Transient and stable transfection of Chinese hamster ovary cells with the recombinant feline erythropoietin gene and expression, purification, and biological activity of feline erythropoietin protein

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Objective—To use transient and stable transfection of Chinese hamster ovary cells to clone the gene encoding feline erythropoietin (feEPO) protein, characterize the expressed protein, and assess its biological activity.

Sample Population—Cultures of Chinese hamster ovary or TF-1 cells.

Procedure—The gene encoding feEPO was cloned into a eukaryotic expression plasmid. Chinese hamster ovary cells were transiently or stably transfected with the plasmid. Expressed recombinant feEPO (rfeEPO) protein was purified from transiently transfected cells. The protein was characterized by use of SDS gel electrophoresis and western blot analysis. Biological activity was assessed by measuring thymidine incorporation by TF-1 erythroleukemic cells.

Results—Purified rfeEPO from supernatants of transiently transfected cells was determined to be 34 to 40 kilodaltons (kd) by use of SDS gel electrophoresis, whereas the molecular weight predicted from the amino acid sequence was 21.5 kd. The banding pattern and high molecular weight suggested the protein was glycosylated. The rfeEPO proteins derived from transient or stable transfections subsequently were determined to be biologically active in vitro.

Conclusions and Clinical Relevance—The gene encoding feEPO can be transfected into eukaryotic cells, and the expressed rfeEPO protein is biologically active in vitro. Cats with chronic renal failure often are anemic as a result of reduced expression of erythropoietin (EPO). Treatment with human-derived EPO stimulates RBCs in anemic cats; however, treatment is often limited by the development of antibodies directed against the recombinant human protein, which can then cross-react with endogenous feEPO. Recombinant feEPO may prove beneficial for use in cats with chronic renal failure. (*Am J Vet Res* 2003; 64:1465–1471)

Erythropoietin (EPO) is a hormone produced by the adult kidneys in response to tissue hypoxia.[1] Impaired production of EPO is a common clinical manifestation in animals with **chronic renal failure (CRF)**.[1-4] Erythropoietin stimulates the production of RBCs. Therefore, impaired production of EPO secondary to CRF can lead to anemia. The current treatment for regenerative anemia is administration of exogenous EPO. **Human EPO (huEPO)** is available for clinical use in companion animals and reportedly[2,4] increases the PCV and improves overall well-being of treated animals. However, a consequence of the use of huEPO in cats or dogs is that the response may decrease over time as a result of the formation of antibodies against the huEPO protein. In some cases, these antibodies can cross-react with the animal's endogenous EPO.[2,3] Therefore, species-specific EPO is needed to improve treatment of animals that have anemia associated with CRF.

A high degree of sequence homology exists between mammalian EPO genes, resulting in immunologic cross-reactivity and in vivo biological activity among several species.[5] Analysis of amino acid sequences deduced from DNA sequences revealed that **canine EPO (caEPO)**[6] and **feline EPO (feEPO)**[7] are 85.8% and 85.0% homologous, respectively, with huEPO. In 1 study,[6] investigators transferred caEPO in Chinese hamster ovary cells and documented that supernatants containing the expressed protein had biological activity in vivo. In another study,[7] investigators used a recombinant **adeno-associated virus (AAV)** vector to transfer the feEPO gene to the muscle of clinically normal cats.

The purpose of the study reported here was to develop an feEPO-expression plasmid. The feEPO plasmid was constructed by inserting the feEPO gene into a plasmid containing the **cytomegalovirus (CMV)** eukaryotic promoter, and the feEPO plasmid was then transiently transfected into Chinese hamster ovary cells. In addition, the feEPO protein from supernatants of transfected cells was characterized, and biological

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activity of this protein was determined. Finally, we selected and characterized a stable line of Chinese hamster ovary cells that expressed feEPO, which has the potential for generating large quantities of the protein.

Materials and Methods

Cloning of cDNA encoding feEPO—The gene encoding feEPO was isolated from feline kidney tissue mRNA by use of a reverse transcriptase-**polymerase chain reaction (PCR)** technique. The PCR primers were designed on the basis of the nucleotide entry for feEPO mRNA, U00685.[7,a] The gene was constructed from fragments of clones from 2 PCR reactions. A clone encoding the full-length feEPO protein was isolated by use of forward (5′–GAG ATG GGG TCG TGC GAA TGT CCT GCC CTG CTG CT–3′) and reverse (5′–GGA GCA CCT GGT CAC CTG TCT CCT CTT CGG CAG GC–3′) primers representing nucleotides 11 to 45 and 569 to 603, respectively, of the U00685 DNA sequence. The PCR conditions were 96°C for 5 minutes; 43 cycles of 96°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes; and then 72°C for 7 minutes. The PCR product was ligated into a pCR2.1 plasmid by use of a commercially available cloning kit[b] that resulted in the production of plasmid TA-feEPO A. The feEPO gene in the TA-feEPO A was sequenced,[c] which revealed sequence differences from U00685 at 3 nucleotide positions. Therefore, 2 nucleotides at the 3′ end of the open reading frame were modified so that the sequence would agree with the U00685 sequence. The third sequence difference (adenine to guanine at nucleotide 144 of the U00685 sequence) was not modified. This latter sequence difference is similar to the single nucleotide substitution in another sequence entry (ie, I46083),[d] resulting in a difference of 1 amino acid (glycine to glutamic acid at position 44 of the full-length, open reading frame) between the 2 published sequences.

To correct the sequences in the 3′ end of the feEPO gene in clone TA-feEPO A, a partial feEPO fragment was isolated from existing kidney cDNA by use of PCR techniques. This was accomplished by use of a forward primer (5′–GAC AGC CGA GTC CTG GAG AGG TAC A–3′) that represented nucleotides 113 to 138 of the U00685 nucleotide sequence. The reverse primer (5′–GCG GTC TAG ATC ACC TGT CTC CTC TTC GGC A–3′) corresponded to nucleotides 572 to 592 of the U00685 nucleotide sequence plus an additional sequence of 10 nonhomologous bases on the 3′ end. The PCR conditions were 96°C for 5 minutes; 43 cycles of 96°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1.5 minutes; and then 72°C for 7 minutes. The partial feEPO PCR fragment was cloned into a pCR2.1 plasmid by use of a cloning kit,[b] which resulted in TA-feEPO B; this product was sequenced.[c] The 3′ sequence of the insert in TA-feEPO B was identical to the U00685 sequence, except for the aforementioned adenine-to-guanine substitution at nucleotide 144. Each clone was digested with *Hin*cII[e] and *Spe*I[f]; the *Hin*cII site is internal to the EPO sequence, and the *Spe*I site is in the vector. Purified fragments were used, and the 3′ end of the EPO gene from TA-feEPO B was substituted for that of TA-feEPO A. This reconstituted clone encoding full-length feEPO (ie, TA-feEPO C) was digested with *Eco*RI,[g] and the feEPO gene was transferred into a eukaryotic expression plasmid, CMVIntA(kan). This plasmid was constructed from CMVIntA(amp)[8] by inserting the kanamycin-resistance gene directly inside the ampicillin sequence. The resulting plasmid was labeled CMVIntA(kan)-feEPO.

Transfection—Chinese hamster ovary cells[h] were seeded at 4×10^6 cells/100-mm dish in **minimal essential medium (MEM)**[i] supplemented with 1mM **nonessential amino acids (NEAA)**[j] and 10% **fetal bovine serum (FBS)**.[k] Chinese hamster ovary cells were incubated at 37°C in a CO_2 incubator until the cells were nearly confluent. Plasmid CMVIntA(kan)-feEPO (7.3 µg) and transfection reagent[l] (73 µg) were each separately diluted in 800 µL of reduced serum MEM[m] and then mixed (DNA:lipid, 1:10 [wt:wt]) and incubated at room temperature (ie, 25°C) for 30 minutes. The DNA-liposome complexes were then diluted in 5.8 mL of reduced-serum MEM, mixed gently, overlaid on the cells, and incubated at 37°C for 5 hours. The transfection mixture was removed from the cells and replaced with 10 mL of MEM supplemented with penicillin-streptomycin[n] and incubated for an additional 48 hours. The supernatants were then harvested and filtered through a 0.22-µm filter to remove cellular debris.

Protein analysis—Purified **recombinant feEPO (rfeEPO)** was denatured and reduced in SDS-PAGE sample buffer, loaded at a rate of 500 ng/lane on a 14% Tris-glycine gel,[o] and resolved by electrophoresis for 1 hour at 200 V. The protein was developed by use of a protein stain.[p]

Western blot analysis—Supernatants obtained from transiently transfected Chinese hamster ovary cells were concentrated 50-fold by use of a microconcentrator.[q] Supernatants from stable transformants were not concentrated. Supernatants were then denatured and reduced in SDS-PAGE sample buffer, loaded on a 4–20% Tris-glycine gel,[r] and electrophoresed at 200 V for 1 hour. Cell pellets were solubilized in SDS-PAGE sample buffer, heated at 95°C for 5 minutes, and centrifuged through an ultrafree filter[s] to remove genomic DNA. **Recombinant huEPO (rhuEPO)**[t] was denatured and reduced in SDS-PAGE sample buffer, loaded at a rate of 5 to 50 ng/lane on a 4–20% Tris-glycine gel, and electrophoresed at 200 V for 1 hour. Purified rfeEPO was denatured and reduced in SDS-PAGE sample buffer, loaded at a rate of 1 µg/lane on a 14% Tris-glycine gel, and subjected to electrophoresis at 200 V for 1 hour. After electrophoresis, proteins were electroblotted to a nitrocellulose membrane[u] at 100 V for 1.5 hours. Membranes were washed and blocked with blocking solution (Tris-buffered saline solution plus 8% milk powder and 0.1% Tween-20) for 30 minutes at 25°C. After blocking, membranes were incubated overnight with a dilution (1:1,000 by use of blocking solution) of rabbit anti-huEPO polyclonal antibody[v] or a rabbit anti-feEPO polyclonal antibody raised against the purified rfeEPO protein. Membranes were then washed in Tris-buffered saline solution plus 0.1% Tween-20 and incubated for 2 hours with phosphatase-linked goat anti-rabbit IgG (H+L)[w] diluted (1:2,000) in blocking solution. Blots were washed once again and developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt.[x]

Expression and purification of rfeEPO from supernatants—Supernatant derived from Chinese hamster ovary cells transiently transfected with CMVIntA(kan)-feEPO was dialyzed exhaustively against 25mM sodium acetate (pH, 4.5) plus 0.02% Tween-20 by use of 3.5-**kilodaltons (kd) molecular-weight-cutoff (MWCO)** dialysis tubing.[y] Dialyzed samples were then loaded onto a cation-exchange column[z] that had been equilibrated by use of buffer A (25mM sodium acetate [pH, 4.5] plus 0.02% Tween-20). Bound protein was eluted with buffer B (buffer A plus 1M NaCl) for the linear gradients of 0% to 40% buffer B over 15 column volumes and 40% to 100% buffer B over 10 column volumes. The SDS gel electrophoresis and western blot analysis detected EPO in the chromatographic fractions containing 150 to 400mM NaCl. Fractions of EPO were pooled and dialyzed against buffer C (20mM Tris [pH, 8.0] plus 0.02% Tween-20). Dialyzed samples were loaded onto an anion-exchange column[aa] that had been equilibrated by use of buffer C. The column was devel-

oped by use of buffer D (buffer C plus 1M NaCl) for the linear gradients of 0% to 50% buffer D over 15 column volumes and 50% to 100% buffer D over 5 column volumes. The rfeEPO protein was detected in the fractions containing 50 to 350mM NaCl. The rfeEPO fractions were concentrated 10-fold by use of a 2-mL, 3-kd MWCO concentrator.[bb] Concentrated samples were loaded onto a gel filtration column[cc] (1 × 30 cm) that had been equilibrated by use of PBS solution (pH, 7.2) plus 0.02% Tween-20. Fractions were analyzed by use of SDS-PAGE, and those that contained feEPO were pooled and dialyzed against PBS solution without Tween-20.

TF-1 proliferation assay—Cultured TF-1 cells[9,dd] were maintained in complete medium consisting of RPMI 1640 medium[ee] supplemented with 2mM L-glutamine,[ff] 1% penicillin-streptomycin,[n] 5% FBS, and **recombinant human granulocyte-macrophage colony-stimulating factor** (**rhuGM-CSF**[gg]; 2 ng/mL). Cells were cultured in 96-well plates[hh] and incubated at 37°C in a humidified incubator with 5% CO_2. The TF-1 cells were seeded at a rate of 0.5×10^4 or 1×10^4 cells/well in the 96-well plates. Purified rfeEPO or supernatants from Chinese hamster ovary cells transiently or stably transfected with the feEPO plasmid were diluted to the aforementioned concentrations in complete media without rhuGM-CSF. Concentration of rfeEPO within the transient- and stable-transfected supernatants was determined by use of an ELISA. Control cells were incubated with rhuGM-CSF (2 ng/mL). Cells were incubated with rfeEPO or 2 sources of rhuEPO[t,ii] at the indicated doses for 48 hours, pulsed with 1 µCi of ^{3}H-thymidine[jj]/well, and then incubated for an additional 18 hours. In some experiments, rfeEPO was mixed with a dilution (1:1,000) of 5% human serum albumin[kk] or a dilution (1:10) of an electrolyte solution plus 5% dextrose.[ll] Contents of the wells were harvested onto filters consisting of glass fibers and counted in a scintillation counter.[mm] Cell proliferation was measured on the basis of the number of counts per minute of ^{3}H–thymidine-labeled DNA.

Measurement of EPO—A quantitative sandwich ELISA was performed by use of anti-rhuEPO antibodies. Mouse anti-rhuEPO monoclonal antibody[nn] (500 µg/mL; 50 µL/well) diluted in 0.1M bicarbonate buffer was added to 96-well microtiter plates[oo] and incubated overnight at 4°C. Plates were then washed with a combination of PBS solution and 0.05% Tween-20 and then blocked for 1 hour at 37°C with a solution of 1% bovine serum albumin in PBS solution and 0.05% Tween-20 (250 µL/well). After washing, solutions containing feEPO or control samples were added to each well, and plates were then incubated for 1 hour at 37°C. Plates were then washed 3 times with PBS solution and 0.05% Tween-20, and 0.1 µg of rabbit anti-rhuEPO polyclonal[pp] was added (2 µg/mL; 50 µL/well). Goat anti-rabbit H+L peroxidase-labeled antibody[qq] (diluted 1:5,000) was added (50 µL/well). Plates were incubated for 1 hour at 37°C. Plates were then washed and developed with tetramethylbenzidine[rr] for 10 minutes at 25°C; the reaction was stopped by the addition of 1M phosphoric acid. Absorbance for each plate was measured at 450 nm on an ELISA plate reader.[ss] Estimates of the number of units of feEPO and amount of purified feEPO protein per milliliter of supernatant were determined by use of rhuEPO[i] (0.1 U/ng) as a standard. The standard curve was linear from 100 to 800 mU/mL (1 to 8 ng/mL).

Identification of a stable line of Chinese hamster ovary cells for production of rfeEPO—Plasmid CMVIntA(kan)-feEPO and neomycin-resistant plasmid pcDNA3[tt] were linearized by use of *Cla*I[uu] and *Eco*RI,[g] respectively. The day before transfection, Chinese hamster ovary cells were plated (0.6×10^6 cells/well) in multiple-well 6-well plates[vv] by use of 2 mL of Dulbecco's MEM,[ww] 10% FBS,[k] and 1mM NEAA.[j] Cells were cotransfected with 3.5 µg of linearized CMVIntA(kan)-feEPO and 3.5 ng of linearized pcDNA3 by use of 8.5 µL of transfection reagent.[l] Media were changed 1 day after transfection. Three days after transfection, media were harvested and assayed by use of an ELISA to confirm expression of rfeEPO. Cells were exposed to trypsin, diluted (1:4) by use of a solution of G418 media (Dulbecco's MEM, 10% FBS,[k] 1mM NEAA,[j] and 500 µg of a G418-selective antimicrobial[xx]/mL), and allocated to four 10-cm dishes.[yy] Media were changed, and cell layers were monitored to detect cell death and colony formation on days 7, 11, 15, and 19. Distinct colonies were visible by day 19. Colonies were transferred to 48-well plates[zz] in G418 media by use of trypsin-soaked, 6-mm-diameter, circular filters.[aaa] Filters were removed 2 days later or at the time cells had expanded onto the plate surface. Clones that continued to expand were transferred to 12-well plates[bbb] and maintained until confluent in G418 media. Supernatants from clones were assayed to detect relative rfeEPO expression by use an ELISA. Selected clones were expanded and assessed for expression in low-serum and serum-free conditions. Clone CE-7 was selected for long-term stable expression in serum-free conditions. Clone CE-7 was plated in flasks[ccc] by use of serum-containing conditions. Once 100% confluence was reached, media were removed, and cell layers were washed once with PBS solution. We then added 70 mL of serum-free media[ddd] that contained 2mM L-glutamine[ff] and 500 µg of G418-selective antimicrobial[xx]/mL to each flask. Media were harvested and replaced at 3-day intervals (through day 15) with fresh serum-free media. Harvested media were stored at –20°C. Proteins were developed for western blot analysis by use of rabbit anti-huEPO antibody, as described previously.

Statistical analysis—Differences between groups were evaluated by use of an ANOVA[eee] appropriate for a 2-factor experiment, with dose, group, and the dose-by-group interaction included as fixed effects. Culture well was included as the experimental unit. When there was a significant dose-by-group interaction, within-dose effects were evaluated by use of a *t* test. Differences were considered significant for values of $P \leq 0.05$.

Results

Sequence of feEPO and huEPO proteins—The feEPO protein sequence determined in the study reported here was compared with the published feEPO and huEPO protein sequences (**Fig 1**). The full-length feEPO protein was identical to the GenBank protein sequence (U00685), except for a single amino acid change at position 44 in which glutamine replaced glycine. This single amino acid change is similar to that of another GenBank feEPO protein sequence (L10606).[7] The percentage identity between the feEPO sequence cloned and reported here (EPOCAT.AMI) and huEPO (EPOHUM.AMI; 224437) was 84.4%, as determined by comparison of the amino acid alignment.

Expression of feEPO in Chinese hamster ovary cells—A cDNA fragment encoding feEPO, including the native signal sequence, was subcloned into the CMV-IntA-(kan) vector to produce the plasmid CMVIntA(kan)-feEPO. Chinese hamster ovary cells were transiently transfected with this construct, and cells and tissue culture supernatants were collected 48 hours after transfection. The SDS-PAGE and western blot analysis of transfected cells and supernatants,

accomplished by use of polyclonal antibodies against rhuEPO, confirmed there was rfeEPO in the supernatant and cells (**Fig 2**). Recombinant feEPO secreted from Chinese hamster ovary cells and detected in the supernatant migrated on SDS-PAGE as a broad band of approximately 30 to 40 kd, whereas nonsecreted feEPO in cell extracts migrated as a concise band at 25 kd. Calculated molecular weight of unglycosylated feEPO is 21.5 kd, consistent with the observed mass of feEPO in the cells. Analysis of these data suggests that the broad, diffuse band observed in the supernatant was heterogeneously glycosylated rfeEPO. Recombinant huEPO was resolved as a 38-kd band, which is slightly higher than the molecular weight of 34 kd reported in another study.[5]

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U00685.AMI          1 MGSCECPALL -LLLSLLLLP LGLPVLGAPP RLICDSRVLE RYILGAREAE
L10606.AMI          1 ----..... -......... ......... ......... ....E.....
EPOCAT.AMI          1 ......... -......... ......... ......... ....E.....
EPOHUM.AMI          1 ..VH....W. W......S.. ......... ......... ..L.E.K...
                        60        70        80        90        100
U00685.AMI         51 NVTMGCAEGC SFSENITVPD TKVNFYTWKR MDVGQQAVEV WQGLALLSEA
L10606.AMI         51 ......... ......... ......... ......... .........
EPOCAT.AMI         51 ......... ......... ......... ......... .........
EPOHUM.AMI         51 .I.T....H. .LN..... ......A... .E........ .........
                       110       120       130       140       150
U00685.AMI        101 ILRGQALLAN SSQPSETLQL HVDKAVSSLR SLTSLLRALG AQKEATSLPE
L10606.AMI        101 ......... ......... ......... ......... .........
EPOCAT.AMI        101 ......... ......... ......... ......... .........
EPOHUM.AMI        101 V.......V. ....W.P... .......G.. ...T...... .....I.P.D
                       160       170       180       190       200
U00685.AMI        151 ATSAAPLRTF TVDTLCKLFR IYSNFLRGKL TLYTGEACRR GDR
L10606.AMI        151 ......... ......... ......... ......... ...
EPOCAT.AMI        151 ......... ......... ......... ......... ...
EPOHUM.AMI        151 .A.......I .A..FR.... V......... K........T ...
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Figure 1—Amino acid sequences of erythropoietin (EPO) for cats and humans. U00685.AMI = Nucleotide entry in GenBank for feline EPO (feEPO) mRNA. L10606.AMI = GenBank feEPO protein sequence. EPOCAT.AMI = Nucleotide sequence for feEPO described in the study reported here. EPOHUM.AMI = Nucleotide sequence for human EPO.

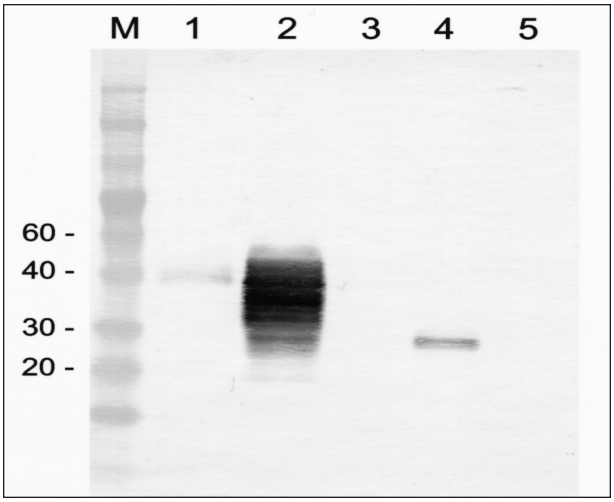

Figure 2—Western blot analysis of the expression of recombinant feEPO (rfeEPO) in Chinese hamster cells transiently transfected with a eukaryotic expression plasmid (eg, CMVIntA[kan]-feEPO) and in supernatants collected from such cells. Cell and supernatant samples were denatured and reduced in SDS-PAGE sample buffer and assayed on a Tris-glycine gel. Proteins were then electroblotted onto a nitrocellulose membrane and analyzed by western blot analysis. The primary antibody was rabbit anti-human EPO polyclonal antibody, and the secondary antibody was goat anti-rabbit IgG (H+L) antibody. Lanes were as follows: M, molecular weight standards; 1, 5 ng of recombinant human EPO (rhuEPO); 2, 500 µL of supernatant collected from Chinese hamster ovary cells transiently transfected with CMVIntA(kan)-feEPO; 3, 500 µL of supernatant collected from Chinese hamster ovary cells transiently transfected with an empty vector; 4, 2×10^5 Chinese hamster ovary cells transiently transfected with CMVIntA(kan)-feEPO; and 5, 2×10^5 Chinese hamster ovary cells transiently transfected with an empty vector. Numbers on the left side represent number of kilodaltons.

Purification of rfeEPO from supernatants—Purification of rfeEPO from supernatant derived from Chinese hamster ovary cells transiently transfected with the CMVIntA(kan)-feEPO plasmid was accomplished by initial dialysis that precipitated out impurities in the supernatant; this was followed by cation-exchange chromatography, anion-exchange chromatography, and size-exclusion chromatography. Approximately 400 µg of pure (> 95%) EPO was derived from 300 mL of supernatant from the transiently transfected Chinese hamster ovary cells.

The SDS-PAGE analysis of rfeEPO protein revealed that it migrated as a broad, diffuse band of approximately 34 to 40 kd (**Fig 3**). A number of lower molecular weight impurities were detected in the overloaded gel. Immunoreactivity with polyclonal antibodies derived against rhuEPO or rfeEPO confirmed that the diffuse protein was EPO.

Selection and characterization of a stable cell line expressing rfeEPO—A stable line of Chinese ovary cells expressing rfeEPO was selected. Chinese hamster ovary cells were cotransfected with CMVIntA(kan)-feEPO and pcDNA3. After selection of drug-resistant clones, each was tested for expression of rfeEPO protein. Clones were isolated and chosen on the basis of the relative amount of rfeEPO expression, and selected clones were expanded. Clone CE-7 was chosen on the

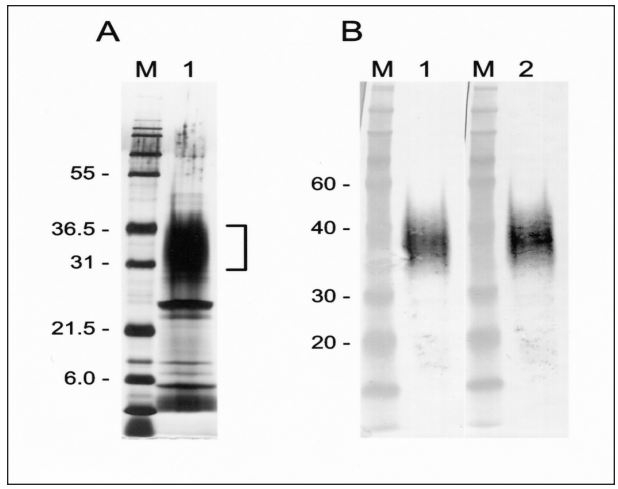

Figure 3—Purified rfeEPO analyzed by use of SDS-PAGE (A) and western blot analysis (B). For the SDS-PAGE analysis, molecular weight standards (lane M) and 2 µg of purified rfeEPO (lane 1) were subjected to SDS-PAGE under reducing conditions and stained by use of a protein stain. For the western blot analysis, samples (lane M, molecular weight standards; lanes 1 and 2, 1 µg of purified rfeEPO) were analyzed by SDS-PAGE under reducing conditions, followed by incubation with antibodies. Primary antibody for lane 1 was rabbit anti-human EPO polyclonal antibody, and primary antibody for lane 2 was rabbit anti-feline EPO polyclonal antibody; secondary antibody for both was goat anti-rabbit IgG (H+L) AP antibody. Brackets indicate rfeEPO protein. Numbers on the left side of each gel represent number of kilodaltons.

basis of its growth characteristics and ability for long-term expression of rfeEPO in serum-free conditions. Successive supernatant aliquots of harvested CE-7 samples were obtained on days 3, 9, 12, and 15 and assayed for protein by use of an ELISA (data not shown) and western blot analysis (**Fig 4**). The relative amount of protein expression by CE-7 cells appeared to increase with time in culture and was comparable to the amount of protein expressed by feEPO-transiently transfected Chinese hamster ovary cells.

Biological activity of rfeEPO in supernatants from transfected cells and in purified rfeEPO—To assess biological activity of the transient and stable cell-expressed rfeEPO and the purified rfeEPO, proliferative effects of these proteins were tested on the growth-factor-dependent, human-erythroleukemic, TF-1 cell line.[9] As expected, TF-1 cells were able to proliferate in the presence of rhuEPO, which was subsequently used as a positive-control sample (**Fig 5**). Supernatants from transiently transfected Chinese hamster ovary cells expressing rfeEPO were able to induce proliferation of TF-1 that was generally not significantly different from induction of proliferation detected for rhuEPO.

Purified rfeEPO induced significant proliferation of TF-1 cells; however, the response was significantly decreased, compared to the response for rhuEPO (**Fig 6**). This decreased activity appeared to be attributable to instability as a result of a lack of a carrier protein.

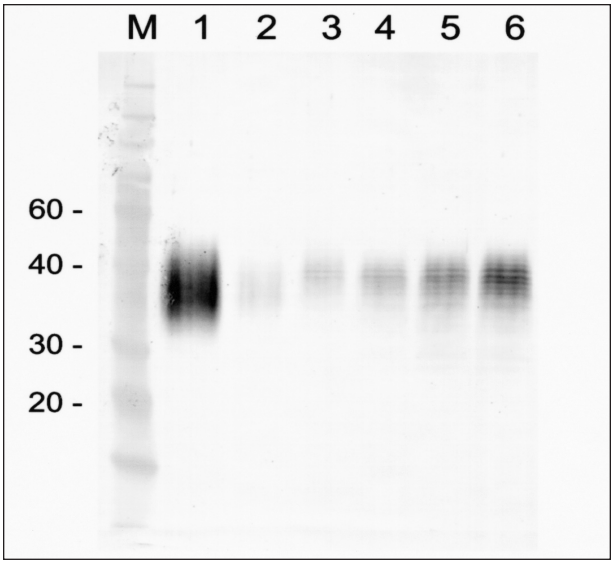

Figure 4—Western blot analysis of the expression of rfeEPO from a stable line of Chinese hamster ovary cells transfected with CMVIntA(kan)-feEPO. Supernatants from a stable cell line that expressed rfeEPO (ie, CE-7) were denatured and reduced in SDS-PAGE sample buffer and assayed on a Tris-glycine gel. Proteins were then electroblotted onto a nitrocellulose membrane and analyzed by use of western blot analysis. Primary antibody was rabbit anti-human EPO polyclonal antibody, and secondary antibody was goat anti-rabbit IgG (H+L) antibody. Lanes were as follows: M, molecular weight standards; 1, 50 ng of purified rfeEPO; 2, 5 ng of purified rfeEPO; 3,10 μL of CE-7 supernatant harvested on day 3 of culture; 4, 10 μL of CE-7 supernatant harvested on day 9 of culture; 5, 10 μL of CE-7 supernatant harvested on day 12 of culture; and 6, 10 μL of CE-7 supernatant harvested on day 15 of culture. Numbers on the left side represent number of kilodaltons.

All aliquots from supernatants collected from cells stably transfected with CMVIntA(kan)-feEPO had similar activity in the TF-1 assay, compared with activity for supernatants collected from cells transiently transfected with CMVIntA(kan)-feEPO (**Fig 7**).

Assessment of biological activity of rfeEPO in various solutions—To determine whether the biological performance of purified rfeEPO could be enhanced by the addition of a stabilizer, purified rfeEPO was mixed with saline (0.9% NaCl) solution, 5% dextrose solution, or a 5% solution of human serum albumin and tested for activity in the TF-1 proliferation assay. Proliferation of TF-1 cells induced by rfeEPO-human serum albumin solution was significantly higher than activity seen for rfeEPO-saline solution (**Fig 8**). The rfeEPO-dextrose solution resulted in significantly increased activity only at the lower doses of protein (6.25 and 12.5 ng/mL). At the higher doses of protein (25 and 50 ng/mL), the addition of dextrose solution significantly reduced pro-

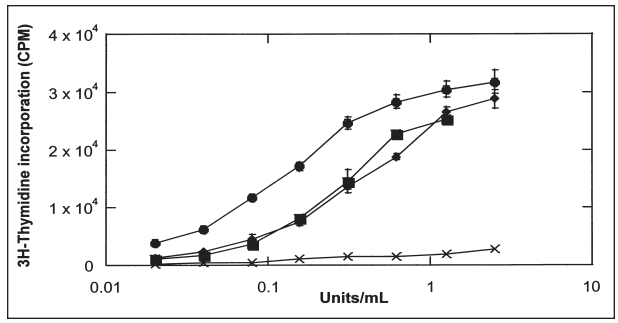

Figure 5—Effects of rhuEPO and supernatant collected from cultured Chinese hamster ovary cells on proliferative activity of TF-1 cells. The TF-1 cells were incubated for 48 hours with 2 commercially available EPO products (rhuEPO[i] [square] or rhuEPO[ii] [circle]), supernatant collected from Chinese hamster ovary cells transiently transfected with CMVIntA-feEPO (diamond), or supernatant collected from Chinese hamster ovary cells transiently transfected with an empty vector (cross). All cells were then pulsed with [^{3}H]thymidine. All assay points were performed in duplicate, and results represent mean ± SD. CPM = Counts per minute.

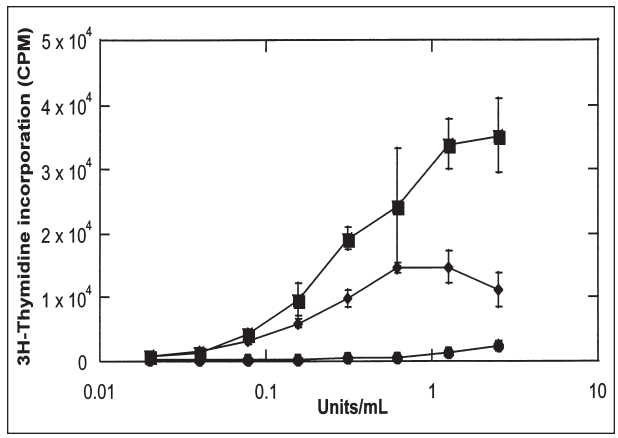

Figure 6—Effects of rhuEPO and purified rfeEPO on proliferative activity of TF-1 cells. The TF-1 cells were incubated for 48 hours with rhuEPO[ii] (square), purified rfeEPO (diamond), or supernatant collected from Chinese hamster ovary cells transiently transfected with an empty vector (circle) for 48 hours. All cells were then pulsed with [^{3}H]thymidine. All assay points were performed in duplicate, and results represent mean ± SD.

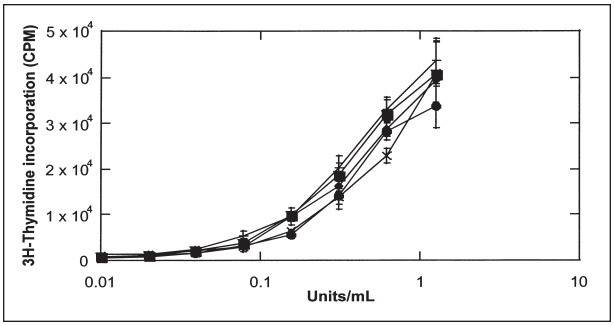

Figure 7—Effect of rfeEPO on proliferative activity as measured in supernatants from a stable line of Chinese hamster ovary cells transfected with CMVIntA(kan)-feEPO (ie, CE-7). Supernatant was harvested on day 3 (circle), 9 (square), 12 (diamond), and 15 (cross) after transfection. Estimated values for rfeEPO in supernatants from stably or transiently transfected Chinese hamster ovary cells were determined by use of an ELISA. All cells were pulsed with [^{3}H]thymidine. All assay points were performed in duplicate, and results represent mean ± SD.

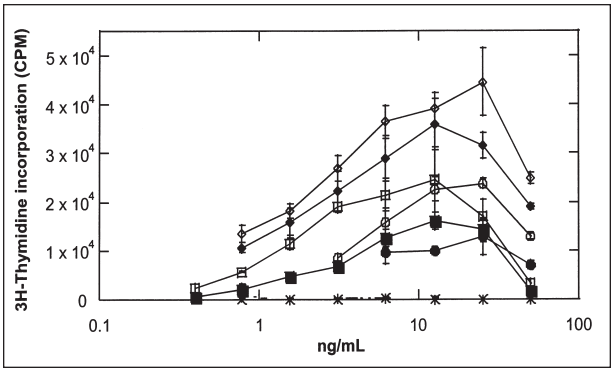

Figure 8—Effects of purified rfeEPO in various solutions on proliferative activity as measured in the TF-1 assay. Purified rfeEPO was mixed in saline (0.9% NaCl) solution and treated at 37°C (solid circle) or 4°C (open circle), 5% dextrose solution and treated at 37°C (solid square) or 4°C (open square), or 5% human serum albumin solution and treated at 37°C (solid diamond) or 4°C (open diamond). The TF-1 cells were also cultured in 5% dextrose solution (cross) and 5% human serum albumin solution (plus sign). All cells were pulsed with [^{3}H]thymidine. All assay points were performed in triplicate, and results represent mean ± SD.

liferation, compared with that for rfeEPO mixed in saline solution. A significant reduction in proliferation was observed among all heat-treated rfeEPO mixtures, compared with proliferation for the corresponding rfeEPO mixtures treated at 4°C.

Discussion

The study reported here described the cloning of the gene that encodes feEPO, expression and purification of rfeEPO protein from transiently transfected Chinese hamster ovary cells, and development of a stable line of Chinese hamster ovary cells that express rfeEPO. Regardless of the expression system used, the resulting rfeEPO was biologically active in vitro.

One of the predominant clinical signs secondary to CRF in cats is anemia.[1,3,4] Progressive destruction of functional renal parenchyma reduces EPO production, resulting in erythroid hypoplasia within the bone marrow.[4] Administration of huEPO can counteract this deficiency and improve the overall well-being of affected animals; however, this treatment cannot reverse the progression of CRF.[1] Treatment with huEPO can alleviate many of the clinical signs associated with anemia in domestic animals with CRF, including fatigue, lethargy, and inappetence.[2] A retrospective study[10] of cats with CRF revealed that 30 of 74 (41%) cats included in that study developed nonregenerative anemia (PCV < 27%). Predominant clinical signs detected by owners of the cats in that study included lethargy, anorexia, and weight loss.

Expression and biological activity of recombinant caEPO[6] and rfeEPO[7] have been reported. Human EPO is approximately 86% identical to caEPO and 85% identical to feEPO. Biological activity of recombinant caEPO expressed in Chinese hamster ovary cells was determined in vitro by measuring stimulation of division in murine erythroid progenitor cells and in vivo by stimulation of reticulocytosis in mice.[6] Recombinant feEPO was expressed from an AAV construct (ie, AAV-feEPO) after transduction in vitro and after injection in vivo.[7] Biological activity of rfeEPO expressed in vivo by AAV-feEPO was determined by use of hematocrit percentages.[7] Recombinant feEPO expressed from the vector successfully increased hematocrit percentages in immunodeficient mice and healthy cats; however, significant increases in the hematocrit percentage following gene therapy required a high dose of AAV-feEPO.[7] Probably the most important disadvantage of AAV-vectored gene therapy is that this form of gene therapy is biologically poorly regulated, making it difficult to consistently deliver the lowest effective therapeutic dose that would be achievable with a purified protein.

In mature humans, huEPO protein consists of 166 amino acids and has a molecular weight of 34 kd; nearly 40% of the molecular weight is carbohydrate. Glycosylation of the huEPO protein includes 1 serine *O*-linked oligosaccharide and 3 asparagine *N*-linked oligosaccharides and is necessary for the biological activity of the protein.[11] The full-length rfeEPO protein described in the study reported here was 192 amino acids, whereas predicted molecular weight of the mature rfeEPO protein in humans (166 amino acids) is only 21.5 kd. The major band of purified rfeEPO was estimated to be 34 to 40 kd, which is larger than the predicted molecular weight attributable to glycosylation. When the purified rfeEPO protein was analyzed by use of western blot analysis, the majority of the protein was estimated to be 36 to 38 kd.

Biological activities of purified rfeEPO and rfeEPO in supernatants of Chinese hamster ovary cells transfected with CMVIntA(kan)-feEPO were determined in vitro by measuring the induction of proliferation in TF-1 cells. Although the erythroleukemic TF-1 cells were of human origin, rfeEPO was capable of binding to the receptors for EPO and inducing a proliferative response. The rfeEPO in supernatants of transfected cells was as effective as rhuEPO at inducing proliferation of TF-1 cells.

Purified rfeEPO was also able to induce proliferation of TF-1 cells. However, purified rfeEPO protein had significantly lower activity than rfeEPO in the crude transiently transfected supernatants. Purified rfeEPO protein may have lost activity as a result of the purification process. Alternatively, exogenous proteins in the cell supernatants may have enhanced the biological activity of rfeEPO. Comparison of rfeEPO

mixed with dextrose, human serum albumin, or saline solution indicated that albumin seemed to restore full biological activity to the purified protein.

Production of EPO from transiently transfected Chinese hamster ovary cells is an arduous task, requiring a large commitment of personnel and reagents. Other investigators have reported[6] a stable line of Chinese hamster ovary cells that expresses caEPO. However, we are not aware of any reports of a stable line of Chinese hamster ovary cells that expresses feEPO. The study reported here describes the development of a stable line of Chinese hamster ovary cells that expresses rfeEPO. Recombinant feEPO protein from this stable cell line was found to be slightly larger than purified rfeEPO, as determined on the basis of western blot analysis, and was effective at inducing a proliferative effect in the TF-1 cell assay equivalent to that seen with transiently expressed feEPO. A stable cell line that expresses rfeEPO could be an important tool for the production of large quantities of rfeEPO. A species-specific EPO protein could facilitate treatment of cats with anemia resulting from CRF.

[a]GenBank sequence entry U00685, NCBI, Bethesda, Md.
[b]TA cloning kit, Invitrogen, Carlsbad, Calif.
[c]ABI PRISM model 377 and reaction kit, PE Applied Biosystems, Foster City, Calif.
[d]GenBank sequence entry I46083, NCBI, Bethesda, Md.
[e]*Hin*c II, New England Biolabs Inc, Beverly, Mass.
[f]*Spe* I, New England Biolabs Inc, Beverly, Mass.
[g]*Eco*R I, New England Biolabs Inc, Beverly, Mass.
[h]Chinese hamster ovary cells, American Type Culture Collection, Manassas, Va.
[i]MEM, Gibco BRL, Gaithersburg, Md.
[j]NEAA, Sigma Chemical Co, St Louis, Mo.
[k]Fetal bovine serum, Summit Biotechnology, Fort Collins, Colo.
[l]LipofectAMINE 2000, Gibco BRL, Gaithersburg, Md.
[m]OPTI-MEM, Gibco BRL, Gaithersburg, Md.
[n]Penicillin-streptomycin, Gibco BRL, Gaithersburg, Md.
[o]14% Tris-glycine gel, Invitrogen, Carlsbad, Calif.
[p]SimplyBlue stain, Invitrogen, Carlsbad, Calif.
[q]Microcon YM-10, Millipore, Bedford, Mass.
[r]4–20% Tris-glycine gel, Invitrogen, Carlsbad, Calif.
[s]Ultrafree filter, Millipore, Bedford, Mass.
[t]rhuEPO, R&D Systems Inc, Minneapolis, Minn.
[u]Protran nitrocellulose membrane, Schleicher & Schuell, Keene, NH.
[v]Rabbit anti-huEPO polyclonal antibody, Accurate Chemical & Scientific Corp, San Diego, Calif.
[w]Phosphatase-linked goat anti-rabbit IgG (H+L), Kirkegaard & Perry Laboratories, Gaithersburg, Md.
[x]BCIP/NBT substrate, Gibco BRL, Gaithersburg, Md.
[y]Spectra/Por 3.5-kd molecular weight cutoff dialysis tubing, Spectrum Laboratories Inc, Rancho Dominguez, Calif.
[z]HiTrap SP sepharose, Amersham Pharmacia, Leppsala, Sweden.
[aa]HiTrap HP sepharose, Amersham Pharmacia, Leppsala, Sweden.
[bb]Centricon, Millipore, Bedford, Mass.
[cc]Superdex 200 column, Amersham Pharmacia, Piscataway, NJ.
[dd]TF-1 cells, R&D Systems Inc, Minneapolis, Minn.
[ee]RPMI 1,640 medium, Sigma Chemical Co, St Louis, Mo.
[ff]L-glutamine, Gibco BRL, Gaithersburg, Md.
[gg]Recombinant human granulocyte-macrophage colony-stimulating factor, R&D Systems Inc, Minneapolis, Minn.
[hh]Costar 96-well plates, Corning Inc, Corning, NY.
[ii]Epogen, Amgen, Thousand Oaks, Calif.
[jj]^{3}H-thymidine, ICN Pharmaceuticals, Irvine, Calif.
[kk]5% human serum albumin, Baxter Healthcare Corp, Glendale, Calif.
[ll]Electrolyte solution plus 5% dextrose, Vedco Inc, St Joseph, Mo.
[mm]MicroBeta Trilux 1450 scintillation counter, Wallac Inc, Gaithersburg, Md.
[nn]Mouse anti-rhuEPO monoclonal antibody, R&D Systems Inc, Minneapolis, Minn.
[oo]Immunlon-2 HB, Dynex Technologies Inc, Chantilly, Va.
[pp]Rabbit anti-rhuEPO polyclonal, R&D Systems Inc, Minneapolis, Minn.
[qq]Goat anti-rabbit H+L peroxidase-labeled antibody, Kirkegaard & Perry Laboratories, Gaithersburg, Md.
[rr]TMB, Kirkegaard & Perry Laboratories, Gaithersburg, Md.
[ss]SPECTRAmax 250 ELISA plate reader, Molecular Devices Corp, Sunnyvale, Calif.
[tt]Neomycin-resistant plasmid pcDNA3, Invitrogen, Carlsbad, Calif.
[uu]*Cla*I, New England Biolabs Inc, Beverly, Mass.
[vv]Falcon multiwell 6-well plate, Becton-Dickinson Co, Franklin Lakes, NJ.
[ww]DMEM, Gibco BRL, Gaithersburg, Md.
[xx]Geneticin selective antibiotic, Gibco BRL, Gaithersburg, Md.
[yy]Falcon 10-cm dishes, Becton-Dickinson Co, Franklin Lakes, NJ.
[zz]Falcon 48-well plates, Becton-Dickinson Co, Franklin Lakes, NJ.
[aaa]6-mm-diameter circular filters, Whatman International Ltd, Maidstone, England.
[bbb]12-well plates, Becton-Dickinson Co, Franklin Lakes, NJ.
[ccc]Falcon tri-level T-500 flasks, Becton-Dickinson Co, Franklin Lakes, NJ.
[ddd]Cellgro-Free media, Mediatech, Herndon, Va.
[eee]SAS, version 8.1, SAS Institute Inc, Cary, NC.

References

1. Giger U. Erythropoietin and its clinical use. *Compend Contin Educ Pract Vet* 1992;14:25–34.

2. Cowgill LD, James KM, Levy JK, et al. Use of recombinant human erythropoietin for management of anemia in dogs and cats with renal failure. *J Am Vet Med Assoc* 1998;212:521–528.

3. Cowgill LD. Erythropoietin: its use in the treatment of chronic renal failure in dogs and cats, in *Proceedings*. Annu Waltham-Ohio State Univ Symp Treat Small Anim Dis 1991;15:65–71.

4. Cowgill LD. Medical management of the anemia of chronic renal failure. In: Osborne CA, Finco DR, eds. *Canine and feline nephrology and urology*. Baltimore: The Williams & Wilkins Co, 1995;538–554.

5. Wen D, Boissel JP, Tracy TE, et al. Erythropoietin structure-function relationships: high degree of sequence homology among mammals. *Blood* 1993;82:1507–1516.

6. MacLeod JN, Tetreault JW, Lorschy KA, et al. Expression and bioactivity of recombinant canine erythropoietin. *Am J Vet Res* 1998; 59:1144–1148.

7. Beall CJ, Phipps AJ, Mathes LE, et al. Transfer of the feline erythropoietin gene to cats using a recombinant adeno-associated virus vector. *Gene Ther* 2000;7:534–539.

8. Osorio JE, Tomlinson CC, Frank RS, et al. Immunization of dogs and cats with a DNA vaccine against rabies virus. *Vaccine* 1999; 17:1109–1116.

9. Kitamura T, Tange T, Terasawa T, et al. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3 or erythropoietin. *J Cell Physiol* 1989; 140:323–334.

10. DiBartola SP, Rutgers HC, Zack PM, et al. Clinicopathologic findings associated with chronic renal disease in cats: 74 cases (1973–1984). *J Am Vet Med Assoc* 1987;190:1196–1202.

11. Wasley LC, Timony G, Murtha P, et al. The importance of N- and O-linked oligosaccharides for the biosynthesis and in vitro and in vivo biologic activities of erythropoietin. *Blood* 1991;77:2624–2632.