATGAGGGTAATAAAATA
2. CCATTATTTTATTACCCTCATGGTTTCTAG
3. ATGGCTCCGCCGCGTCTGATCTGCGAC
4. CTCGAGTCGCAGATCAGACGCGGCGGAG
5. TCGAGAGTTCTGGAACGTTACCTGCTG
6. CTTCCAGCAGGTAACGTTCCAGAACT
7. GAAGCTAAAGAAGCTGAAAACATC
8. GTGGTGATGTTTTTCAGCTTCTTTAG
9. ACCACTGGTTGTGCTGAACACTGTTC
10. CAAAGAACAGTGTTTCAGCACAAcca
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTTTCGTT

FIG. 11

XbaI
EcoRI 1 3
AATTCTAG AAACCATGAG GGTAATAAAA TAATGGCTCC GCCGCGTCTG
GATC TTTGGTACTC CCATTATTTT ATTACCGAGG CGGCGCAGAC
2 4

5
ATCTGCGACT CGAGAGTTCT GGAACGTTAC CTGCTGGAAG CTAAAGAAGC
TAGACGCTGA GCTCTCAAGA CCTTGCAATG GACGACCTTC GATTTCTTCG
6

7 9 11
TGAAAACATC ACCACTGGTT GTGCTGAACA CTGTTCTTTG AACGAAAACA
ACTTTTGTAG TGGTGACCAA CACGACTTGT GACAAGAAAC TTGCTTTTGT
8 10

0
KpnI BamHI
TTACGGTACC G
AATGCCATGG CCTAG
12

FIG. 12

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

FIG. 13

ECORI **KpnI**
A **ATT**CGGTACC **AGAC**CCAG **GTTAA**CTTCT **ACG**CTTGGAA **ACG**TATGGAA
 GCCATGG **TCT**GTGGTTC **CAA**TTGAAGA **TGCG**AACTT **TGC**ATACCTT

⁵
 GTTGGTCAAC AAGCAGTTGA AGTTTGGCAG GGTCTGGCAC TGCTGAGCGA
⁶ CAACCCACTTG TTCTGTCAACT TCAAACCGTC CCAGACCGTG ACGACTCGCT
⁷
⁸

GGCTGTACTG CGTGGCCAGG CACTGCTGGT AAACCTCCTCT CAGCCGTGGG
CCGACATGAC GCACCGGTCC GTGACGACCA TTGAGGAGA GTCGGCACCC

<u>13</u>	<u>15</u>	<u>Bgl</u> <u>III</u>	<u>Bam</u> <u>HI</u>
AAACCGCTGCA	GCTGCATGTT	GACAAAGCAG	TATCTGGCCT
TTGGCGACGT	CGACGTACAA	CTGTTTCGTC	GAGATCTG
<u>14</u>	<u>16</u>		
		ATAGACCGGA	CTCTAGACCTAC

FIG. 14

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

TATACTGGCG AAGCATGCCG TACTGGTGAC CGCTAATAG SalI
 ATATGACCGC TTCGTACGGC ATGACCACTG GCGATTATC AGCT
14 16

FIG. 16

1. AATTCAAGCTTGGATAAAAGAGCT
2. GTGGAGCTCTTTTATCCAAGCTTG
3. CCACCAAGATTGATCTGTGACTC
4. TCTCGAGTCACAGATCAATCTTG
5. GAGAGTTTTGGAAAGATACTTGTTG
6. CTTCCAACAAGTATCTTTCCAAAAC
7. GAAGCTAAAGAAGCTGAAAACATC
8. GTGGTGATGTTTTTCAGCTTCTTTAG
9. ACCACTGGTTGTGCTGAACACTGTTC
10. CAAAGAACAGTGTTTCAGCACAACCA
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTTTCGTT

FIG. 17

EcoRI HindIII 1
AATTCA AGCTTGGATA
GT TCGAACCTAT
2

3
AAAGAGCTCC ACCAAGATTG ATCTGTGACT CGAGAGTTTT
TTTCTCGAGG TGGTTCTAAC TAGACACTGA GCTCTCAAAA
4

5 7
GGAAAGATAC TTGTTGGAAG CTAAAGAAGC TGAAAACATC ACCACTGGTT
CCTTTCTATG AACAACTTC GATTCTTTCG ACTTTTGTAG TGGTGACCAA
6 8

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

FIG. 18

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

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FIG. 19

KpnI

EcoRI 1
A ATTCGGTACC AGACACCAAG
GCCATGG TCTGTGGTTC
2

3 5
GTTAACTTCT ACGCTTGGAA ACGTATGGAA GTTGGTCAAC AAGCTGTTGA
CAATTGAAGA TCGGAACCTT TGCATACCTT CAACCAGTTG TTCGACAACT
4 6

7 9
AGTTTGGCAA GGTTTGGCCT TGTTATCTGA AGCTGTTTTG AGAGGTCAAG
TCAAACCGTT CCAAACCGGA ACAATAGACT TCGACAAAAC TCTCCAGTTC
8 10

11 13
CCTTGTTGGT TAACTCTTCT CAACCATGGG AACCATTGCA ATTGCACGTC
GGAACAACCA ATTGAGAAGA GTTGGTACCC TTGGTAACGT TAACGTGCAG
12 14

15 BglIII BamHI
GATAAAGCCG TCTCTGGTTT GAGATCTG
CTATTTCGGC AGAGACCAAA CTCTAGACCTA G
16

FIG. 20

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

FIG. 21

BamHI

BglII

1

GATC

CAGATCTTTG

ACTACTTTGT

TGAGAGCTTT

GTCTAGAAAC

TGATGAAACA

ACTCTCGAAA

2

3

GGGTGCTCAA

AAGGAAGCCA

TTTCCCCACC

AGACGCTGCT

TCTGCCGCTC

CCCACGAGTT

TTCCTTCGGT

AAAGGGGTGG

TCTGCGACGA

AGACGGCGAG

4

5

6

7

CATTGASAAC

CATCACTGCT

GATACCTTCA

GAAAGTTATT

CAGAGTTTAC

GTAACCTCTT

GTAGTGACGA

CTATGGAAGT

CTTTCAATAA

GTCTCAAATG

8

9

10

11

12

13

TCCAACCTTCT

TGAGAGGTAA

ATTGAAGTTG

TACACCGGTG

AAGCCTGTAG

AGGTTGAAGA

ACTCTCCATT

TAACCTCAAC

ATGTGGCCAC

TTCGGACATC

14

15

16

17

AACTGGTGAC

AGATAAGCCC

GACTGATAAC

AACAGTGTAG

TTGACCACTG

TCTATTCGGG

CTGACTATTG

TTGTCACATC

19

SalI

ATGTAACAAA

G

TACATTGTTT

CAGCT

20

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PRODUCTION OF ERYTHROPOIETIN

This is a continuation of my U.S. patent application Ser. No. 07/113,179 filed Oct. 23, 1987 and issued as U.S. Pat. No. 5,441,868 on Aug. 15, 1995, which was a continuation of U.S. patent application Ser. No. 06/675,298, Nov. 30, 1984, and issued as U.S. Pat. No. 4,703,008 on Oct. 27, 1987, which was a continuation-in-part of U.S. patent application Ser. No. 06/655,841, filed Sep. 28, 1984, now abandoned, which was a continuation-in-part of U.S. patent application Ser. No. 06/582,185, filed Feb. 21, 1984, now abandoned, and which was a continuation-in-part of U.S. patent application Ser. No. 06/561,024, filed Dec. 13, 1983, now abandoned.

BACKGROUND

The present invention relates generally to the manipulation of genetic materials and, more particularly, to recombinant procedures making possible the production of polypeptides possessing part or all of the primary structural conformation and/or one or more of the biological properties of naturally-occurring erythropoietin.

A. Manipulation Of Genetic Materials

Genetic materials may be broadly defined as those chemical substances which program for and guide the manufacture of constituents of cells and viruses and direct the responses of cells and viruses. A long chain polymeric substance known as deoxyribonucleic acid (DNA) comprises the genetic material of all living cells and viruses except for certain viruses which are programmed by ribonucleic acids (RNA). The repeating units in DNA polymers are four different nucleotides, each of which consists of either a purine (adenine or guanine) or a pyrimidine (thymine or cytosine) bound to a deoxyribose sugar to which a phosphate group is attached. Attachment of nucleotides in linear polymeric form is by means of fusion of the 5' phosphate of one nucleotide to the 3' hydroxyl group of another. Functional DNA occurs in the form of stable double stranded associations of single strands of nucleotides (known as deoxyoligonucleotides), which associations occur by means of hydrogen bonding between purine and pyrimidine bases [i.e., "complementary" associations existing either between adenine (A) and thymine (T) or guanine (G) and cytosine (C)]. By convention, nucleotides are referred to by the names of their constituent purine or pyrimidine bases, and the complementary associations of nucleotides in double stranded DNA (i.e., A-T and G-C) are referred to as "base pairs". Ribonucleic acid is a polynucleotide comprising adenine, guanine, cytosine and uracil (U), rather than thymine, bound to ribose and a phosphate group.

Most briefly put, the programming function of DNA is generally effected through a process wherein specific DNA nucleotide sequences (genes) are "transcribed" into relatively unstable messenger RNA (mRNA) polymers. The mRNA, in turn, serves as a template for the formation of structural, regulatory and catalytic proteins from amino acids. This mRNA "translation" process involves the operations of small RNA strands (tRNA) which transport and align individual amino acids along the mRNA strand to allow for formation of polypeptides in proper amino acid sequences. The mRNA "message", derived from DNA and providing the basis for the tRNA supply and orientation of any given one of the twenty amino acids for polypeptide "expression", is in the form of triplet "codons"—sequential groupings of three nucleotide bases. In one sense, the formation of a protein is the ultimate form of "expression" of the programmed genetic message provided by the nucleotide sequence of a gene.

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"Promoter" DNA sequences usually "precede" a gene in a DNA polymer and provide a site for initiation of the transcription into mRNA. "Regulator" DNA sequences, also usually "upstream" of (i.e., preceding) a gene in a given DNA polymer, bind proteins that determine the frequency (or rate) of transcriptional initiation. Collectively referred to as "promoter/regulator" or "control" DNA sequence, these sequences which precede a selected gene (or series of genes) in a functional DNA polymer cooperate to determine whether the transcription (and eventual expression) of a gene will occur. DNA sequences which "follow" a gene in a DNA polymer and provide a signal for termination of the transcription into mRNA are referred to as transcription "terminator" sequences.

A focus of microbiological processing for the last decade has been the attempt to manufacture industrially and pharmaceutically significant substances using organisms which either do not initially have genetically coded information concerning the desired product included in their DNA, or (in the case of mammalian cells in culture) do not ordinarily express a chromosomal gene at appreciable levels. Simply put, a gene that specifies the structure of a desired polypeptide product is either isolated from a "donor" organism or chemically synthesized and then stably introduced into another organism which is preferably a self-replicating unicellular organism such as bacteria, yeast or mammalian cells in culture. Once this is done, the existing machinery for gene expression in the "transformed" or "transfected" microbial host cells operates to construct the desired product, using the exogenous DNA as a template for transcription of mRNA which is then translated into a continuous sequence of amino acid residues.

The art is rich in patent and literature publications relating to "recombinant DNA" methodologies for the isolation, synthesis, purification and amplification of genetic materials for use in the transformation of selected host organisms. U.S. Pat. No. 4,237,224 to Cohen, et al., for example, relates to transformation of unicellular host organisms with "hybrid" viral or circular plasmid DNA which includes selected exogenous DNA sequences. The procedures of the Cohen, et al. patent first involve manufacture of a transformation vector by enzymatically cleaving viral or circular plasmid DNA to form linear DNA strands. Selected foreign ("exogenous" or "heterologous") DNA strands usually including sequences coding for desired product are prepared in linear form through use of similar enzymes. The linear viral or plasmid DNA is incubated with the foreign DNA in the presence of ligating enzymes capable of effecting a restoration process and "hybrid" vectors are formed which include the selected exogenous DNA segment "spliced" into the viral or circular DNA plasmid.

Transformation of compatible unicellular host organisms with the hybrid vector results in the formation of multiple copies of the exogenous DNA in the host cell population. In some instances, the desired result is simply the amplification of the foreign DNA and the "product" harvested is DNA. More frequently, the goal of transformation is the expression by the host cells of the exogenous DNA in the form of large scale synthesis of isolatable quantities of commercially significant protein or polypeptide fragments coded for by the foreign DNA. See also, e.g., U.S. Pat. Nos. 4,264,731 (to Shine), 4,273,875 (to Manis), 4,293,652 (to Cohen), and European Patent Application 093,619, published Nov. 9, 1983.

The development of specific DNA sequences for splicing into DNA vectors is accomplished by a variety of techniques, depending to a great deal on the degree of

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"foreignness" of the "donor" to the projected host and the size of the polypeptide to be expressed in the host. At the risk of over-simplification, it can be stated that three alternative principal methods can be employed: (1) the "isolation" of double-stranded DNA sequence from the genomic DNA of the donor; (2) the chemical manufacture of a DNA sequence providing a code for a polypeptide of interest; and (3) the in vitro synthesis of a double-stranded DNA sequence by enzymatic "reverse transcription" of mRNA isolated from donor cells. The last-mentioned methods which involve formation of a DNA "complement" of mRNA are generally referred to as "cDNA" methods.

Manufacture of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. DNA manufacturing procedures of co-owned, co-pending U.S. patent application Ser. No. 483,451, by Alton, et al., (filed Apr. 15, 1983 and corresponding to PCT US83/00605, published Nov. 24, 1983 as W083/04053), for example, provide a superior means for accomplishing such highly desirable results as: providing for the presence of alternate codons commonly found in genes which are highly expressed in the host organism selected for expression (e.g., providing yeast or *E.coli* "preference" codons); avoiding the presence of untranslated "intron" sequences (commonly present in mammalian genomic DNA sequences and mRNA transcripts thereof) which are not readily processed by procaryotic host cells; avoiding expression of undesired "leader" polypeptide sequences commonly coded for by genomic DNA and cDNA sequences but frequently not readily cleaved from the polypeptide of interest by bacterial or yeast host cells; providing for ready insertion of the DNA in convenient expression vectors in association with desired promoter/regulator and terminator sequences; and providing for ready construction of genes coding for polypeptide fragments and analogs of the desired polypeptides.

When the entire sequence of amino acid residues of the desired polypeptide is not known, direct manufacture of DNA sequences is not possible and isolation of DNA sequences coding for the polypeptide by a cDNA method becomes the method of choice despite the potential drawbacks in ease of assembly of expression vectors capable of providing high levels of microbial expression referred to above. Among the standard procedures for isolating cDNA sequences of interest is the preparation of plasmid-borne cDNA "libraries" derived from reverse transcription of mRNA abundant in donor cells selected as responsible for high level expression of genes (e.g., libraries of cDNA derived from pituitary cells which express relatively large quantities of growth hormone products). Where substantial portions of the polypeptide's amino acid sequence are known, labelled, single stranded DNA probe sequences duplicating a sequence putatively present in the "target" cDNA may be employed in DNA/DNA hybridization procedures carried out on cloned copies of the cDNA which have been denatured to single stranded form. [See, generally, the disclosure and discussions of the art provided in U.S. Pat. No. 4,394,443 to Weissman, et al. and the recent demonstrations of the use of long oligonucleotide hybridization probes reported in Wallace, et al., *Nuc.Acids Res.*, 6, pp. 3543-3557 (1979), and Reyes, et al., *P.N.A.S. (U.S.A.)*, 79, pp. 3270-3274 (1982), and Jaye, et al., *Nuc.Acids Res.*, 11, pp. 2325-2335 (1983). See also, U.S. Pat. No. 4,358,535 to Falkow, et al., relating to DNA/DNA hybridization procedures in effecting diagnosis; published European Patent Application Nos. 0070685 and 0070687 relating to light-emitting labels on single stranded polynucleotide probes;

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Davis, et al., "A Manual for Genetic Engineering, Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1980) at pp. 55-58 and 174-176, relating to colony and plaque hybridization techniques; and, New England Nuclear (Boston, Mass.) brochures for "Gene Screen" Hybridization Transfer Membrane materials providing instruction manuals for the transfer and hybridization of DNA and RNA, Catalog No. NEF-972.]

Among the more significant recent advances in hybridization procedures for the screening of recombinant clones is the use of labelled mixed synthetic oligonucleotide probes, each of which is potentially the complete complement of a specific DNA sequence in the hybridization sample including a heterogenous mixture of single stranded DNAs or RNAs. These procedures are acknowledged to be especially useful in the detection of cDNA clones derived from sources which provide extremely low amounts of mRNA sequences for the polypeptide of interest. Briefly put, use of stringent hybridization conditions directed toward avoidance of non-specific binding can allow, e.g., for the autoradiographic visualization of a specific cDNA clone upon the event of hybridization of the target DNA to that single probe within the mixture which is its complete complement. See generally, Wallace, et al., *Nuc.Acids Res.*, 9, pp. 879-897 (1981); Suggs, et al., *P.N.A.S. (U.S.A.)*, 78, pp. 6613-6617 (1981); Choo, et al., *Nature*, 299, pp. 178-180 (1982); Kurachi, et al., *P.N.A.S. (U.S.A.)*, 79, pp. 6461-6464 (1982); Ohkubo, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 2196-2200 (1983); and Kornblihtt, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 3218-3222 (1983). In general, the mixed probe procedures of Wallace, et al. (1981), supra, have been expanded upon by various workers to the point where reliable results have reportedly been obtained in a cDNA clone isolation using a 32-member mixed "pool" of 16-base-long (16-mer) oligonucleotide probes of uniformly, varying DNA sequences together with a single 11-mer to effect a two-site "positive" confirmation of the presence of cDNA of interest. See, Singer-Sam, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 802-806 (1983).

The use of genomic DNA isolates is the least common of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures. This is especially true in the area of recombinant procedures directed to securing microbial expression of mammalian polypeptides and is due, principally to the complexity of mammalian genomic DNA. Thus, while reliable procedures exist for developing phage-borne libraries of genomic DNA of human and other mammalian species origins [See, e.g., Lawn, et al., *Cell*, 15, pp. 1157-1174 (1978) relating to procedures for generating a human genomic library commonly referred to as the "Maniatis Library"; Karn, et al., *P.N.A.S. (U.S.A.)*, 77, pp. 5172-5176 (1980) relating to a human genomic library based on alternative restriction endonuclease fragmentation procedure; and Blattner, et al., *Science*, 196, pp. 161-169 (1977) describing construction of a bovine genomic library] there have been relatively few successful attempts at use of hybridization procedures in isolating genomic DNA in the absence of extensive foreknowledge of amino acid or DNA sequences. As one example, Fiddes, et al., *J.Mol. and App.Genetics*, 1, pp. 3-18 (1981) report the successful isolation of a gene coding for the alpha subunit of human pituitary glycoprotein hormones from the Maniatis Library through use of a "full length" probe including a complete 621 base pair fragment of a previously-isolated cDNA sequence for the alpha subunit. As another example, Das, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 1531-1535 (1983) report isolation of human genomic clones for human HLA-DR using a 175 base pair synthetic oligo-

nucleotide. Finally, Anderson, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 6838-6842 (1983) report the isolation of genomic clone for bovine pancreatic trypsin inhibitor (BPTI) using a single probe 86 base pairs in length and constructed according to the known amino acid sequence of BPTI. The authors note a determination of poor prospects for isolating mRNA suitable for synthesis of a cDNA library due to apparent 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 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 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, 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 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 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 by the gene sought.

B. Erythropoietin As A Polypeptide Of Interest

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

ing the conversion of primitive precursor cells in the bone marrow into proerythroblasts 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.*, 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., *Exp.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., *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 process of red blood cell formation, the hormone has potential useful application in both the diagnosis and the 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 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 corrective 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 availability of erythropoietin in quantity would allow for 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 provided a basis for projection of efficacy of erythropoietin 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.Gynecol.*, 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).

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 extracts containing erythropoietin.

U.S. Pat. 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.

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

Another method of purifying human erythropoietin from urine of patients with aplastic anemia is described in Miyake, et al., *J.Biol.Chem.*, Vol. 252, No. 15 (Aug. 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. Pat. 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. Pat. 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); Hagiwara, 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. 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 hybrid-

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ization 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 of the preparation and use of a monoclonal, antierythropoietin 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. Pat. 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 erythropoietin, a substance for which no substantial amino acid sequence information has been published. In co-owned, co-pending U.S. patent application Ser. No. 463,724, filed Feb. 4, 1983, by J. Egrie, published Aug. 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-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 immunoas-

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says 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 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 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 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 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 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 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 Ser. Nos. 561,024 and 582,185, there has appeared a single report of the cloning and expression of what is asserted to have been human erythropoietin cDNA in *E.coli*. Briefly put, a number

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of cDNA clones were inserted into *E.coli* plasmids and β -lactamase fusion products were noted to be immunoreactive with a monoclonal antibody to an unspecified "epitope" of human erythropoietin. See, Lee-Huang, *Proc. Nat. Acad. Sci. (USA)*, 81, pp. 2708-2712 (1984).

BRIEF SUMMARY

The present invention provides, for the first time, novel purified and isolated polypeptide products 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., *Saccharomyces 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 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.

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 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 immunological 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 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 conformation of erythropoietins of monkey and human species origins.

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Also provided by the present invention are novel biologically functional viral and circular plasmid DNA vectors incorporating DNA sequences of the invention and 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 transfected microbial hosts under conditions facilitative of large scale expression of the exogenous, vector-borne DNA sequences 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 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 FIGS. 5 and 6 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 coded for by portions of the DNA complement to the top strand human genomic DNA sequence of FIG. 6 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

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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}I) to provide reagents useful in detection and quantification of 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 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

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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.

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.

Reference is made to FIGS. 1 through 21, wherein: FIG. 1 is a graphic representation of a radioimmunoassay analysis of products of the invention; FIG. 2 shows vector pDSVL-MkE.

FIG. 3 shows vector pSVgHuEPO.

FIG. 4 shows vector pDSVL-gHuEPO.

FIG. 5A, 5B and 5C (collectively referred to as FIG. 5) show the sequence of monkey EPO cDNA and the encoded EPO.

FIGS. 6A, 6B, 6C, 6D and 6E (collectively referred to as FIG. 6) show the sequence of human genomic EPO DNA and the encoded EPO.

FIG. 7 shows the sequence of the ECEPO gene.

FIG. 8 shows the sequence of the SCEPO gene.

FIG. 9 shows a comparison of the human and monkey EPO polypeptides.

FIG. 10 shows the ECEPO section 1 oligonucleotides.

FIG. 11 shows section 1 of the ECEPO gene.

FIG. 12 shows the ECEPO section 2 oligonucleotides.

FIG. 13 shows section 2 of the ECEPO gene.

FIG. 14 shows the ECEPO section 3 oligonucleotides.

FIG. 15 shows section 3 of the ECEPO gene.

FIG. 16 shows the SCEPO section 1 oligonucleotides.

FIG. 17 shows section 1 of the SCEPO gene.

FIG. 18 shows the SCEPO section 2 oligonucleotides.

FIG. 19 shows section 2 of the SCEPO gene.

FIG. 20 shows the SCEPO section 3 oligonucleotides.

FIG. 21 shows the section 3 of the SCEPO gene.

DETAILED DESCRIPTION

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 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.

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 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 sequence information provided by enzymatic fragmentation and sequencing a small sample of human EPO.

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

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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 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.

EXAMPLE 1

A. Human EPO Fragment Amino Acid Sequencing

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

Fragments were arbitrarily assigned numbers and were analyzed for amino acid sequence by microsequence 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 determined.

TABLE I

Fragment No.	Sequence Analysis Result
T4a	A-P-P-R
T4b	G-K-L-K
T9	A-I-G-A-Q-K
T13	V-L-E-R
T16	A-V-S-G-L-R
T18	L-F-R
T21	K-L-F-R
T25	Y-I-L-E-A-K
T26a	L-I-C-D-S-R
T26b	L-Y-T-G-E-A-C-R
T27	T-I-T-A-D-T-F-R
T28	E-A-I-S-P-D-A-A-M-A-A-P-L-R
T30	E-A-E-X-I-T-T-G-X-A-E-H-X-S-L-N-E-X-I-T-V-P
T31	V-Y-S-N-F-L-R
T33	S-L-T-T-L-L-R
T35	V-N-F-Y-A-W-K
T38	G-Q-A-L-L-V-X-S-S-Q-P-W-E-P-L-Q-L-H-V-D-K

B. Design and Construction of Oligonucleotide Probe Mixtures

The amino acid sequences set out in Table I were reviewed in the context of the degeneracy of the genetic code for the purpose of ascertaining whether mixed probe procedures could be applied to DNA/DNA hybridization procedures on cDNA and/or genomic DNA libraries. This analysis revealed that within Fragment No. T35 there existed a series of 7 amino acid residues (Val-Asn-Phe-Tyr-Ala-Trp-Lys) which could be uniquely characterized as encoded for by one of 128 possible DNA sequences spanning 20 base pairs. A first set of 128 20-mer oligonucleotides was therefore synthesized by standard phosphoramidite methods (See, e.g., Beaucage, et al., *Tetrahedron Letters*, 22, pp. 1859–1862 (1981) on a solid support according to the sequence set out in Table II, below.

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TABLE II

Residue	Val	Asn	Phe	Tyr	Ala	Trp	Lys
3'	CAA T G C	TTG A	AAG A	ATG A	CGA T G C	ACC	TT - 5'

Further analysis revealed that within fragment No. T38 there existed a series of 6 amino acid residues (Gln-Pro-Trp-Glu-Pro-Leu) on the basis of which there could be prepared a pool of 128 mixed oligonucleotide 17-mer probes as set out in Table III, below.

TABLE III

Residue	Gln	Pro	Trp	Glu	Pro	Leu
3'	GTT C	GGA T G C	ACC	CTT C	GGA T G C	GA - 5'

Oligonucleotide probes were labelled at the 5' end with gamma-³²P-ATP, 7500–8000 Ci/mmol (ICN) using T₄ polynucleotide kinase (NEN).

EXAMPLE 2

A. Monkey Treatment Procedures Female Cynomolgus monkeys *Macaca fascicularis* (2.5–3 kg, 1.5–2 years old) were treated subcutaneously with a pH 7.0 solution of phenylhydrazine hydrochloride at a dosage level of 12.5 mg/kg on days 1, 3 and 5. The hematocrit was monitored prior to each injection. On day 7, or whenever the hematocrit level fell below 25% of the initial level, serum and kidneys were harvested after administration of 25 mg/kg doses of ketamine hydrochloride. Harvested materials were immediately frozen in liquid nitrogen and stored at –70° C.

B. RIA for EPO

Radioimmunoassay procedures applied for quantitative detection of EPO in samples were conducted according to the following procedures:

An erythropoietin standard or unknown sample was incubated together with antiserum for two hours at 37° C. After the two hour incubation, the sample tubes were cooled on ice, ¹²⁵I-labelled erythropoietin was added, and the tubes were incubated at 0° C. for at least 15 more hours. Each assay tube contained 500 µl of incubation mixture consisting of 50 µl of diluted immune sera, 10,000 cpm of ¹²⁵I-erythropoietin, 5 µl trasylol and 0–250 µl of either EPO standard or unknown sample, with PBS containing 0.1% BSA making up the remaining volume. The antiserum used was the second test bleed of a rabbit immunized with a 1% pure preparation of human urinary erythropoietin. The final antiserum dilution on the assay was adjusted so that the antibody-bound ¹²⁵I-EPO did not exceed 10–20% of the input total counts. In general, this corresponded to a final antiserum dilution of from 1:50,000 to 1:100,000.

The antibody-bound ¹²⁵I-erythropoietin was precipitated by the addition of 150 µl Staph A. After a 40 min. incubation, the samples were centrifuged and the pellets were washed two times with 0.75 ml 10 mM Tris-HCl pH 8.2 containing 0.15M NaCl, 2 mM EDTA, and 0.05% Triton X-100. The washed pellets were counted in a gamma counter to determine the percent of ¹²⁵I-erythropoietin bound. Counts bound

by pre-immune sera were subtracted from all final values to correct for nonspecific precipitation. The erythropoietin content of the unknown samples was determined by comparison to the standard curve.

The above procedure was applied to monkey serum obtained in Part A, above, as well as to the untreated monkey serum. Normal serum levels were assayed to contain approximately 36 mU/ml while treated monkey serum contained from 1000 to 1700 mU/ml.

EXAMPLE 3

A. Monkey cDNA Library Construction

Messenger RNA was isolated from normal and anemic monkey kidneys by the guanidinium thiocyanate procedure of Chirgwin, et al., *Biochemistry*, 18, p. 5294 (1979) and poly (A)⁺mRNA was purified by two runs of oligo(dT)-cellulose column chromatography as described at pp. 197-198 in Maniatis, et al., "Molecular Cloning, A Laboratory Manual" (Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y., 1982). The cDNA library was constructed according to a modification of the general procedures of Okayama, et al., *Mol. and Cell.Biol.*, 2, pp. 161-170 (1982). The key features of the presently preferred procedures were as follows: (1) pUC8 was used as the sole vector, cut with PstI and then tailed with oligo dT of 60-80 bases in length; (2) HincII digestion was used to remove the oligo dT tail from one end of the vector; (3) first strand synthesis and oligo dG tailing was carried out according to the published procedure; (4) BamHI digestion was employed to remove the oligo dG tail from one end of the vector; and (5) replacement of the RNA strand by DNA was in the presence of two linkers (GATCTAAAGACCGTCCCCCCCC and ACGGTCTTTA) in a three-fold molar excess over the oligo dG tailed vector.

B. Colony Hybridization Procedures For Screening Monkey cDNA Library

Transformed *E.coli* were spread out at a density of 9000 colonies per 10x10 cm plate on nutrient plates containing 50 micrograms/ml Ampicillin. GeneScreen filters (New England Nuclear Catalog No. NEF-972) were pre-wet on a BHI-CAM plate (Bacto brain heart infusion 37 g/L, Casamino acids 2 g/L and agar 15 g/L, containing 500 micrograms/ml Chloramphenicol) and were used to lift the colonies off the plate. The colonies were grown in the same medium for 12 hours or longer to amplify the plasmid copy numbers. The amplified colonies (colony side up) were treated by serially placing the filters over 2 pieces of Whatman 3 MM paper saturated with each of the following solutions:

- (1) 50 mM glucose—25 mM Tris-HCl (pH 8.0)—10 mM EDTA (pH 8.0) for five minutes;
- (2) 0.5M NaOH for ten minutes; and
- (3) 1.0M Tris-HCl (pH 7.5) for three minutes.

The filters were then air dried in a vacuum over at 80° C. for two hours.

The filters were then subjected to Proteinase K digestion through treatment with a solution containing 50 micrograms/ml of the protease enzyme in Buffer K [0.1M Tris-HCl (pH 8.0)—0.15M NaCl—10 mM EDTA (pH 8.2)—0.2% SDS]. Specifically, 5 ml of the solution was added to each filter and the digestion was allowed to proceed at 55° C. for 30 minutes, after which the solution was removed.

The filters were then treated with 4 ml of a prehybridization buffer (5xSSPE—0.5% SDS—100 micrograms/ml SS *E.coli* DNA—5xBFP). The prehybridization treatment was carried out at 55° C., generally for 4 hours or longer, after which the prehybridization buffer was removed.

The hybridization process was carried out in the following manner. To each filter was added 3 ml of hybridization buffer (5xSSPE—0.5% SDS—100 micrograms/ml yeast tRNA) containing 0.025 picomoles of each of the 128 probe sequences of Table II (the total mixture being designated the EPV mixture) and the filters were maintained at 48° C. for 20 hours. This temperature was 2° C. less than the lowest of the calculated dissociation temperatures (Td) determined for any of the probes.

Following hybridization, the filters were washed three times for ten minutes on a shaker with 6xSSC—0.1% SDS at room temperature and washed two to three times with 6xSSC—1% SOS at the hybridization temperature (48° C.).

Autoradiography of the filters revealed seven positive clones among the 200,000 colonies screened.

Initial sequence analysis of one of the putative monkey cDNA clones (designated clone 83 deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. under deposit accession No. A.T.C.C. 67545 on Oct. 20, 1987.) was performed for verification purposes by a modification of the procedure of Wallace, et al., *Gene*, 16, pp. 21-26 (1981). Briefly, plasmid DNA from monkey cDNA clone 83 was linearized by digestion with EcoRI and denatured by heating in a boiling water bath. The nucleotide sequence was determined by the dideoxy method of Sanger, et al., *P.N.A.S.(U.S.A.)*, 74, pp. 5463-5467 (1977). A subset of the EPV mixture of probes consisting of 16 sequences was used as a primer for the sequencing reactions.

C. Monkey EPO cDNA Sequencing

Nucleotide sequence analysis of clone 83 was carried out by the procedures of Messing, *Methods in Enzymology*, 101, pp. 20-78 (1983). Set out in Table IV is a preliminary restriction map analysis of the approximately 1600 base pair EcoRI/HindIII cloned fragment of clone 83. Approximate locations of restriction endonuclease enzyme recognition sites are provided in terms of number of bases 3' to the EcoRI site at the 5' end of the fragment. Nucleotide sequencing was carried out by sequencing individual restriction fragments with the intent of matching overlapping fragments. For example, an overlap of sequence information provided by analysis of nucleotides in a restriction fragment designated C113 (Sau3A at ~111/SmaI at ~324) and the reverse order sequencing of a fragment designated C73 (AluI at ~424/BstEII at ~203).

TABLE IV

Restriction Enzyme Recognition Site	Approximate Location(s)
EcoRI	1
Sau3A	111
SmaI	180
BstEII	203
SmaI	324
KpnI	371
RsaI	372
AluI	424
PstI	426
AluI	430
HpaI	466
AluI	546
PstI	601
PvuII	604
AluI	605
AluI	782
AluI	788
RsaI	792
PstI	807
AluI	841
AluI	927

TABLE IV-continued

Restriction Enzyme Recognition Site	Approximate Location(s)
NcoI	946
Sau3A	1014
AluI	1072
AluI	1115
AluI	1223
PstI	1301
RsaI	1343
AluI	1384
HindIII	1449
AluI	1450
HindIII	1585

Sequencing of approximately 1342 base pairs (within the region spanning the Sau3A site 3' to the EcoRI site and the HindIII site) and analysis of all possible reading frames has allowed for the development of DNA and amino acid sequence information set out in FIG. 5, comprising portions 5A, 5B and 5C. In the FIG., the putative initial amino acid residue of the amino terminal of mature EPO (as verified by correlation to the previously mentioned sequence analysis of twenty amino terminal residues) is designated by the numeral +1. The presence of a methionine-specifying ATG codon (designated -27) "upstream" of the initial amino terminal alanine residue as the first residue designated for the amino acid sequence of the mature protein is indicative of the likelihood that EPO is initially expressed in the cytoplasm in a precursor form including a 27 amino acid "leader" region which is excised prior to entry of mature EPO into circulation. Potential glycosylation sites within the polypeptide are designated by asterisks. The estimated molecular weight of the translated region was determine to be 21,117 daltons and the M.W. of the 165 residues of the polypeptide constituting mature monkey EPO was determined to be 18,236 daltons.

The polypeptide sequence of FIG. 5 may readily be subjected to analysis for the presence of highly hydrophilic regions and/or secondary conformational characteristics indicative of potentially highly immunogenic regions by, e.g., the methods of Hopp, et al., *P.N.A.S.(U.S.A.)*, 78, pp. 3824-3828 (1981) and Kyte et al., *J.Mol.Biol.*, 157, pp. 105-132 (1982) and/or Chou, et al., *Biochem.*, 13, pp. 222-245 (1974) and *Advances in Enzymology*, 47, pp. 45-47 (1978). Computer-assisted analysis according to the Hopp, et al. method is available by means of a program designated PEP Reference Section 6.7 made available by Intelligenetics, Inc., 124 University Avenue, Palo Alto, Calif.

EXAMPLE 4

A. Human Genomic Library A Ch4A phage-borne human fetal liver genomic library prepared according to the procedures of Lawn, et al., *Cell*, supra (1979) was obtained and maintained for use in a plaque hybridization assay.

B. Plaque Hybridization Procedures for Screening Human Genomic Library

Phage particles were lysed and the DNAs were fixed on filters (50,000 plaques per filter) according to the procedures of Woo, *Methods In Enzymology*, 68, pp. 389-395 (1979) except for the use of GeneScreen Plus filters (New England Nuclear Catalog No. NEF-972) and NZYAM plates (NaCl, 5 g; MgCl₂—6H₂O, 2 g; NZ-Amine A, 10g; yeast extract, 5 g; casamino acids, 2 g; maltose; 2 g; and agar, 15 g per liter).

The air-dried filters were baked at 80° C. for 1 hour and then digested with Proteinase K as described in Example 3,

Part B. Prehybridization was carried out with a 1M NaCl—1% SDS buffer for 55° C. for 4 hours or more, after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Part B. Both the mixture of 128 20-mer probes designated EPV and the mixture of 128 17-mer probes of Table III (designated the EPQ mixture) were employed. Hybridization was carried out at 48° C. using the EPV probe mixture. EPQ probe mixture hybridization was carried out at 46° C.—4 degrees below the lowest calculated Td for members of the mixture. Removal of the hybridized probe for rehybridization was accomplished by boiling with 1×SSC—0.1% SDS for two minutes. Autoradiography of the filters revealed three positive clones (reactive with both probe mixtures) among the 1,500,000 phage plaques screened. Verification of the positive clones as being EPO-encoding was obtained through DNA sequencing and electron micrographic visualization of heteroduplex formation with the monkey cDNA of Example 3. This procedure also gave evidence of multiple introns in the genomic DNA sequence.

EXAMPLE 5

Nucleotide sequence analysis of one of the positive clones (designated λhE1), deposited with the American Type Culture Collection, 12301 Parklawn drive, Rockville, Md. under deposit accession No. A.T.C.C. 40381 on Oct. 20, 1987, was carried out and results obtained to date are set out in FIG. 6, comprising portions 6A, 6B, 6C, 6D and 6E.

In FIG. 6 Table VI, the initial continuous DNA sequence designates a top strand of 620 bases in what is apparently an untranslated sequence immediately preceding a translated portion of the human EPO gene. More specifically, the sequence appears to comprise the 5' end of the gene which leads up to a translated DNA region coding for the first four amino acids (-27 through -24) of a leader sequence ("presequence"). Four base pairs in the sequence prior to that encoding the beginning of the leader have not yet been unambiguously determined and are therefore designated by an "X". There then follows an intron of about 639 base pairs (439 base pairs of which have been sequenced and the remaining 200 base pairs of which are designated "LS.") and immediately preceding a codon for glutamic acid which has been designated as residue -23 of the translated polypeptide. The exon sequence immediately following is seen to code for amino acid residues through an alanine residue (designated as the +1 residue of the amino acid sequence of mature human EPO) to the codon specifying threonine at position +26, whereupon there follows a second intron consisting of 256 bases as specifically designated. Following this intron is an exon sequence for amino acid residues 27 through 55 and thereafter a third intron comprising 612 base pairs commences. The subsequent exon codes for residues 56 through 115 of human EPO and there then commences a fourth intron of 134 bases as specified. Following the fourth intron is an exon coding for residue Nos. 116 through 166 and a "stop" codon (TGA). Finally, FIG. 6 identifies a sequence of 568 base pairs in what appears to be an untranslated 3' region of the human EPO gene, two base pairs of which ("X") have not yet been unambiguously sequenced.

FIG. 6 thus serves to identify the primary structural conformation (amino acid sequence) of mature human EPO as including 166 specified amino acid residues (estimated M.W.=18,399). Also revealed in the FIG. is the DNA sequence coding for a 27 residue leader sequence along with 5' and 3' DNA sequences which may be significant to

promoter/operator functions of the human gene operon. Sites for potential glycosylation of the mature human EPO polypeptide are designated in the FIG. by asterisks. It is worthy of note that the specific amino acid sequence of FIG. 6 likely constitutes that of a naturally occurring allelic form of human erythropoietin. Support for this position is found in the results of continued efforts at sequencing of urinary isolates of human erythropoietin which provided the finding that a significant number of erythropoietin molecules therein have a methionine at residue 126 as opposed to a serine as shown in the FIG.

FIG. 9 below, illustrates the extent of polypeptide sequence homology between human and monkey EPO. In the upper continuous line of the FIG. single letter designations are employed to represent the deduced translated polypeptide sequences of human EPO commencing with residue -27 and the lower continuous line shows the deduced polypeptide sequence of monkey EPO commencing at assigned residue number -27. Asterisks are employed to highlight the sequence homologies. It should be noted that the deduced human and monkey EPO sequences reveal an "additional" lysine (K) residue at (human) position 116. Cross-reference to FIG. 6 indicates that this residue is at the margin of a putative mRNA splice junction in the genomic sequence. Presence of the lysine residue in the human polypeptide sequence was further verified by sequencing of a cDNA human sequence clone prepared from mRNA isolated from COS-1 cells transformed with the human genomic DNA in Example 7, infra.

EXAMPLE 6

The expression system selected for initial attempts at microbial synthesis of isolatable quantities of EPO polypeptide material coded for by the monkey cDNA provided by the procedures of Example 3 was one involving mammalian host cells (i.e., COS-1 cells, A.T.C.C. No. CRL-1650). The cells were transfected with a "shuttle" vector capable of autonomous replication in *E.coli* host (by virtue of the presence of pBR322-derived DNA) and the mammalian hosts (by virtue of the presence of SV40 virus-derived DNA).

More specifically, an expression vector was constructed according to the following procedures. The plasmid clone 83 provided in Example 3 was amplified in *E.coli* and the approximately 1.4 kb monkey EPO-encoding DNA was isolated by EcoRI and HindIII digestion. Separately isolated was an approximately 4.0 kb, HindIII/SalI fragment from pBR322. An approximately 30 bp, EcoRI/SalI "linker" fragment was obtained from M13mp10 RF DNA (P and L Laboratories). This linker included, in series, an EcoRI sticky end, followed by SstI, SmaI, BamHI and XbaI recognition sites and a SalI sticky end. The above three fragments were ligated to provide an approximately 5.4 kb intermediate plasmid ("pERS") wherein the EPO DNA was flanked on one side by a "bank" of useful restriction endonuclease recognition sites. pERS was then digested with HindIII and SalI to yield the EPO DNA and the EcoRI to SalI (M13mp10) linker. The 1.4 kb fragment was ligated with an approximately 4.0 kb BamHI/SalI of pBR322 and another M13mp10 HindIII/BamHI RF fragment linker also having approximately 30 bp. The M13 linker fragment was characterized by a HindIII sticky end, followed by PstI, SalI, XbaI recognition sites and a BamHI sticky end. The ligation product was, again, a useful intermediate plasmid ("pBR-EPO") including the EPO DNA flanked on both sides by banks of restriction site.

The vector chosen for expression of the EPO DNA in COS-1 cells ("pDSVL1") had previously been constructed

to allow for selection and autonomous replication in *E.coli*. These characteristics are provided by the origin of replication and Ampicillin resistance gene DNA sequences present in the region spanning nucleotides 2448 through 4362 of pBR322. This sequence was structurally modified by the addition of a linker providing a HindIII recognition immediately adjacent nucleotide 2448 prior to incorporation into the vector. Among the selected vector's other useful properties was the capacity to autonomously replicate in COS-1 cells and the presence of a viral promoter sequence functional in mammalian cells. These characteristics are provided by the origin of replication DNA sequence and "late gene" viral promoter DNA sequence present in the 342 bp sequence spanning nucleotide numbers 5171 through 270 of the SV40 genome. A unique restriction site (BamHI) was provided in the vector and immediately adjacent the viral promoter sequence through use of a commercially available linker sequence (Collaborative Research). Also incorporated in the vector was a 237 base pair sequence (derived as nucleotide numbers 2553 through 2770 of SV40) containing the "late gene" viral mRNA polyadenylation signal (commonly referred to as a transcription terminator). This fragment was positioned in the vector in the proper orientation vis-a-vis the "late gene" viral promoter via the unique BamHI site. Also present in the vector was another mammalian gene at a location not material to potential transcription of a gene inserted at the unique BamHI site, between the viral promoter and terminator sequences. [The mammalian gene comprised an approximately 2,500 bp mouse dihydrofolate reductase (DHFR) minigené isolated from plasmid pMG-1 as in Gasser, et al., *P.N.A.S.(U.S.A.)*, 79, pp. 6522-6526, (1982).] Again, the major operative components of plasmid pDSVL1 comprise nucleotides 2448 through 4362 of pBR322 along with nucleotides 5171 through 270 (342 bp) and 2553 through 2770 (237 bp) of SV40 DNA.

Following procedures described, e.g., in Maniatis, et al., supra, the EPO-encoding DNA was isolated from plasmid pBR-EPO as a BamHI fragment and ligated into plasmid pDSVL1 cut with BamHI. Restriction enzyme analysis was employed to confirm insertion of the EPO gene in the correct orientation in two of the resulting cloned vectors (duplicate vectors H and L). See FIG. 2, illustrating plasmid pDSVL-MkE. Vectors with EPO genes in the wrong orientation (vectors F, X and Ce) were saved for use as negative controls in transfection experiments designed to determine EPO expression levels in hosts transformed with vectors having EPO DNA in the correct orientation.

Vectors H, L, F, X and G were combined with carrier DNA (mouse liver and spleen DNA) were employed to transfect duplicate 60 mm plates by calcium phosphate microprecipitate methods. Duplicate 60 mm plates were also transfected with carrier DNA as a "mock" transformation negative control. After five days all culture media were tested for the presence of polypeptides possessing the immunological properties of naturally-occurring EPO.

EXAMPLE 7

A. Initial EPO Expression System Involving COS-1 Cells
The system selected for initial attempts at microbial synthesis of isolatable quantities of human EPO polypeptide material coded for by the human genomic DNA EPO clone, also involved expression in mammalian host cells (i.e., COS-1 cells, A.T.C.C. No. CRL-1650). The human EPO gene was first sub-cloned into a "shuttle" vector which is capable of autonomous replication in both *E.coli* hosts (by virtue of the presence of pBR322 derived DNA) and in the mammalian cell line COS-1 (by virtue of the presence of

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SV40 virus derived DNA). The shuttle vector, containing the EPO gene, was then transfected into COS-1 cells. EPO polypeptide material was produced in the transfected cells and secreted into the cell culture media.

More specifically, an expression vector was constructed according to the following procedures. DNA isolated from lambda clone λ HE1, containing the human genomic EPO gene, was digested with BamHI and HindIII restriction endonucleases, and a 5.6 Kb DNA fragment known to contain the entire EPO gene was isolated. This fragment was mixed and ligated with the bacterial plasmid pUC8 (Bethesda Research Laboratories, Inc.) which had been similarly digested, creating the intermediate plasmid "pUC8-HuE", providing a convenient source of this restriction fragment.

The vector chosen for expression of the EPO DNA in COS-1 cells (pSV4SEt) had previously been constructed. Plasmid pSV4SEt contained DNA sequences allowing selection and autonomous replication in *E. coli*. These characteristics are provided by the origin of replication and Ampicillin resistance gene DNA sequences present in the region spanning nucleotides 2448 through 4362 of the bacterial plasmid pBR322. This sequence was structurally modified by the addition of a linker providing a HindIII recognition site immediately adjacent to nucleotide 2448. Plasmid pSV4SEt was also capable of autonomous replication in COS-1 cells. This characteristic was provided by a 342 bp fragment containing the SV40 virus origin of replication (nucleotide numbers 5171 through 270). This fragment had been modified by the addition of a linker providing an EcoRI recognition site adjacent to nucleotide 270 and a linker providing a SalI recognition site adjacent nucleotide 5171. A 1061 bp fragment of SV40 was also present in this vector (nucleotide numbers 1711 through 2772 plus a linker providing a SalI recognition site next to nucleotide number 2772). Within this fragment was a unique BamHI recognition sequence. In summary, plasmid pSV4SEt contained unique BamHI and HindIII recognition sites, allowing insertion of the human EPO gene, sequences allowing replication and selection in *E. coli*, and sequences allowing replication in COS-1 cells.

In order to insert the EPO gene into pSV4SEt, plasmid pUC8-HuE was digested with BamHI and HindIII restriction endonucleases and the 5.6 kb EPO encoding DNA fragment isolated. pSV4SEt was also digested with BamHI and HindIII and the major 2513 bp fragment isolated (preserving all necessary functions). These fragments were mixed and ligated, creating the final vector "pSVgHuEPO". (See, FIG. 3.) This vector was propagated in *E. coli* and vector DNA isolated. Restriction enzyme analysis was employed to confirm insertion of the EPO gene.

Plasmid pSVgHuEPO DNA was used to express human EPO polypeptide material in COS-1 cells. More specifically, pSVgHuEPO DNA was combined with carrier DNA and transfected into triplicate 60 mm plates of COS-1 cells. As a control, carrier DNA alone was also transfected into COS-1 cells. Cell culture media were sampled five and seven days later and tested 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

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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 pre-peptide 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 (CHO) DHFR- cells and the selectable marker, DHFR. [For discussion of related expression systems, see U.S. Pat. No. 4,399,216 and European Patent Applications 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 and 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 resistant 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 DHFR 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 CHO 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 pOSVL-gHuEPO was subjected to a series of increasing MTX concentrations of 30 nM, 50 nM, 100 nM, 200 nM, 1 μ M, and 5 μ M MTX. A representative 3-day culture media sample from the 100 nM MTX step contained human EPO at 3089 \pm 129 u/ml as judged by RIA. Representative 48 hour cultural medium samples from the 100 nM and 1 μ M MTX steps contained, respectively, human EPO at 466 and 1352 U/ml as judged by RIA (average of triplicate assays). In these procedures, 1 \times 10⁶ 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 μ M MTX, respectively, provided actual yields of 2335 U/plate and 6750 U/plate. The average cell numbers per plate were 1.94 \times 10⁶ and 3.12 \times 10⁶ cells, respectively. The effective production rates for these culture conditions were thus 1264 and 2167 U/10⁶ 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 homogeneous 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", Jun. 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 cm² 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/cm², each 850 cm² 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/10⁶ cells/48 hours.

Culture fluids from cell strain CHO 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 membrane 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 (C₄) 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 recombinant 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 encod-

ing 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 below 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. Moreover, 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 isolating the section as an XbaI/KpnI fragment from RF DNA generated in *E.coli*, likely due to methylation of the KpnI recognition site bases within the host. Single-stranded phage DNA was therefore isolated and rendered into double-stranded form in vitro by primer extension and the desired double-stranded fragment was thereafter readily isolated.

ECEPO gene Sections 2 and 3 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 MD. 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 C₁₈₅₇ 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 [Asn², des-Pro² through Ile⁶]hEPO and [His⁷]hEPO, as described below.

A. [Asn², des-Pro² through Ile⁶]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 Asp⁸ through the second base of the Arg¹⁰ 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 plasmidborne DNA sequence encoding *E.coli* expression of the Met⁻¹ form of the desired analog.

B. [His⁷]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. 10 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/gIII, 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 copending U.S. patent application Ser. No. 487,753, filed Apr. 22, 1983, by Grant A. Bitter, published Oct. 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 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/Sall digestion of plasmid p α C3. From the resulting plasmid p α C3/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_{1857} gene. Cultures of cells in LB broth (Ampicillin 50 μ g/ml and kanamycin 5 μ g/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 24,000xg 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 Ala¹]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.

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 [Asn², des-Pro² through Ile⁶]hEPO expression products were approximately 11,000 U/mg and 6,000 U/mg protein, respectively, while the assay values for [His⁷]hEPO were about 41,000 U/mg and 14,000 U/mg protein, respectively, indicating that the analog products were from one-fourth to one-tenth as "active" as the "parent" expression product in the assays.

In the expression system designed for use of *S. cerevisiae* host cells, plasmid pYE/SCEPO was transformed into two

different strains, YSDP4 (genotype α pep4-3 trp1) and RK81 (genotype α 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 μ g/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 μ g/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 p α C3 and pYE in HB101 *E. coli* cells were deposited in accordance with the Rules of Practice of the U.S. Patent Office on Sep. 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. 39932, 39934, and 39933, respectively. *Saccharomyces cerevisiae* strains YSDP4 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 heretofore attributed in vivo to EPO, e.g., stimulation of reticulocyte response, development of ferrokinetic effects (such as plasma iron turnover effects and marrow transit time effects), erythrocyte mass changes, stimulation of hemoglobin C synthesis (see, Eschbach, et al., supra) and, as indicated in Example 10, increasing hematocrit levels in mammals. Included within the class of humans treatable with products of the invention are patients generally requiring blood transfusions and including trauma victims, surgical patients, renal disease patients including dialysis patients, and patients with a variety of blood composition affecting disorders, such as hemophilia, sickle cell disease, physiologic anemias, and the like. The minimization of the need for transfusion therapy through use of EPO therapy can be expected to result in reduced transmission of infectious agents. Products of the invention, by virtue of their production by recombinant methods, are expected to be free of pyrogens, natural inhibitory substances, and the like, and are thus likely to provide enhanced overall effectiveness in therapeutic processes vis-a-vis naturally derived products. Erythropoietin therapy with products of the present invention is also expected to be useful in the enhancement of

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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) pg/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 testosterone, 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); Paviovic-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, Jul. 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 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 mate-

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rials 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 strong positive response to hEPO 144-166, as measured by capacity of rabbit serum antibodies to immunoprecipitate ¹²⁵I-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 FIG. 5 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 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 FIGS. 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 micro-

bial 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 [Asn², des-Pro² through Ile⁶]hEPO, [des-Thr¹⁶³ through Arg¹⁶⁶]hEPO and "Δ27-55hEPO", the latter having the residues coded for by an entire exon deleted; or which are more stable to hydrolysis (and, therefore, may have more pronounced or longer lasting effects than naturally-occurring EPO); or which have been altered to delete one or more potential sites for glycosylation (which may result in higher activities for yeast-produced products); or which have one or more cysteine residues deleted or replaced by, e.g., histidine or serine residues (such as the analog [His⁷]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 [Phe¹⁵]hEPO, [Phe⁴⁹]hEPO, and [Phe¹⁴⁵]hEPO) and may bind more or less readily to EPO receptors on target cells. Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within mature EPO, which fragments may possess one activity of EPO (e.g., receptor binding) and not others (e.g., erythropoietic activity). Especially significant in this regard are those potential fragments of EPO which are elucidated upon consideration of the human genomic DNA sequence of FIG. 6, i.e., "fragments" of the total continuous EPO sequence which are delineated by intron sequences and which may constitute distinct "domains" of biological activity. It is noteworthy that the absence of in vivo activity for any one or more of the "EPO products" of the invention is not wholly preclusive of therapeutic utility (see, Weiland, et al., supra) or of utility in other contexts, such as in EPO assays or EPO antagonism. Antagonists of erythropoietin may be quite useful in treatment of polycythemias or cases of overproduction of EPO [see, e.g., Adamson, *Hosp.Practice*, 18(12), 49-57 (1983), and Hellmann, et al., *Clin.Lab.Haemat.*, 5, 335-342 (1983)].

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

lian 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 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 mammlian 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. 15 161-165 (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 nylonbased filters such as Gene-Screen 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 abundance 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 10⁶ 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 10⁶ cells in 48 hours.

3. Vertebrate cells according to claim 1 capable of producing in excess of 1000 U erythropoietin per 10⁶ 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 10⁶ 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 10⁶ cells in 48 hours.

6. Vertebrate cells according to claim 4 capable of producing in excess of 1000 U erythropoietin per 10⁶ 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.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,756,349
DATED : May 26, 1998
INVENTOR(S) : Fu-Kuen Lin

Page 1 of 4

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,

Delete entire Section [63] and substitute

--[63] Continuation of Ser. No. 113,179, Oct. 23, 1987, Pat. No. 5,441,868, which is a continuation of Ser. No. 675,298, Nov. 30, 1984, Pat. No. 4,703,008, which is a continuation-in-part of Ser. No. 655,841, Sept. 28, 1984, abandoned, which is a continuation-in-part of Ser. No. 582,185, Feb. 21, 1984, abandoned, which is a continuation-in-part of Ser. No. 561,024, Dec. 13, 1983, abandoned . --

Column 1,

Page 4, line 59, delete "Homone" and substitute -- Hormone --.

Column 1,

Page 8, line 15, delete "Chines" and substitute -- Chinese --.

Column 2,

Page 9, line 37, delete "expressin" and substitute -- expression --.

Fig. 13,

Line 7, delete CAACCACTTG and substitute -- CAACCAGTTG --.

Column 1,

Line 3, after the word "my" insert -- co-pending --.

Line 52, delete "atranscribed" and substitute -- transcribed --.

Column 3,

Line 30, delete "cONA" and substitute -- cDNA --.

Line 48, delete "cONA" and substitute -- cDNA --.

Column 4,

Line 33, delete "cONA" and substitute -- cDNA --.

Column 5,

Line 11, delete "mixedsequence" and substitute -- mixed sequence --.

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Column 9,

Line 26, delete "cONA" and substitute -- cDNA --.

Column 13,

Line 26, delete "genoimic" and substitute -- genomic --.

Column 16,

Line 29, after the word "Procedures" insert -- . --.

Column 18,

Line 13, delete "SOS" and substitute -- SDS --.

Line 20, after "1987" delete -- . --.

Line 17, delete "83deposited" and substitute -- 83 deposited --.

Column 19,

Line 53, after the word "Library", insert -- . -- .

Line 55, delete "(1979)".

Column 20,

Line 26, delete "drive" and substitute -- Drive --.

Line 30, delete "Table VI".

Column 21,

Line 40, delete "virusderived" and substitute -- virus derived --.

Column 22,

Line 44, delete "Ce" and substitute -- G --.

Column 23,

Line 28, delete "fraoment" and substitute -- fragment --.

Line 48, delete "liaated" and substitute -- ligated --.

Column 24,

Line 16, delete "SAII" and substitute -- SaII --.

Column 26,

Line 30, delete "pOSVL-" and substitute -- pDSVL- --.

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Page 3 of 4

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Column 27,

Line 52, delete "membranc" and substitute -- membrane --.

Column 29,

Line 42, delete "amolified" and substitute -- amplified --.

Line 63, delete "MD." and substitute -- No. --.

Column 30,

Lines 21-23, delete

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

and substitute

-- 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' --.

Line 30, delete "plasmidborne" and substitute -- plasmid-borne --.

Lines 36-39, delete

"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
 TAC GTA CTG AGCT-5' "

and substitute

-- XbaI +1 2 3 4 5 6 7 8 9 XhoI
 Met Ala Pro Pro 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' --

Line 46, delete "10" and substitute -- 16 -- .

Line 48, after "20" insert --) --.

Line 50, after "21" insert --) --.

Line 57, delete "KpnI/gII" and substitute -- KpnI/BglII --.

Line 66, delete "reaion" and substitute -- region --.

Column 32,

Line 22, delete "μMW1" and substitute -- pMW1 --.

UNITED STATES PATENT AND TRADEMARK OFFICE
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Page 4 of 4

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Column 33,

Line 14, delete "pg/kg" and substitute -- $\mu\text{g/kg}$ --.

Line 28, delete "Paviovic" and substitute -- Pavlovic --.

Line 56, delete "unlablled" and substitute -- unlabeled --.

Column 34,

Line 26, delete "resopnse" and substitute -- response --.

Line 42, delete "y" and substitute -- γ --.

Column 36,

Line 45, delete "cONA" and substitute -- cDNA --.

Column 37,

Line 10, delete "15".

Line 19, delete "nylonbase" and substitute -- nylon base --.

Signed and Sealed this

Thirteenth day of November, 2001

Attest:

Nicholas P. Godici

Attesting Officer

NICHOLAS P. GODICI
Acting Director of the United States Patent and Trademark Office

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Line 26, delete "genoimic" and substitute -- genomic --.

Column 16,

Line 29, after the word "Procedures" insert -- . --.

Column 18,

Line 13, delete "SOS" and substitute -- SDS --.

Line 20, after "1987" delete -- . --.

Line 17, delete "83deposited" and substitute -- 83 deposited --.

Column 19,

Line 53, after the word "Library", insert -- . -- .

Line 55, delete "(1979)".

Column 20,

Line 26, delete "drive" and substitute -- Drive --.

Line 30, delete "Table VI".

Column 21,

Line 40, delete "virusderived" and substitute -- virus derived --.

Column 22,

Line 44, delete "Ce" and substitute -- G --.

Column 23,

Line 28, delete "fraoment" and substitute -- fragment --.

Line 48, delete "liaated" and substitute -- ligated --.

Column 24,

Line 16, delete "SAII" and substitute -- SalI --.

Column 26,

Line 30, delete "pOSVL-" and substitute -- pDSVL- --.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,756,349
 DATED : May 26, 1998
 INVENTOR(S) : Fu-Kuen Lin

Page 3 of 4

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 27,

Line 52, delete "membranc" and substitute -- membrane --.

Column 29,

Line 42, delete "amolified" and substitute -- amplified --.

Line 63, delete "MD." and substitute -- No. --.

Column 30,

Lines 21-23, delete

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

and substitute

-- 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' --.

Line 30, delete "plasmidborne" and substitute -- plasmid-borne --.

Lines 36-39, delete

"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
 TAC GTA CTG AGCT-5' "

and substitute

-- XbaI +1 2 3 4 5 6 7 8 9 XhoI
 Met Ala Pro Pro 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' --

Line 46, delete "10" and substitute -- 16 -- .

Line 48, after "20" insert --) --.

Line 50, after "21" insert --) --.

Line 57, delete "KpnI/gIII" and substitute -- KpnI/BglII --.

Line 66, delete "reaion" and substitute -- region --.

Column 32,

Line 22, delete "μMW1" and substitute -- pMW1 --.

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Page 4 of 4

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 33,

Line 14, delete "pg/kg" and substitute -- $\mu\text{g/kg}$ --.

Line 28, delete "Paviovic" and substitute -- Pavlovic --.

Line 56, delete "unlablled" and substitute -- unlabeled --.

Column 34,

Line 26, delete "resopnse" and substitute -- response --.

Line 42, delete "y" and substitute -- γ --.

Column 36,

Line 45, delete "cONA" and substitute -- cDNA --.

Column 37,

Line 10, delete "15".

Line 19, delete "nylonbase" and substitute -- nylon base --.

Signed and Sealed this

Twenty-seventh Day of November, 2001

Attest:

Nicholas P. Godici

Attesting Officer

NICHOLAS P. GODICI
Acting Director of the United States Patent and Trademark Office