EXHIBIT A

PRODUCTION OF RECOMBINANT HUMAN ERYTHROPOIETIN IN MAMMALIAN CELLS: HOST-CELL DEPENDENCY OF THE BIOLOGICAL ACTIVITY OF THE CLONED GLYCOPROTEIN

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Erythropoietin (EPO), a heavily glycosylated protein, is a major stimulatory factor in erythropoiesis. The human EPO gene was engineered for expression in animal cells. Recombinant EPOs produced in two kinds of cells were isolated and their properties compared with those of human urinary EPO. The results indicated that the carbohydrates attached to the EPO peptide are responsible for the different biological activities of these proteins. The biological activities of recombinant glycoproteins produced in heterologous systems may vary depending on the host cells in which the proteins are modified.

Erythropoietin (EPO) stimulates red blood cell production by promoting both the growth of late erythroid precursor cells and their maturation into proerythroblasts, in which globin synthesis starts. It is widely accepted that EPO is a major physiological regulator of the process of erythroid differentiation and that it controls the maintenance of physiological levels of the circulating erythrocyte mass (reviewed in rfs. 1–3). This hormone is produced mainly by the kidney in human adults[2,4,5] and anemia associated with renal failure often results from a decreased level of EPO. It therefore appears indicated for use in the therapeutic treatment of anemia[4,6–8]. Human EPO has been isolated from the urine of patients with anemia[9–11], and the gene and cDNA of EPO have been cloned[12–14]. Human EPO is a glycoprotein with a molecular weight of 35,000. Thirty–50% of its mass is made up by carbohydrates[10,12,15].

For clinical applications of a recombinant glycoprotein, the carbohydrate chains may be important. They may protect the glycoprotein from proteolytic degradation, resulting in an increased lifetime in circulating blood, or the glycoprotein may be removed rapidly from the blood by binding to hepatic receptors when the termini of the carbohydrate chains are residues that are recognized by these receptors[16]. The kind of carbohydrate chain may also affect the antigenicity of the recombinant product. When recombinant glycoproteins are produced in heterologous animal cells, their amino acid sequences govern the positions onto which carbohydrate chains can be attached, but the maturation of the chains may entirely depend on the host cells. It is of interest, therefore, to compare the properties of recombinant glycoproteins produced in different heterologous cells with those of naturally occurring ones.

We have cloned human EPO gene and engineered it for expression in two different mammalian cell lines. The biological activity and carbohydrate composition of EPOs isolated from human urine and culture supernatants of the cells carrying the EPO gene were compared.

RESULTS

Isolation and expression of EPO gene. Peptides derived from urinary EPO (u-EPO) were sequenced to prepare synthetic DNA probes for screening a human fetal liver genomic library for EPO gene clones. Screening with single long probes (30 nucleotides/probe) deduced from the sequences of three peptides resulted in the identification of three phage plaques that hybridized to these probes. Restriction endonuclease mapping and Southern analysis showed the gene fragments contained in these phage clones to be identical. One, designated λEPO41, was used for sequence analysis. Although the nucleotide sequences in the exons agreed with those published previously[12], some nucleotide bases in the introns did not (data not shown). The clone λEPO41 contained a whole EPO coding region, but half of 3′-untranslated region was missing.

A vector for the expression of EPO in animal cells was constructed by the procedures diagrammed in Figure 1. The EPO expression vector, pZIP-NeoSV(X)1-EPO, contained the EPO gene preceded by a long terminal repeat (LTR) functioning as transcriptional promoter and followed by DNA sequences derived from the transposon Tn5 (Neo fragment in Fig. 1) that confers G418 resistance upon mammalian cells. The expression vector was introduced into two mammalian cells, BHK 21 cells established from baby hamster kidney, and ψ2 cells derived from N1H/3T3[19]. EPO-producing cells were isolated by selection in G418 media and by two rounds of limiting dilution. Established EPO-producing cells showed anchorage-dependent growth as the original cell lines did. The EPO levels in culture supernatants of EPO-producing cells assayed by radioimmunoassay (RIA) were ~150 U/ml for BHK cells and ~300 U/ml for ψ2 cells.

Purification of recombinant EPO. Two kinds of recombinant EPO (r-EPO), r-EPO-ψ and r-EPO-B, were purified from the culture supernatants of ψ2 and BHK cells bearing the EPO gene by use of immunoaffinity and a molecular sieve that yielded pure u-EPO with high recovery[10,11]. This method was also effective for purification of r-EPOs (Table 1), but the EPO preparations obtained from a G-100 column were not homogeneous as assessed by SDS-polyacrylamide gel electrophoresis. We therefore

used hydroxyapatite column-chromatography for the final purification. The r-EPO-B appeared in the flowthrough of a column equilibrated with 10 mM NaPi, but r-EPO-ψ was eluted by 50 mM NaPi. Figure 2 shows the electrophoretic patterns of r-EPOs and u-EPO on SDS-polyacrylamide gels. Purified u-EPO showed two bands (lane 1). The minor one with the lower molecular weight, is the asialylated form of u-EPO[10,11]. Both r-EPOs migrated as a single band, but they differed in electrophoretic mobility (lanes 2 and 3). Recombinant EPO from ψ2 cells migrated slightly faster than u-EPO, and the r-EPO-B band was broader than those of r-EPO-ψ and u-EPO. Thirty amino acids in the NH_2-terminal portion of r-EPO-ψ and r-EPO-B were consistent with those of u-EPO and also with those deduced from the cDNA sequence[12]. The amino acid sequence in the COOH-terminal region of both r-EPOs and u-EPO determined by the carboxypeptidase method was Gly-Asp-Arg-COOH, identical to that of u-EPO[20].

Rabbit antiserum against u-EPO was examined for reactivity to both r-EPOs by Ouchterlony double diffusion (Fig. 3). A single precipitation line was formed between anti u-EPO antiserum and each r-EPO, and the lines fused symmetrically with no spur. This indicates the presence of similar antigenic determinants in the r-EPOs.

Biological activity of isolated EPOs and their carbohydrate compositions. The *in vivo* and *in vitro* activities of three purified EPOs were assayed (Table 2). The *in vitro* activity was assayed measuring the stimulation by EPO of ^{3}H-thymidine incorporation into the DNA of fetal mouse liver cells. The stimulatory effect of the EPO preparations were completely repressed by incubation with antiserum[21] against EPO, indicating that the mitogenic effect of a contaminant, if any, did not contribute to the *in vitro* activity. In fact, the endotoxin content (1–5 ng/mg protein) of the purified EPO preparations had no effect on the assay used here. The *in vivo* activity of r-EPO-B was equivalent to that of u-EPO, and its *in vitro* activity was slightly higher than that of u-EPO. The *in vitro* activity of r-EPO-ψ was 1.6-fold that of u-EPO and of r-EPO-B, but surprisingly, the *in vivo* activity was only 1/4 that of other EPOs. The *in vitro* activities of the three EPO preparations increased somewhat after sialidase treatment, but their *in vivo* activities were totally abolished.

Comparison of the amino acid sequences in the NH_2- and COOH-terminal regions of u-EPO and r-EPOs indicated that they were identical in terms of peptide chains, although r-EPO-ψ differed from the others in its biological activity *in vivo* and *in vitro*. To find what caused this difference, we analysed the carbohydrate composition of three EPO preparations (Table 3). All of the preparations contained fucose, galactose, mannose, N-acetylhexosamine, and sialic acid. Both r-EPOs contained more neutral sugar and N-acetylhexosamine than u-EPO. The greatest difference was in sialic acid; u-EPO and r-EPO-B contained 11 and 19 molecules of sialic acid/mole of protein, respectively, but r-EPO-ψ contained only 5. Most of the N-acetylhexosamine in the EPOs from the three sources was N-acetylglucosamine but about 1/10 of the total was always N-acetylgalactosamine (unpublished data), suggesting that urinary and recombinant EPOs contained O-linked oligosaccharides.

DISCUSSION

One major use of eukaryotic cells in the production of recombinant proteins is for the production of glycoproteins that have carbohydrate structures similar or, preferably, identical to naturally occurring ones, because the carbohydrate chains can affect the biological properties of the proteins. However, only a few papers have appeared reporting the analysis of the carbohydrate chains of recombinant products. Human γ-interferon produced in Chinese hamster ovary cells had partially, biantennary chains of N-glycosidic carbohydrate that were found in several human secreted proteins[22]. It is not known if these carbohydrate chains are identical to those of naturally occurring interferon, because the carbohydrate structures of the latter have not yet been analysed. Chicken ovalbumin synthesized in mouse L cells contain carbohydrate chains that are not present in natural ovalbumin[23], showing that there is host-dependent variation in the oligosaccharides attached to the recombinant protein produced in heterologous cells.

Naturally occurring EPO is a heavily glycosylated protein. There are three N-glycosylation sites (Asn-X-Ser/Thr)[12,15,20]. Human genomic EPO gene was expressed in ψ2 and BHK cells using the same expression vector, and recombinant EPOs were isolated from the culture supernatants of cells and compared with urinary EPO in biological and structural properties. Both recombinant EPOs had the same amino acid sequences in their NH_2-terminal and COOH-terminal regions as u-EPO, indicating that the recombinant products had the same primary structure as u-EPO. Differences between purified r-EPOs in their electrophoretic mobility on SDS-polyacrylamide gel, therefore, is attributable to the difference in the carbohydrate chains.

Recombinant EPOs and u-EPO contained the same molecular species of carbohydrate residues but in different amounts. The low *in vivo* activity of r-EPO-ψ is probably due to the small amount of sialic acid, because the removal of sialic acid by sialidase treatment of all EPOs abolished *in vivo* activity. Loss of *in vivo* activity is caused by the removal of asialoglycoproteins from the circulation by the liver[16]. Thus, it is likely that the *in vivo* lifetime of r-EPO-B is longer than that of r-EPO-ψ.

The *in vitro* activity of u-EPO and both r-EPOs was enhanced by sialidase treatment. The rule seems to be that the lower the amount of sialic acid, the higher the *in vitro*

TABLE 1 Purification of recombinant erythropoietins

EPO from	Purification procedures	Protein (A_{280})	Activity* (units × 10^{-4})	Specific activity (units/A_{280})	Purification
BHK cells (r-EPO-B)	Concentrated culture supernatant	892,000	1,250	14	1
	Immunoadsorbent column	707	897	12,700	907
	Sephadex G-100	103	834	81,000	5,790
	Hydroxylapatite	77	631	82,000	5,860
ψ cells (r-EPO-ψ)	Concentrated culture supernatant	784,000	1,027	13	1
	Immunoadsorbent column	862	853	9,900	756
	Sephadex G-100	140	798	57,000	4,350
	Hydroxylapatite	52	567	109,000	8,320

*Found by radioimmunoassay.

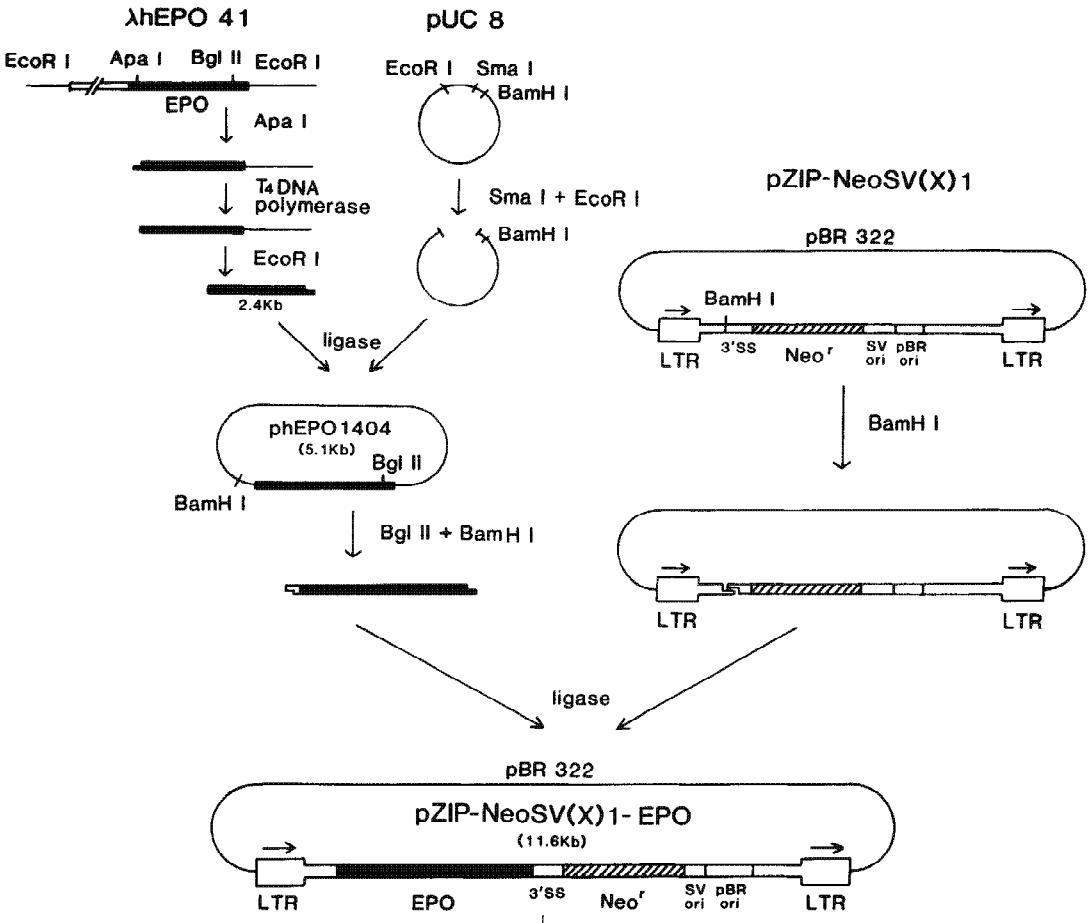

FIGURE 1 Construction of erythropoietin expression plasmid. The ApaI site of the EPO gene in λhEPO41 is a unique site located at 62 bp upstream from the translation initiation codon and the EcoRI site is for inserting human DNA fragments into the bacteriophage cloning vector, Charon 4A[17]. The BglII site of the EPO gene in phEPO1404 is 185 bp downstream from the translation stop codon. The details of the functions and sizes of the fragments in pZIP-NeoSV(X)1 have been described elsewhere[18]. LTR, Long terminal repeat of Molony murine leukemia virus; 3'SS, 3' splicing signal; Neo^r, sequence derived from the transposon Tn*5*, which encodes G418 resistance in mammalian cells; SV *ori*, sequence of SV40 origin; pBR *ori*, sequence of pBR322 origin.

activity. The asialo-hormone may have an increased affinity to the target cells, if the cell membrane is negatively charged. The high *in vitro* activity of r-EPO-ψ is puzzling. Not only the desialated r-EPO-ψ but also the native hormone has higher activity than any other EPO. The high activity of the native r-EPO-ψ as compared with other native EPOs may be partly due to the low content of sialic acid, but sialic acid content cannot account for the fact that the activity of desialated r-EPO-ψ is significantly higher than those of desialated u-EPO and r-EPO-B. Structures of carbohydrate chains in recombinant EPOs and u-EPO need to be analysed to discover what confers the high *in vitro* activity to r-EPO-ψ, as well as to establish to what extent the carbohydrate chains of r-EPO-B resembles those of u-EPO.

EXPERIMENTAL PROTOCOL

Isolation of human genomic EPO clones. EPO isolated from human urine was fragmented by cleavage with proteases and CNBr and the resulting peptides were purified for sequencing with an Applied Biosystems gas-phase sequenator. The entire amino acid sequence of EPO was determined (unpublished). The sequences of the three peptides were used to design single long synthetic DNA probes[24] for hybridization screening of a DNA library to identify EPO gene clones; probe I (5' CCTGGAGTCGCAGATCAGCCTGGGGGGGGC), probe II (5' CTGCCACACCTCCACGGCCTGCTGGCCCAC), and probe III (5' CCTGCAGGCCTGGCCGGTGTACAGCTTCAG) were deduced from sequences I (Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg), II (Val-Gly-Gln-Gln-Ala-Val-Glu-Val-Trp-Gln), and III (Leu-Lys-Leu-Tyr-Thr-Gly-Gln-Ala-Cys-Arg), respectively. Sequences I, II, and III correspond to the amino acid numbers of EPO of 1–10, 55–65, and 153–162. Oligonucleotide probes were synthesized with an Applied Biosystems 380A DNA synthesizer and labeled at the 5' end with T4 polynucleotide kinase (Takara shuzo) and [γ-^{32}P]ATP (Amersham). A human fetal genomic library in bacteriophage λ[17] was screened for the EPO gene with the use of the labeled probes[25]. Three phage plaques that hybridized with all probes were found in the library containing 1×10^6 phage colonies.

Construction of EPO expression plasmids and transfection of mammalian cells. Phage DNA from a clone (λhEP041 in Fig. 1) harboring the EPO gene was digested with ApaI and single-stranded ends were filled in by treatment with T4 polymerase. After the digestion of the DNA with EcoRI, a DNA fragment (2.4 kilobases) containing the EPO gene was purified by agarose gel electrophoresis and inserted into pUC8 that had been digested with EcoRI and SmaI. The 2.4 kilobase BamHI-Bg1II fragment of the resulting plasmid, phEp1404, was ligated into pZIP-NeoSV(X)1 vector[18] digested with BamHI (Fig. 1). ψ2 Cells and BHK-21 cells were transfected with the expression vector, pZIP-NeoSV(X)1-EPO, using the calcium-phosphate method[26]. Transfected cells were selected in G418 (GIBCO) media.

Cell culture and production of EPO. BHK cells were cultured in Eagle basal medium (GIBCO) supplemented with 10% calf serum (Flow Lab.) and 10% Bacto Tryptose Phosphate Broth (DIFCO). The ψ2 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum. Cell cultures for EPO production were started at a cell density of 2×10^5 cells/ml in plastic culture trays (Cell Factory, 6000 cm^2,

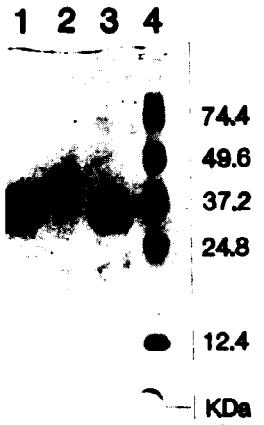

FIGURE 2 Human urinary and recombinant erythropoietins were analysed with SDS-polyacrylamide gel electrophoresis. Lanes 1, 2, and 3 are erythropoietins (2 μg of each) from urine (u-EPO), the culture supernatants of BHK cells (r-EPO-B), and ψ2 cells (r-EPO-ψ). M^r standards are shown in lane 4. The gel was silver-stained as described by the manufacturer (Bio-Rad).

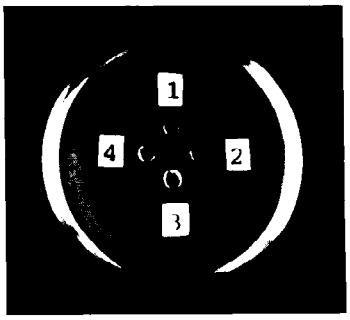

FIGURE 3 Ouchterlony double-immunodiffusion experiment. Wells 1, 2, 3, and 4 contained EPO (2 μg) from the culture supernatant of BHK cells (r-EPO-B), 40 μl of antiserum against human urinary EPO, EPO (2 μg) from the culture supernatant of ψ2 cells (r-EPO-ψ), and 40 μl of control serum, respectively.

TABLE 2 Biological activities of human erythropoietins.

EPO	Treatment	EPO activity (units/A_{280}) *in vitro*	EPO activity (units/A_{280}) *in vivo*
u-EPO	Untreated	77,000	72,000
	Treated with sialidase	99,000	0
r-EPO-B	Untreated	96,000	72,000
	Treated with sialidase	109,000	0
r-EPO-ψ	Untreated	153,000	18,000
	Treated with sialidase	173,000	0

TABLE 3 Carbohydrate composition of erythropoietins.

Sugar	Sugar content (moles/mole of EPO) r-EPO-B	r-EPO-ψ	u-EPO
Fucose	3.3	3.2	2.6
Galactose	15.7	20.6	13.3
Mannose	10.1	11.4	8.1
N-acetylhexosamine	28.9	26.8	22.1
N-acetylneuraminic acid	18.7	5.0	10.7

NUNC) containing 2 liters of the medium. After 4 days of culture, the cells grew to be nearly confluent. The culture medium was replaced with the low-serum medium (1.5% calf serum) and culture supernatants were harvested every 3 days.

Purification of recombinant EPO. For purification of r-EPO from the culture supernatants of EPO-producing cells, immunoaffinity column chromatography with monoclonal antibody against u-EPO[10,11,27] was used. Briefly, 100 liters of culture supernatant was filtered under suction and concentrated to 2 liters by ultrafiltration on a hollow-fiber device (Amicon DC-10) with a nominal Mr cutoff of 10,000. To the concentrated culture supernatant, solid sodium dodecyl sulfate (SDS) was added to a final concentration of 2%. The mixture was heated in a boiling water bath for 10 min with stirring and kept on ice for 2 days. The precipated SDS was removed by centrifugation and the supernatant put on an immunoadsorbent column (3.2×6.2 cm) equilibrated with phosphate-buffered saline (PBS) containing 2 g of monoclonal antibody fixed on Affigel 10 (Bio-Rad). The column was extensively washed with 5 liters of PBS, 800 ml of a mixture of 10 mM NaPi (pH 7.4) and 0.5 M NaCl, and 800 ml of 0.15 M NaCl, in this order, and then eluted by reverse flow of 200 ml of a solution of 0.2 M acetate (pH 2.5) and 0.15 M NaCl. The eluted fraction was neutralized and the protein was precipitated with 90% ethanol. The ethanol precipitate was dissolved in 5 ml of PBS and put on a Sephadex G-100 column (2.2×94 cm) equilibrated with PBS. The column was developed with PBS. EPO in the fractions (140–180 ml) was precipitated with 90% ethanol. The precipitate was dissolved in 10 mM NaPi, pH 6.8, containing 0.01 mM $CaCl_2$ and loaded on a hydroxyapatite column (100×7.8 mm) (Bio-Rad, HPHT) equilibrated with the same buffer. The adsorbed proteins were eluted by increasing concentration of NaPi.

Assay for EPO. To screen cells producing EPO and to purify the r-EPOs, RIA for EPO was done as described previously[28]. In brief, 10 μl of a sample diluted to about 1 unit of EPO/ml with PBS containing 1% bovine serum albumin (BSA) was mixed with 10,000 cpm of ^{125}I-u-EPO (about 100 μCi/μg), rabbit anti-u-EPO antiserum[21], and rabbit γ-globulin (Cappel) as the carrier in 500 μl of PBS containing 0.1% BSA, 0.05% Tween 20, 1 mM EDTA, and 0.01% NaN_3. The mixture was incubated at 4°C overnight and sheep anti-rabbit γ-globulin (Cappel) was added. The mixture was left for 2 hr at 4°C, and then immunocomplexes were precipitated by centrifugation at 1,000×g for 30 min at 4°C. The precipitate was rinsed with 2 ml of PBS and the radioiodine in it was counted with a Packard γ-counter model 5650. The amounts of EPO in the sample were estimated from a standard curve (2–128 mU/assay). The Second International Reference Preparation for human EPO obtained from the WHO International Laboratory for Biological Standards, National Institute for Medical Research, London[29] was used as the standard. An *in vitro* EPO assay that used the stimulatory effect of EPO on the incorporation of ^{3}H-thymidine into DNA[30] in cultured mouse fetal liver cells was done as described elsewhere[31]. EPO activity was assayed *in vivo* with the use of starved rats (4 rats/sample)[32].

Carbohydrate analysis. To analyze carbohydrate residues, each EPO preparation was hydrolyzed by 2.5 M trifluoroacetic acid at 100°C for 6 hr in an evacuated sealed tube[33]. The monosaccharides obtained were analyzed by HPLC on a Shimazu Model LC-2A apparatus with a column of ISA-07/S2504 (4×250 mm) as described previously[34]. To measure sialic acid content of EPO, the isolated EPO (10 mg) was incubated with 0.1 units of neuraminidase (Sigma, Type X) at pH 5.0 for 4 hr at 37°C and the sialic acid released was measured by the 2-thiobarbituric acid method[35].

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