

## **EXHIBIT 3**

## Erythropoietin: Gene Cloning, Protein Structure, and Biological Properties

J.K. BROWNE, A.M. COHEN, J.C. EGRIE, P.H. LAI, F.-K. LIN,  
T. STRICKLAND, E. WATSON, AND N. STEBBING  
*Amgen, Thousand Oaks, California 91320*

The successful cloning of the gene for human erythropoietin (EPO) (Jacobs et al. 1985; Lin et al. 1985) has yielded information on the genetic organization and protein structure of this hormone and has allowed assessment of its biological properties. Biological studies have clearly indicated the clinical potential for this hormone in treatment of various anemias, and initial clinical studies of recombinant-DNA-produced human EPO (r-hEPO)<sup>1</sup> are now under way.

EPO, a sialylglycoprotein hormone, is responsible for regulating the rate of red blood cell formation and for maintaining the red blood cell mass (Krantz and Jacobson 1970; Graber and Krantz 1978; Spivak and Graber 1980). EPO is produced primarily by the kidney in adults and by the liver during fetal life and is secreted into the circulation (Jacobsen et al. 1957; Fried 1972; Zanjani et al. 1981). Circulating levels of EPO are approximately 20 mU/ml (Koeffler and Goldwasser 1981; Cotes 1982; Garcia et al. 1982). Serum levels of EPO increase under conditions of tissue hypoxia and decrease under conditions of hyperoxia. The kidney responds to anemia by increasing the rate of EPO production, resulting in an increase of as much as 100-fold in serum EPO levels (Eschbach and Adamson 1985). Damage to the kidney, as found in chronic renal failure, results in anemia primarily due to a deficiency in EPO production (Brown 1965; Naets 1975; Erslev et al. 1980). Although postulated at the turn of the century (Carnat and Defandre 1906), EPO was first partially purified in 1971 from anemic sheep plasma (Goldwasser and Kung 1971). Human EPO was first purified to homogeneity in 1977 from the urine of aplastic anemia patients (Miyake et al. 1977). Purified human urinary EPO has an apparent molecular weight of about 34,000 and can be separated into two forms, termed  $\alpha$  and  $\beta$ , which differ in their carbohydrate content (Dordal et al. 1985). A specific activity of 70,000 U/mg has been reported for purified human urinary EPO (Miyake et al. 1977).

Isolation of a gene for human EPO proved to be particularly problematical because there was no known source of mRNA. No cell lines had been characterized

that produced significant amounts of EPO that could provide enriched sources of mRNA. Moreover, although the kidney had been identified as the probable site of synthesis, there remained considerable uncertainty as to whether induction of EPO represented de novo synthesis, release, or activation of an inactive precursor (Fyhrquist et al. 1984). In addition, there was no reliable, convenient assay that could be used to screen cell lines or clones rapidly in an expression system. Thus, the overall cloning strategy was based on obtaining some amino acid sequence information from the very limited amount of purified human urinary EPO that was available. Mixed DNA probes, based on all possible coding sequences, could then be used to screen a human genomic library, and candidate DNA would be expressed in mammalian cells. A cDNA clone, isolated separately, would also be required to unambiguously assign intron/exon boundaries.

Due to the lack of a suitable source of human tissue mRNA, a heterologous approach was employed in which mixed oligonucleotide probes, based on human EPO amino acid sequence information, were used to identify EPO mRNA isolated from various tissues from normal animals or animals made anemic experimentally. This approach required that the amino acid sequence be essentially identical over the region for which the oligonucleotide probes were constructed. For this reason, primates, namely, cynomolgus monkeys, were used because of their relatively close relationship to man.

Gas-phase microsequencing of a small amount of purified human urinary EPO yielded some definitive amino acid assignments but also some uncertainties in the first amino-terminal 23 positions. Due to the redundancy in the genetic code and uncertainties in the amino acid sequence, oligonucleotide probes corresponding to this region proved not to be useful for isolating the EPO gene. Amino acid sequences from internal peptide regions with lower genetic code redundancy were obtained by trypsin digestion of EPO. The amino-terminal sequence did allow, however, preparation of a monoclonal antibody directed against a synthetic peptide from this region (Egrie 1983), and this material, together with antibodies against intact EPO, proved useful in the overall cloning strategy. Because of the heterogeneous nature of urinary EPO preparations, it seemed that either there was considerable secondary

<sup>1</sup>r-hEPO is being jointly developed by Amgen (Thousand Oaks, California), Cilag (Schaffhausen, Switzerland), Kirin Brewery Co., Ltd. (Tokyo, Japan), and Ortho Pharmaceutical Corp. (Raritan, New Jersey).

modification of the protein or the material was heterogeneous in amino acid sequence and was perhaps coded by a gene family. Thus, considerable effort was directed toward characterization of urinary EPO with the realization that, at the very least, it could confirm primary structure features predictable from the coding gene sequence(s). As it transpired, almost complete characterization of the primary structure of human urinary EPO was achieved by direct analysis within the time taken to characterize the genes (Lai et al. 1986). The direct structural analyses proved important in resolving various structural features of this hormone. EPO proved to be a posttranslationally modified product of a single-copy gene that is highly conserved within the mammals.

#### EXPERIMENTAL PROCEDURES

**Isolation of EPO.** Human EPO was purified from the urine of aplastic anemia patients as described previously (Miyake et al. 1977). r-hEPO was produced by Chinese hamster ovary (CHO) cells stably transformed with the human gene (Lin et al. 1985) and purified to homogeneity from conditioned culture media.

**Isolation and analyses of gene clones.** Cloning of the human and cynomolgus monkey EPO genes, RNA isolation, cDNA cloning, and Northern and Southern blot analyses have been described recently (Lin et al. 1985, 1986). Nucleic acid sequence analysis was carried out primarily by the dideoxy method (Sanger et al. 1977), with a few regions sequenced by the chemical cleavage method (Maxam and Gilbert 1980).

**DNA-mediated gene transfer.** DNA-mediated gene transfer was carried out using the calcium phosphate microprecipitation method (Graham and van der Eb 1973) as modified by Wigler et al. (1978). For gene transfer into 293 cells (Graham et al. 1977), cells were treated for 4 hours with the calcium-DNA precipitate. COS-1 cells (Gluzman 1981) (ATCC no. CRL 1650) were incubated for 16 hours with the precipitate. Media were sampled for EPO 3-7 days posttransfection.

**Amino acid sequence analysis.** The sequencing of human EPO was described by Lai et al. (1986).

**EPO assays.** A radioimmunoassay (RIA) for EPO using purified urinary <sup>125</sup>I-labeled EPO and rabbit sera raised against a partially purified preparation of urinary EPO was described recently by Egrie et al. (1986). RIAs using recombinant reagents were performed as described by J.C. Egrie et al. (in prep.). The in vivo exhypoxic polycythemic mouse bioassay for EPO (Cotes and Bangham 1961) and the in vitro rat bone-marrow assay (Goldwasser et al. 1975) were used to assess the biological activity of various EPO preparations. Western blot analysis of EPO, which used a mouse monoclonal antibody raised to a synthetic peptide corresponding to the amino terminus of EPO (Egrie 1983), was described previously (Egrie et al. 1985, 1986).

**Physical analyses of EPO.** The carbohydrate composition of r-hEPO was determined by methanolysis, trifluoroacetylation, and separation by gas chromatography (Zanetta et al. 1972). Endoglycosidase F (New England Nuclear), neuraminidase, and O-glycanase (Genzyme) digestions were performed according to the manufacturers procedures. Analytical ultracentrifuge analysis, dry-weight determination, and measurement of the partial specific volume of EPO were performed as described by J. Davis et al. (in prep.).

**Treatment of dogs with r-hEPO.** Young adult male and female beagles (six of each sex per group) were dosed intravenously via the cephalic vein for 3 weeks, three times per week, with purified r-hEPO. The animals received either excipient control or a dose of 280 or 2800 U/kg EPO. Blood samples were taken 1 week prior to the study and weekly thereafter.

**Pharmacokinetic studies.** Pharmacokinetic analysis of r-hEPO in rats will be described in detail separately (A.C. Cohen et al., in prep.). CD rats (Charles Rivers Breeding Laboratories) had a polyethylene cannula implanted into the left carotid artery and a silastic cannula implanted into the right jugular vein 3 days prior to the study. To determine the effect of renal failure on the pharmacokinetics of EPO, both renal pedicles were ligated in one group of animals on the day of the study. r-hEPO was metabolically labeled with [<sup>35</sup>S]methionine and cysteine and purified. <sup>35</sup>S-labeled r-hEPO was administered via the jugular cannula at 1 μCi (370 units r-hEPO) per kilogram of body weight. Blood samples were collected periodically via the carotid cannula, and ethanol precipitable counts were determined. The best-fit lines were determined by a Gauss-Newton curve-fitting algorithm, and pharmacokinetic parameters were determined by standard methods.

**Subtotal nephrectomized animal study.** The system developed by Anagnostou et al. (1977) was used to study r-hEPO in an animal model of renal failure. One group of rats were subjected to a two-step surgical removal of all of one kidney and one half of the second kidney. Sham-operated controls were subjected to concurrent laparotomy. Starting 1 week after surgery, the two groups of animals were injected intramuscularly with 0.1 ml of saline control solution or 10 units of r-hEPO five times a week for 2 weeks. Body weight, hematocrit, and plasma urea nitrogen were determined at the beginning and end of the study (A.C. Cohen, in prep.).

**Inflammatory disease animal model.** The anemia of adjuvant inflammation was induced according to the procedure of Lukens et al. (1967). Rats were given a single injection of Freund's complete adjuvant in the left hind footpad. Ten days later, treatments were begun with 0.1-ml intraperitoneal injections of either saline (control solution) or 40 units of r-hEPO. After 10 days of treatment, blood samples were obtained for determination of hematocrit, red cell mass, hemoglobin

concentration, plasma iron, and iron-binding capacity. Similar determinations were made on nonadjuvant treated rats concurrently given injections of either saline or r-hEPO.

## RESULTS

### Cloning of the Human EPO Gene

The strategy employed to isolate a human EPO genomic gene clone and a cynomolgus monkey cDNA is outlined in Figure 1. A human EPO gene clone was isolated from a  $\lambda$  bacteriophage-borne human genomic library using oligonucleotide probes (Lin et al. 1985). Two sets of mixed oligonucleotide probes were used, each containing a pool of 128 sequences: One pool was a mixture of 20-nucleotide-long oligonucleotides containing all possible coding sequences for an internal hexapeptide, and the other pool was a mixture of 17-nucleotide-long oligonucleotides directed against the coding sequence for a heptapeptide. Using probes directed to two nonoverlapping regions of the EPO gene allowed rapid confirmation of putative clones, eliminating a great number of the false positives obtained with either probe alone.

DNA sequence analysis demonstrated that three of four independent genomic clones had sequences corresponding to the known EPO tryptic peptide amino acid sequences. Proof that a clone contained a com-

plete functional gene encoding human EPO was obtained by its expression in mammalian cells, to produce a gene product with the immunological and biological properties of EPO. A 5.4-kb *Bam*HI-*Hind*III restriction fragment from clone  $\lambda$ HE1 was identified as potentially carrying the entire EPO gene by Southern blot analysis (Southern 1975) using mixed nucleotide probes. This fragment was subcloned into the plasmid pUC8 (Vieira and Messing 1982) and transiently transfected into 293 cells. These cells are a human embryonic kidney line stably transformed with adenovirus type 5 (Ad5). 293 cells constitutively express the Ad5 EIA gene products that act as *trans*-acting enhancers of expression of DNA introduced into these cells by transfection.

RIA analysis demonstrated the presence of EPO in culture medium samples from transfected cells; the expressed EPO produced a dose-response curve identical to that of urinary EPO. Medium samples from cultures transfected with the plasmid lacking the EPO gene insert were uniformly negative in the RIA. The *Bam*HI-*Hind*III fragment was inserted into a shuttle vector, containing the SV40 origin of replication, and transiently transfected into COS-1 cells. RIA analysis again demonstrated that EPO was secreted into the culture media; this material was also shown to be biologically active as determined by the *in vitro* rat bone-marrow assay and the *in vivo* exhypoxic polycythemic mouse bioassay. Control cultures were uniformly negative in

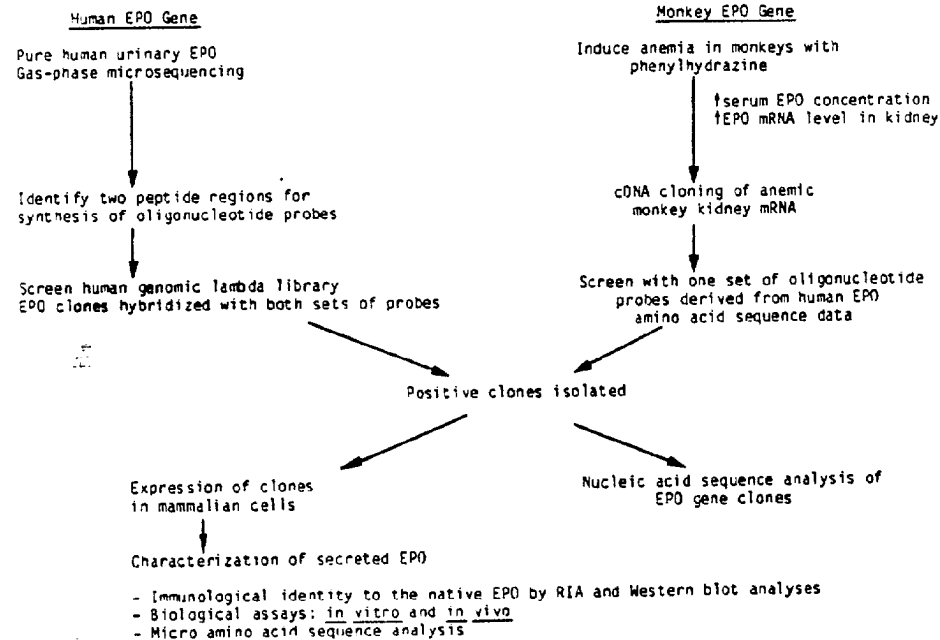


Figure 1. Strategy employed to clone, express, and characterize the human and monkey EPO genes.

AM 27 021118

AM-ITC 00461054

these assays. The r-hEPO produced in COS-1 cells is also indistinguishable from urinary EPO by Western blot analysis (Egrie et al. 1985). Polyadenylated RNA isolated from COS-1 cells 48 hours after transfection with this construction was used to prepare a cDNA library from which human EPO cDNA clones were isolated.

The amino acid sequence of the 193-amino-acid primary translation product was deduced from the nucleic acid sequence as shown in Figure 2. The mature hormone is 166 amino acids in length (calculated *M<sub>r</sub>* of 18,399). There are three potential N-linked glycosylation sites, as indicated. The first 27 amino acids predicted by the DNA coding sequence are consistent with this being a hydrophobic leader peptide. The coding portion of the gene is divided by four intervening sequences. The amino terminus of the mature protein was assigned directly from amino-terminal amino acid sequencing of urinary and r-hEPO. Intron/exon junction assignments, which all conform to consensus splice rules (Mount 1982), were made by comparison of a monkey cDNA clone, a human EPO cDNA clone prepared from mRNA isolated from COS-1 cells transfected with the genomic gene clone, and, ultimately, complete amino acid sequence analysis of human urinary EPO (Lai et al. 1986) and recombinant EPO produced in cell culture (P.H. Lai, unpubl.).

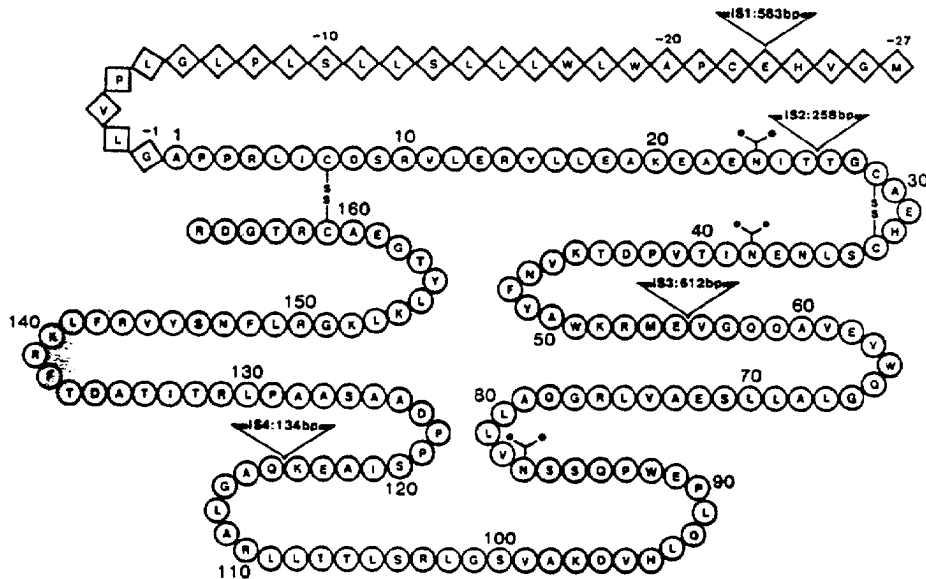
Southern blot analysis (Southern 1975) was used to

analyze the human EPO gene. The restriction fragment pattern of human DNA probes with a human EPO cDNA clone and the results of low-stringency hybridizations with this probe demonstrate that there is a single copy of the human EPO gene and that there are no apparent closely related genes or pseudogenes (Lin et al. 1985). Computer searches of protein and nucleic acid databases failed to reveal a significant homology with any published sequence. In particular, there is no homology with angiotensinogen, which has been suggested as a possible precursor of EPO (Fyhrquist et al. 1984). In addition, the structure of the human EPO gene and cDNA clones and the amino acid sequence results are consistent with EPO being secreted as an active hormone rather than an inactive precursor.

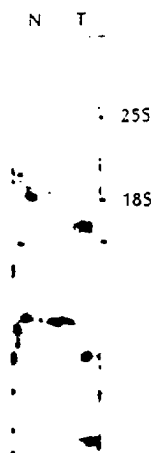
**Cloning of a Monkey EPO cDNA**

A cynomolgus monkey EPO cDNA was isolated using the strategy outlined in Figure 1 (Lin et al. 1986). Anemia was induced in monkeys by treatment with phenylhydrazine. As a result of the anemia, serum EPO levels were found to increase in anemic monkeys as measured by RIA and Western blot analyses (Egrie et al. 1985).

A Northern blot analysis of polyadenylated mRNA isolated from normal and anemic monkey kidneys is shown in Figure 3. One of the pools of mixed oligonu-



**Figure 2.** Amino acid sequence of the 193-amino-acid primary translation product of the human EPO gene is presented in one-letter code. The 27 amino-terminal amino acids of the putative signal peptide are boxed, and the residues in the 166-amino-acid mature hormone are circled. Positions of the two disulfide bands (S-S) and the three N-linked glycosylation sites (Y) are noted. Positions at which the four intervening sequences (IS) interrupt the coding position of the gene are given, along with the length of each IS sequence.



**Figure 3.** Northern blot analysis of cynomolgus monkey kidney poly(A) containing RNA. <sup>32</sup>P-end-labeled mixed oligonucleotide probes to EPO were used to develop the blot. (N) RNA isolated from normal monkey kidneys; (T) RNA isolated from the kidneys of monkeys made anemic by phenylhydrazine treatment. Positions of the ribosomal RNA markers are indicated.

cleotides corresponding to the human EPO amino acid sequence was used as the probe. Positive results were obtained only with mRNA from phenylhydrazine-treated animals (Fig 3, lane T). There was no detectable EPO-specific mRNA in normal monkey kidneys (Fig. 3, lane N). The size of the EPO-specific mRNA is approximately 1600 nucleotides in length. These Northern blot analysis results demonstrate that the steady-state level of EPO mRNA in the kidney is dramatically increased upon induction of anemia. The rise in serum EPO levels is therefore apparently due to increased EPO synthesis mediated by an increased transcription rate of the EPO gene and/or an increase in EPO mRNA stability as opposed to release of previously synthesized and sequestered EPO.

A monkey EPO cDNA clone was isolated from a cDNA library prepared from mRNA from anemic monkey kidneys. Only one of the two pools of mixed oligonucleotide probes proved useful for Northern blot analysis or cDNA cloning. It was subsequently determined that the second probe mixture failed to hybridize with monkey EPO due to a single-amino-acid difference between human and monkey EPO in the region corresponding to the probe. The cDNA clone sequence codes for a 192-amino-acid protein, differing at 15 residues from the human EPO gene sequence. Monkey EPO lacks lysine at position 116, when aligned with the human EPO sequence. Lysine at position 116 in the human gene is the first amino acid in the fourth exon of the human gene. The monkey gene presumably uses an

alternative splice junction for the fourth exon, and the DNA sequence is consistent with this. The amino terminus of mature-cell-culture-produced recombinant monkey EPO is at position -3 relative to human EPO. Thus, monkey EPO has a 24-amino-acid signal peptide, and the mature hormone is 168 amino acids in length. A substitution of a proline for a leucine at position -2 (relative to the human gene) in monkey EPO may be the cause of the difference in the signal peptide cleavage site.

#### Structural Features of EPO

To verify the primary amino acid sequence determined by gene cloning and to assess the nature of secondary modifications, purified human urinary EPO was characterized beyond the initial sequencing involved in designing gene probes. A total of 565  $\mu$ g of urinary EPO was used to determine the primary structure of the molecule (Lai et al. 1986). About 30  $\mu$ g was used to determine the amino-terminal amino acid sequence and this allowed assignments at 42 of 50 cycles of degradation. Cyanogen bromide cleavage fragments (100  $\mu$ g) allowed assignment of 45 additional positions from a total of 77 cycles. About 200  $\mu$ g was then utilized for sequencing tryptic digests, and about the same amount was used for V8-protease digests. The only residues not assigned directly were the asparagines at positions 24, 38, and 83. The absence of signals for these three residues during direct sequencing is consistent with the presence of carbohydrate moieties at these positions.

The positions of the disulfides were determined by the copurification of peptides predicted from the primary amino acid sequence to be separate after protease treatments (see Fig. 2). A PTH-cysteine was detected in the seventh step of Edman degradation, and sequencing of a reduced preparation of the peptide indicated the presence of the 7-161 disulfide. Assignment of the 29-33 disulfide was indirect and based on the following observations: (1) by Ellman's reaction and attempted labeling with [<sup>3</sup>H]iodoacetic acid, EPO was found to contain no free thiol residues; (2) sequencing of EPO showed no residues at positions 29 and 33, but performic-acid-oxidized material showed cysteic acid at these positions; and (3) the Glu-31, His-32 bond in oxidized r-hEPO was not hydrolyzed by V8 protease, indicating some conformational abnormality in the structure around these residues.

The molecular weight of purified r-hEPO, produced in CHO cells stably transfected with the human EPO gene inserted in an expression vector, was determined by analytical ultracentrifuge analysis. To carry out this analysis, the extinction coefficient of r-hEPO was measured by dry-weight determination, and the partial-specific volume was determined by the mechanical oscillation technique. This analysis yielded a result of 29,900  $\pm$  400 daltons for the mass of r-hEPO (J. Davis et al., in prep.). The carbohydrate portion of the molecule, assuming a molecular weight of 18,399 for the

protein portion of r-hEPO, comprises  $38 \pm 1\%$  of the total weight.

Human urinary EPO and CHO-cell-derived r-hEPO migrate identically in SDS-polyacrylamide gels, indicating that both molecules are glycosylated to a similar extent. The carbohydrate composition of r-hEPO was determined as described by Zanetta et al. (1972) and compared to literature values for human urinary EPO (Dordal et al. 1985). The carbohydrate composition of r-hEPO was essentially the same as that of urinary EPO (T.W. Strickland et al., in prep.). Trace amounts of *N*-acetylgalactosamine were found in r-hEPO, indicating the presence of O-linked glycosylation.

Shown in Figure 4 are the results of a deglycosylation experiment that indicates that both r-hEPO and urinary EPO contain both N-linked and O-linked carbohydrates in similar amounts. Both r-hEPO and urinary EPO were analyzed by Western blot analysis after sequential glycosylase digestion. Figure 4 (lanes 1 and 5) shows urinary EPO and r-hEPO, respectively, prior to treatment. After treatment with endoglycosidase F, which removes N-linked carbohydrate, the apparent molecular weight of both r-hEPO and urinary EPO is shifted to approximately 19,500 with a minor band at about 18,400 (lanes 2 and 6). Following further treatment, first with sialidase (lanes 3 and 7) and then by *O*-glycanase (lanes 4 and 8), which remove O-linked carbohydrate, both r-hEPO and urinary EPO migrated as a single band with an apparent molecular weight of 18,400. Although the presence of *N*-acetylgalactosamine had not been detected previously (Dordal et al. 1985), these results demonstrate that urinary EPO, as well as r-hEPO, contains O-linked carbohydrate. In addition, direct carbohydrate analysis of endoglycosidase-F-treated r-hEPO yields galactose, sialic acid, and

*N*-acetyl galactosamine, confirming the presence of O-linked carbohydrate (T.W. Strickland et al., in prep.). As shown in Figure 4, the proportion of EPO containing O-linked carbohydrate is comparable in urinary EPO and r-hEPO.

Samples of r-hEPO and urinary EPO taken over the course of endoglycosidase-F digestion were analyzed by Western blot analysis. Two clear partial digestion products, in addition to the final product, were revealed, indicating that all three potential N-linked glycosylation sites are utilized (data not shown).

The immunoreactivity of r-hEPO and urinary EPO were evaluated by comparing the dose-response curves of each preparation in a series of RIAs. The first RIA compared the ability of r-hEPO or urinary EPO to compete the binding of urinary  $^{125}\text{I}$ -labeled EPO by a rabbit polyclonal antibody raised against a 1% pure preparation of urinary EPO. In this assay, identical dose-response curves were obtained with the recombinant and natural hormones (Egrie et al. 1985, 1986). Identical dose-response curves were also obtained for each source of hormone when  $^{125}\text{I}$ -labeled r-hEPO was used as the tracer or when rabbit polyclonal sera to r-hEPO was used in combination with either tracer (data not shown; J.C. Egrie et al., in prep.). These experiments demonstrate that there are no epitopes present on one hormone preparation that are not present on the other.

#### Biological Effects of EPO

The biological activities of r-hEPO and urinary EPO were also indistinguishable, as measured by the dose-response curve of each preparation in both in vitro and in vivo biological assays (Egrie et al. 1986). The exhypoxic polycythemic mouse bioassay (Cotes and Bangham 1961) measures the incorporation of  $^{59}\text{Fe}$  into red blood cells in mice made polycythemic by exposure to low-oxygen conditions. Due to the polycythemia, these animals have a reduced rate of red blood cell synthesis after return to normal atmospheric conditions and therefore have a low background rate of incorporation of iron into red blood cells. However, these animals will respond to exogenous administration of EPO, in a dose-dependent manner, by increasing the rate of red blood cell synthesis and incorporation of  $^{59}\text{Fe}$ . The biological activity of EPO was also measured by the incorporation of  $^{59}\text{Fe}$  into heme in primary cultures of rat bone-marrow cells. In each of these assays (Egrie et al. 1986), as well as in mouse BFU-e and CFU-e assays (S.B. Krantz, pers. comm.), r-hEPO and urinary EPO have indistinguishable dose-response curves. These results indicate that r-hEPO produced in CHO cells has all the biological and immunological properties of natural EPO that can be measured by these assays. In addition, r-hEPO has the same activity in the RIAs and in vitro and in vivo bioassays, indicating that it is fully biologically active (Egrie et al. 1986).

The biological and immunological activities of multiple lots of purified r-hEPO have been measured.

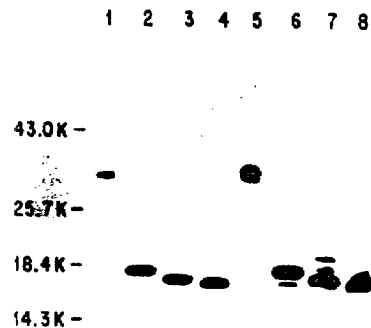


Figure 4. Western blot analysis of enzymatically deglycosylated urinary EPO (lanes 1-4) and r-hEPO (lanes 5-8). Samples were digested sequentially with glycosidases, and the products were separated on a 12.5% SDS-polyacrylamide gel under reducing conditions. An EPO-specific monoclonal antibody was used to visualize EPO after transfer to a nitrocellulose membrane. (1,5) No treatment; (2,6) endoglycosidase F; (3,7) endoglycosidase F plus neuraminidase; (4,8) endoglycosidase F, neuraminidase plus *O*-glycanase.

## STRUCTURE AND PROPERTIES OF EPO

699

These assays are standardized with respect to the second international reference preparation of EPO (IRP#2) (Annable et al. 1972). r-hEPO has a constant specific activity of 174,000 units/A<sub>280</sub> ± 5%, which is approximately 2.5 times the reported specific activity of urinary EPO (Miyake et al. 1977). Perhaps the harsh conditions required for purification of EPO from concentrated urine are responsible for the difference in specific activity.

Because of earlier notions that EPO may not be the only factor involved in production of red blood cell mass, we determined whether EPO alone could stimulate red blood cell production in normal mice, rats, and dogs. In all three species, there was a dose-dependent increase in reticulocyte counts and an increase in red blood cells. The results in Figure 5 show the effect on reticulocyte and red blood cell counts in dogs treated three times per week for 3 weeks. No effects were observed in excipient-treated controls, but doses of 280 U/kg and 2800 U/kg gave dose-dependent increases in both reticulocyte and red blood cell counts. It is noteworthy that the reticulocyte counts began to drop be-

fore the end of treatment and then dropped rapidly after treatment ceased, whereas the red blood cell counts continued to rise and then showed a gradual decrease. This particular study involved very high doses of EPO and thus allowed assessment of potential toxicity effects of EPO; however, no adverse effects of any note were observed. Overall, EPO was well tolerated and showed no histological abnormalities. Demonstration of an increase in red blood cells with highly purified EPO in normal, intact experimental animals indicates, for the first time, that EPO alone is capable of this effect and that other factors are not limiting in stimulating red blood cell production.

Previous studies of the pharmacokinetic features of EPO in experimental animals have involved use of biologically inactive, iodinated materials (Emmanouel et al. 1984). Production of EPO in a defined and controllable system has allowed labeling with <sup>35</sup>S-labeled amino acids during manufacture. This material has been found to be fully biologically active and its clearance is comparable to that of iodinated EPO, in terms of the rate of distribution and elimination. There was a modest reduction in clearance characteristics in functionally nephrectomized rats (A.C. Cohen et al., in prep.). These observations are encouraging in terms of the potential clinical use of EPO in patients with end-stage renal disease.

In view of the potential utility of EPO in patients with end-stage renal disease, we examined the responsiveness of nephrectomized rats to r-hEPO. Control animals were subjected to laparotomy without ligation of the kidneys. These animals showed no changes in blood urea nitrogen or hematocrit. Subtotal nephrectomized rats showed, as expected, increased blood urea nitrogen. Frank anemia was not observed in these animals presumably because insufficient renal mass had been removed. Both control (sham operated) and subtotal nephrectomized rats were responsive to EPO treatment in terms of increased hematocrits, as indicated by the results in Table I. Because this occurred without significant changes in body weight, we conclude that the hematocrit increase is due to an increase in red blood cells and not simply contraction of intravascular fluid space.

Anemias associated with inflammatory diseases, such as rheumatoid arthritis, provide other possible indications for an agent such as EPO. In this case, abnormalities of iron turnover and compartmentalization might override the effects of EPO. Thus, we examined the effect of EPO on the anemia of adjuvant-linked inflammation in rats. Data (Table 2) show that this experimental anemia is corrected by treatment with EPO. Specifically, this type of anemia is a consequence of impaired recirculation of iron from the liver to the marrow. This was evidenced by the decrease in plasma iron and hemoglobin concentration in the adjuvant-treated rats. EPO corrected this situation by stimulating the production of erythrocytes. In the process, plasma iron was decreased further as it diverted into hemoglobin synthesis, as evidenced by a return of

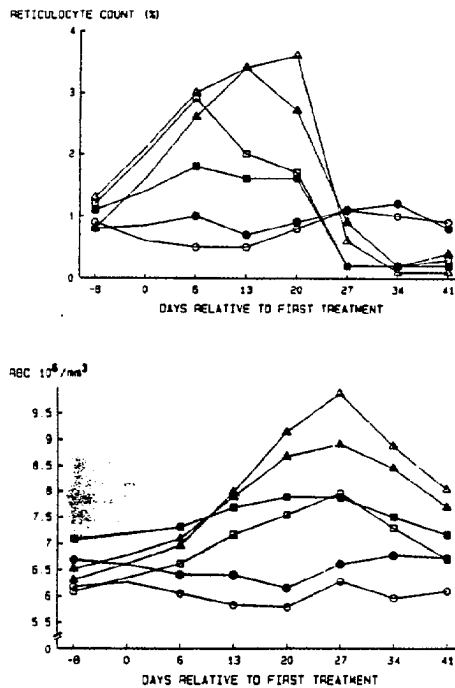


Figure 5. Effect of treatment with r-hEPO on the reticulocyte and red blood cell count of beagles. Young adult male (O, □, △) and female (●, ■, ▲) beagles were injected intravenously three times a week for 3 weeks with either a buffered saline control solution (O, ●) or a dose of r-hEPO at 280.0 U/kg (□, ■) or 2800 U/kg (△, ▲). The last treatment with r-hEPO was on day 20.

AM 27 021122

AM-ITC 00461058



Table 1. Treatment of Subtotally Nephrectomized Rats with r-hEPO

	Pretreatment		Posttreatment			
	sham	nephx	sham +	sham +	nephx +	nephx +
			0 EPO	10 units EPO	0 EPO	10 units EPO
Body weight (g)	210 ± 15	199 ± 16	234 ± 20	236 ± 11	218 ± 23	229 ± 12
Hematocrit	42.4 ± 2.2	40.8 ± 2.0	39.7 ± 1.1	46.8 ± 4.4 <sup>a</sup>	41.9 ± 3.8	48.2 ± 3.2 <sup>a</sup>
Blood urea nitrogen (mg/dl)	24.1 ± 4.6	40.1 ± 8.0 <sup>b</sup>	29.5 ± 4.0	26.0 ± 5.4	45.5 ± 2.9 <sup>b</sup>	47.0 ± 0.4 <sup>b</sup>

<sup>a</sup>*p* < 0.05 vs. respective treatment control (0 EPO).<sup>b</sup>*p* < 0.05 vs. sham control.

hemoglobin concentrations to normal values, with a concomitant decrease in the percentage of saturation. Total iron-binding capacity (TIBC) was unaffected by EPO.

#### DISCUSSION

Paucity of structural information, tissue of origin, and specific assays rendered the cloning of human EPO particularly difficult and required refinement of methods to allow identification of a single-gene copy in the genome using multiple DNA probes. The structural heterogeneity of urinary EPO, as exemplified by its disperse nature on SDS-PAGE, was ambiguous in terms of whether there was a family of related proteins and/or variable secondary modifications of the primary protein structure. The successful cloning and expression of the protein, as well as characterization of natural urinary EPO, has indicated that there is a single unique gene for this protein. The cloning approach used was risky in that it relied on considerable homology between human EPO and monkey EPO in the sequences used to construct probes. An alternative strategy involving use only of a human fetal cDNA library (Jacobs et al. 1985) was confounded by the possibility of a distinct fetal EPO gene. Current data indicate that there is only one gene for human EPO, and if there is also a distinct fetal gene, it is only distantly related in structure to the EPO gene described here. Although it is probable that EPO can be secreted by tissues other than the kidney (anephric individuals retain the ability to produce low amounts of EPO), the results of the screening of monkey tissue mRNA clearly indicate that the kidney responds to anemia by increasing the rate of synthesis of EPO.

The EPO gene seems to be highly conserved in mammals. The amino acid sequences of the primary trans-

lation products in human and cynomolgus monkey EPO differ at 15 positions and the monkey protein is one residue shorter: The lysine at position 116 in human EPO is missing. Mature monkey EPO and human EPO also differ at the site of cleavage of the signal peptide. There are 33 positions different between mouse EPO and human EPO (McDonald et al. 1986). Mouse EPO has only three cysteine residues (position 33 is not a cysteine), so that the small loop maintained by a disulfide in human EPO (see Fig. 3) would not seem to be essential for activity. Estimates of evolution rates have been made from the three known mammalian EPO sequences and these indicate  $1.3 \times 10^{-8}$  amino acid substitutions per site per year. This is comparable (although slightly lower) to the rate of evolution of rapidly evolving proteins, such as the fibrinopeptides ( $9 \times 10^{-8}$  substitutions/site/year), and much more rapid than the most slowly evolving proteins, such as histones ( $0.006 \times 10^{-8}$  substitutions/site/year) (McDonald et al. 1986).

It is noteworthy that the N-linked glycosylation sites are conserved in human, monkey, and mouse EPO sequences. This is distinct from cases in which glycosylation sites in closely related proteins, even in the same species, are not conserved. For example, some subtypes of the human interferon- $\alpha$  family of proteins are glycosylated, but the sites are nonhomologous and distinct also from the glycosylation sites in human interferon- $\beta$ , which is closely related in amino acid sequence to interferon- $\alpha$  (Stebbing 1986). A remarkable feature of human EPO produced in CHO cells is the similarity in the carbohydrate modifications that occur. The same broad spread of material on SDS-PAGE occurs with urinary EPO and CHO cell-derived EPO, and deglycosylation experiments yielded the same ratio of the N- and O-linked species. Furthermore, the car-

Table 2. Effect of r-hEPO on the Anemia of Adjuvant-induced Inflammation in Rats

	Untreated		Adjuvant-treated	
	saline	EPO	saline	EPO
PCV (%)	43.6 ± 0.8	54.9 ± 1.3	43.2 ± 0.6	52.9 ± 0.8
RBCV (ml/100 g)	2.02 ± 0.02	2.75 ± 0.06	2.10 ± 0.07	2.72 ± 0.04
Hemoglobin (g/dl)	13.89 ± 0.36	14.87 ± 0.69	11.62 ± 0.31	13.60 ± 1.05
Plasma iron (μg/dl)	145.9 ± 12.8	50.7 ± 26.7	124.4 ± 9.5	31.0 ± 8.3
TIBC (μg/dl)	505.6 ± 18.1	476.3 ± 16.1	491.4 ± 23.3	522.0 ± 29.4
% Saturation	28.9 ± 2.3	10.4 ± 5.4	25.3 ± 1.6	5.8 ± 1.4

AM 27 021123

AM-ITC 00461059

## STRUCTURE AND PROPERTIES OF EPO

701

bohydrate composition of r-hEPO and urinary EPO is very similar. The mechanisms controlling these secondary modifications remain unclear. Recombinant EPO produced in *Escherichia coli*, and therefore lacking glycosylation, or r-hEPO deglycosylated enzymatically has greatly decreased *in vivo* activity, although *in vitro*, its biological activity is preserved. The reasons why glycosylation is important for biological activity are unclear.

A clinical use of EPO in end-stage renal disease is obvious from the biology reviewed here. Treatment with EPO in end-stage renal disease constitutes replacement therapy. As such, clinical schedules and doses should prove easier to establish than for other recombinant-DNA-derived materials, such as the various lymphokines now in clinical trials. The extent to which EPO may be useful in other forms of anemia remains to be established, but the preclinical studies carried out so far are promising with regard to anemia of cancers and inflammatory diseases.

## ACKNOWLEDGMENTS

We thank Dr. Peter Dukas for performing the *in vivo* mouse exhypoxic polycythemic EPO bioassay, Joan Bennett and Sandi Olson for preparation of the manuscript, Jeanne Fitzgerald and Julie Heuston for the artwork, and Daniel Vapnek for critical reading of the manuscript.

## REFERENCES

- Anagnostou, A., J. Barone, A. Kedo, and W. Fried. 1977. Effect of erythropoietin therapy on the red cell volume of uraemic and non-uraemic rats. *Br. J. Haematol.* 37: 85.
- Annable, L., M. Cotes, and M.V. Musset. 1972. The second international reference preparation of erythropoietin, human urinary, for bioassay. *Bull. W.H.O.* 47: 99.
- Brown, R. 1963. Plasma erythropoietin in chronic uremia. *Br. Med. J.* 2: 1036.
- Carnot, P. and C. Deflandre. 1906. Sur l'activite hematopoietique des differents organes au cours de la regeneration du sang. *C.R. Acad. Sci. D.* 143: 432.
- Cotes, P.M. 1982. Immunoreactive erythropoietin in serum. *Br. J. Haematol.* 50: 427.
- Cotes, P.M. and D.R. Bangham. 1961. Bioassay of erythropoietin in mice made polycythemic by exposure to air at reduced pressure. *Nature* 191: 1065.
- Dordal, M.S., F.F. Wang, and E. Goldwasser. 1985. The role of carbohydrate in erythropoietin action. *Endocrinology* 116: 2293.
- Egrie, J.C. 1983. Monoclonal antibodies to the N-terminal region of human erythropoietin. *Hybridoma* 2: 136.
- Egrie, J.C., J.K. Browne, P. Lai, and F.-K. Lin. 1985. Characterization of recombinant monkey and human erythropoietin. In *Experimental approaches for the study of hemoglobin switching* (ed. G. Stamatoyanopoulos and A.W. Nienhuis), p. 339. A.R. Liss, New York.
- Egrie, J.C., T.W. Strickland, J. Lane, K. Aoki, A.M. Cohen, R. Smalling, G. Trail, F.-K. Lin, J.K. Browne, and D.K. Hines. 1986. Characterization and biological effects of recombinant human erythropoietin. *Immunobiology* (in press).
- Emmanouel, D.S., E. Goldwasser, and A.I. Katz. 1984. Metabolism of pure human erythropoietin in the rat. *Am. J. Physiol.* 247: F168.
- Erslev, A.J., J. Caro, E. Kansu, and R. Silver. 1980. Renal and extrarenal erythropoietin production in anemic rats. *Br. J. Haematol.* 45: 65.
- Eschbach, J.W. and J.W. Adamson. 1985. Anemia of end stage renal disease (ESRD). *Kidney Int.* 28: 1.
- Fried, W. 1972. The liver as a source of extrarenal erythropoietin. *Blood* 40: 671.
- Fyhrquist, F., K. Rosenlof, C. Gronhagen-Riska, L. Hortling, and I. Tikkanen. 1984. Is renin substrate an erythropoietin precursor? *Nature* 308: 649.
- Garcia, J.F., S.N. Ebbe, L. Hollander, H.O. Cutting, M.E. Miller, and E.P. Cronkite. 1982. Radioimmunoassay of erythropoietin: Circulating levels in normal and polycythemic human beings. *J. Lab. Clin. Med.* 99: 624.
- Gluzman, Y. 1981. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* 23: 175.
- Goldwasser, E. and C.K.H. Kung. 1971. Purification of erythropoietin. *Proc. Natl. Acad. Sci.* 68: 697.
- Goldwasser, E., J.F. Eliason, and D. Sikkema. 1975. An assay for erythropoietin *in vitro* at the milliunit level. *Endocrinology* 97: 315.
- Graber, S.E. and S.B. Krantz. 1978. Erythropoietin and the control of red cell production. *Annu. Rev. Med.* 29: 51.
- Graham, F.L. and A.J. van der Eb. 1973. A new technique for the assay of infectivity of adenovirus 5 DNA. *Virology* 52: 456.
- Graham, F.L., J. Smiley, W.C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from adenovirus type 5. *J. Gen. Virol.* 36: 59.
- Jacobs, K., C. Shoemaker, R. Rurensdorf, S.D. Neill, R.J. Kaufman, H. Mufson, J. Seehra, S.S. Jones, R. Hewick, E.F. Fritsch, M. Kawakita, T. Shimizu, and T. Miyake. 1985. Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature* 313: 806.
- Jacobson, L.O., E. Goldwasser, W. Fried, and L. Plzak. 1957. Studies on erythropoiesis. VII. The role of the kidney in the production of erythropoietin. *Trans. Assoc. Am. Physicians* 70: 305.
- Koeffler, H.P. and E. Goldwasser. 1981. Erythropoietin radioimmunoassay in evaluating patients with polycythemia. *Ann. Intern. Med.* 94: 44.
- Krantz, S.B. and L.O. Jacobsen. 1970. *Erythropoietin and the regulation of erythropoiesis*, p. 325. University of Chicago Press, Chicago, Illinois.
- Lai, P.-H., R. Everett, F.F. Wang, T. Arakawa, and E. Goldwasser. 1986. The primary structure of human erythropoietin. *J. Biol. Chem.* 261: 3116.
- Lin, F.-K., C.H. Lin, P.-H. Lai, J.K. Browne, J.C. Egrie, R. Smalling, G.M. Fox, K.K. Chen, M. Castro, and S. Suggs. 1986. Monkey erythropoietin gene: Cloning, expression and comparison with the human erythropoietin gene. *Gene* 44: 201.
- Lin, F.-K., S. Suggs, C.-H. Lin, J.K. Browne, R. Smalling, J.C. Egrie, K.K. Chen, G.M. Fox, F. Martin, Z. Stabinisky, S.M. Badrawi, P.-H. Lai, and E. Goldwasser. 1985. Cloning and expression of the human erythropoietin gene. *Proc. Natl. Acad. Sci.* 82: 7580.
- Lukens, J.N., G.E. Cartwright, and M.M. Wintrobe. 1967. Anemia of adjuvant-induced inflammation in rats. *Proc. Soc. Exp. Biol. Med.* 126: 346.
- Maxam, A.M. and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65: 499.
- McDonald, J.D., F.-K. Lin, and E. Goldwasser. 1986. Cloning, sequencing, and evolutionary analysis of the mouse erythropoietin gene. *Mol. Cell. Biol.* 6: 842.
- Miyake, T., C.K.H. Kung, and E. Goldwasser. 1977. Purification of human erythropoietin. *J. Biol. Chem.* 252: 5558.
- Mount, S.M. 1982. A catalog of splice junction sequences. *Nucleic Acids Res.* 10: 459.
- Naets, J.P. 1975. Hematological disorders in renal failure. *Nephron* 14: 181.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA se-

AM 27 021124

AM-ITC 00461060

- quencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74: 5463.
- Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503.
- Spivak, J.L. and S.E. Graber. 1980. Erythropoietin and the regulation of erythropoiesis. *Johns Hopkins Med. J.* 146: 311.
- Stebbing, N. 1986. Mechanisms of action of interferons: Evidence from studies with recombinant DNA derived subtypes and analogs. In *Mechanisms of interferon actions* (ed. L.M. Pfeffer). CRC Press, Boca Raton, Florida. (In press.)
- Vieira, J. and J. Messing. 1982. The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19: 259.
- Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* 14: 725.
- Zanetta, J.P., W.C. Breckenridge, and G. Vincendon. 1972. Analysis of monosaccharides by gas liquid chromatography of O-methyl glucosides as trifluoroacetate derivatives. Application to glycoproteins and glycolipids. *J. Chromatogr.* 69: 291.
- Zanjani, E.D., J.L. Ascensao, P.B. McGlave, M. Banisadre, and R.C. Ash. 1981. Studies on the liver to kidney switch of erythropoietin production. *J. Clin. Invest.* 67: 1183.

AM 27 021125

AM-ITC 00461061