TABLE I

T0300X

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

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B. Design and Construction of Oligonucleotide Probe Mixtures

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

TC310X

TABLE II

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

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

TC311X

TABLE III

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

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Oligonucleotide probes were labelled at the 5' end with gamma - ^{32}P-ATP, 7500-8000 Ci/mmole (ICN) using T_4 polynucleotide kinase (NEN).

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EXAMPLE 2

A. Monkey Treatment Procedures ~~and RIA Analysis~~

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

B. RIA for EPO

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

An erythropoietin standard or unknown sample was incubated together with antiserum for two hours at 37°C.
25 After the two hour incubation, the sample tubes were cooled on ice, ^{125}I-labelled erythropoietin was added, and the tubes were incubated at 0°C for at least 15 more hours. Each assay tube contained 500 ul of incubation mixture consisting of 50 ul of diluted immune sera,
30 10,000 cpm of ^{125}I-erythropoietin, 5 µl trasylol and 0-250 µl of either EPO standard or unknown sample, with PBS containing 0.1% BSA making up the remaining volume. The antiserum used was the second test bleed of a rabbit

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immunized with a 1% pure preparation of human urinary erythropoietin. The final antiserum dilution on the assay was adjusted so that the antibody-bound ^{125}I-EPO did not exceed 10-20% of the input total counts. In general, this corresponded to a final antiserum dilution of from 1:50,000 to 1:100,000.

The antibody-bound ^{125}I-erythropoietin was precipitated by the addition of 150 µl Staph A. After a 40 min. incubation, the samples were centrifuged and the pellets were washed two times with 0.75 ml 10 mM Tris-HCl pH 8.2 containing 0.15M NaCl, 2mM EDTA, and 0.05% Triton X-100. The washed pellets were counted in a gamma counter to determine the percent of ^{125}I-erythropoietin bound. Counts bound by pre-immune sera were subtracted from all final values to correct for nonspecific precipitation. The erythropoietin content of the unknown samples was determined by comparison to the standard curve.

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

EXAMPLE 3

A. Monkey cDNA Library Construction

Messenger RNA was isolated from normal and anemic monkey kidneys by the guanidinium thiocyanate procedure of Chirgwin, et al., Biochemistry, 18, p. 5294 (1979) and poly (A)$^+$ mRNA was purified by two runs of oligo(dT)-cellulose column chromatography as described at pp. 197-198 in Maniatis, et al., "Molecular Cloning, A Laboratory Manual" (Cold Springs Harbor Laboratory, Cold Springs, Harbor, N.Y., 1982). The cDNA library was constructed according to a modification of the general pro-

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cedures of Okayama, et al., Mol. and Cell.Biol., 2, pp. 161-170 (1982). The key features of the presently preferred procedures were as follows: (1) pUC8 was used as the sole vector, cut with PstI and then tailed with
5 oligo dT of 60-80 bases in length; (2) HincII digestion was used to remove the oligo dT tail from one end of the vector; (3) first strand synthesis and oligo dG tailing was carried out according to the published procedure; (4) BamHI digestion was employed to remove the oligo dG tail
10 from one end of the vector; and (5) replacement of the RNA strand by DNA was in the presence of two linkers (GATCTAAAGACCGTCCCCCCCCC and ACGGTCTTTA) in a three-fold molar excess over the oligo dG tailed vector.

15 B. Colony Hybridization Procedures For Screening Monkey cDNA Library

Transformed E.coli were spread out at a density of 9000 colonies per 10 x 10 cm plate on nutrient plates containing 50 micrograms/ml Ampicillin. GeneScreen
20 filters (New England Nuclear Catalog No. NEF-972) were pre-wet on a BHI-CAM plate (Bacto brain heart infusion 37 g/L, Casamino acids 2 g/L and agar 15 g/L, containing 500 micrograms/ml Chloramphenicol) and were used to lift the colonies off the plate. The colonies were grown in the
25 same medium for 12 hours or longer to amplify the plasmid copy numbers. The amplified colonies (colony side up) were treated by serially placing the filters over 2 pieces of Whatman 3 MM paper saturated with each of the following solutions:
30 (1) 50 mM glucose - 25 mM Tris-HCl (pH 8.0) - 10 mM EDTA (pH 8.0) for five minutes;

(2) 0.5 M NaOH for ten minutes; and

(3) 1.0 M Tris-HCl (pH 7.5) for three minutes.

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

The filters were then subjected to Proteinase K

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

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

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

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

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

Initial sequence analysis of one of the putative monkey cDNA clones (designated clone 83) was performed for verification purposes by a modification of the procedure of Wallace, et al., Gene, 16, pp. 21-26 (1981).
35 Briefly, plasmid DNA from monkey cDNA clone 83 was linearized by digestion with EcoRI and denatured by

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heating in a boiling water bath. The nucleotide sequence was determined by the dideoxy method of Sanger, et al., P.N.A.S. (U.S.A.), 74, pp. 5463-5467 (1977). A subset of the EPV mixture of probes consisting of 16 sequences was used as a primer for the sequencing reactions.

C. Monkey EPO cDNA Sequencing

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

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TABLE IV

T0370X

	Restriction Enzyme Recognition Site	Approximate Location(s)
5	EcoRI	1
	Sau3A	111
	SmaI	180
	BstEII	203
	SmaI	324
10	KpnI	371
	RsaI	372
	AluI	424
	PstI	426
	AluI	430
15	HpaI	466
	AluI	546
	PstI	601
	PvuII	604
	AluI	605
20	AluI	782
	AluI	788
	RsaI	792
	PstI	807
	AluI	841
25	AluI	927
	NcoI	946
	Sau3A	1014
	AluI	1072
	AluI	1115
30	AluI	1223
	PstI	1301
	RsaI	1343
	AluI	1384
	HindIII	1449
35	AluI	1450
	HindIII	1585

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

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TABLE V

Translation of Monkey EPO cDNA

```
Sau3A
GATCCCGCGCCCCCTGGACAGCCGCCCTCTCCTCCAGGCCCGTGGGGCTGGCCCTGCCC

CGCTGAACTTCCCGGGATGAGGACTCCCGGTCTGGTCACCGCGCGCCTAGGTCGCTGAG

                          -27                      -20
                          Met Gly Val His Glu Cys Pro Ala Trp
GGACCCCGGCCAGGCGCGGAGATG GGG GTC CAC GAA TGT CCT GCC TGG

                                    -10
Leu Trp Leu Leu Leu Ser Leu Val Ser Leu Pro Leu Gly Leu Pro
CTG TGG CTT CTC CTG TCT CTC GTG TCG CTC CCT CTG GGC CTC CCA

        -1  +1                                  10
Val Pro Gly Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu
GTC CCG GGC GCC CCA CCA CGC CTC ATC TGT GAC AGC CGA GTC CTG

                            20              *
Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Val Thr Met
GAG AGG TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT GTC ACG ATG

        30                              *   40
Gly Cys Ser Glu Ser Cys Ser Leu Asn Glu Asn Ile Thr Val Pro
GGC TGT TCC GAA AGC TGC AGC TTG AAT GAG AAT ATC ACC GTC CCA
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