TABLE XI

ECEPO SECTION 2

TABLE XII

ECEPO SECTION 3

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

20

25

TABLE XIII

ECEPO SECTION 3

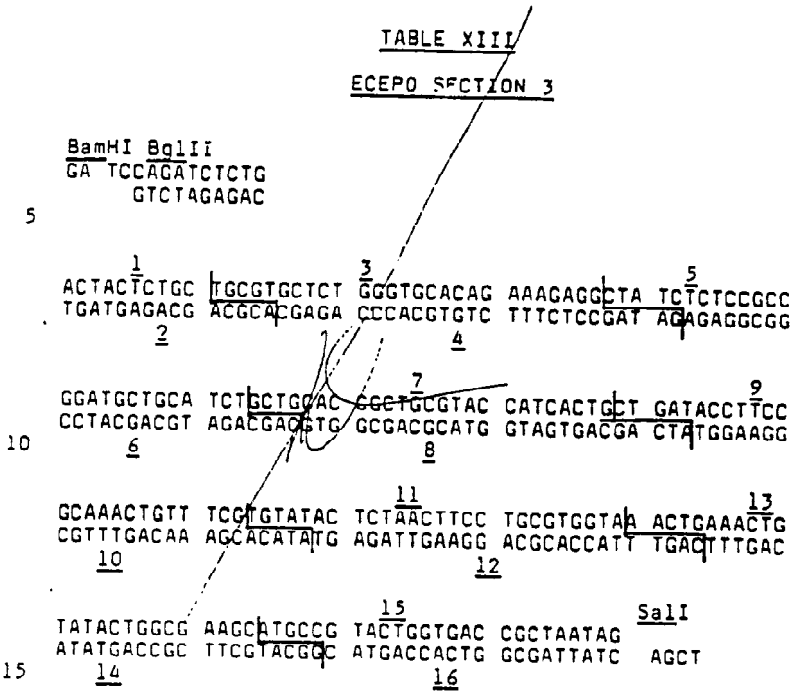

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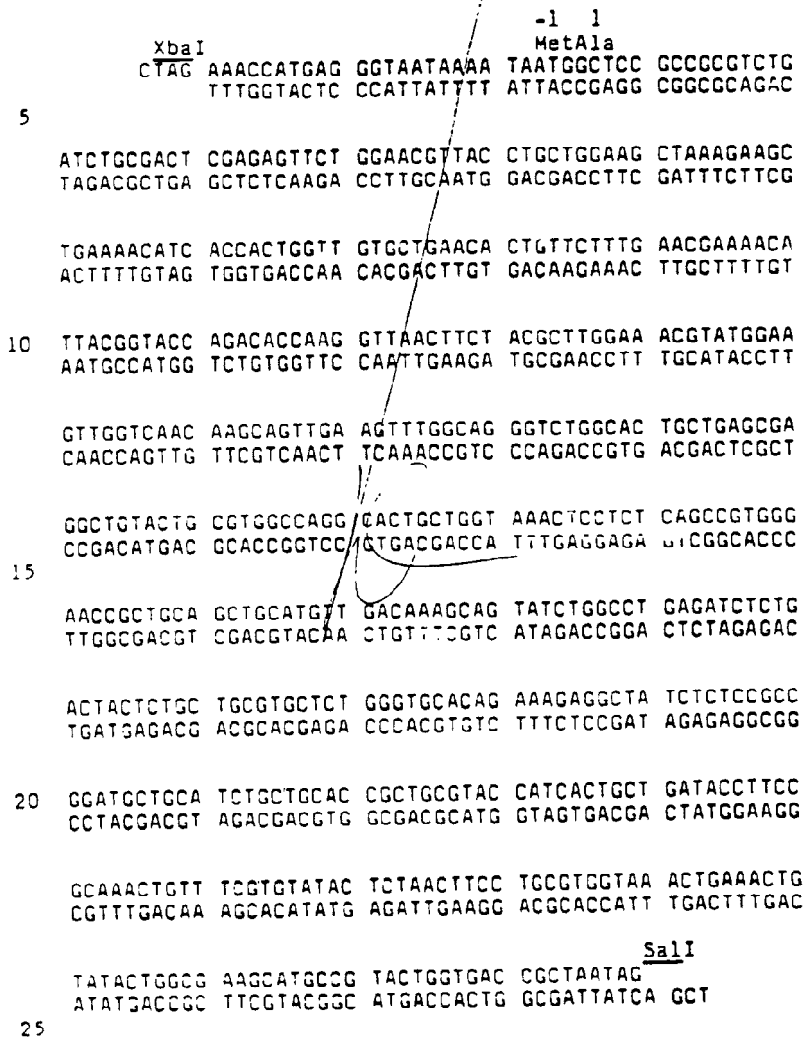
TABLE XIV

ECEPO GENE



More particularly, Figure 10 illustrates oligonucleotides employed to generate the Section 1 of the ECEPO gene encoding amino terminal residues of the human species polypeptide. Oligonucleotides were assembled into duplexes (1 and 2, 3 and 4, etc.) and the duplexes were then ligated to provide ECEPO Section 1 as in Figure 11. Note that the assembled section includes respective terminal EcoRI and BamHI sticky ends, that "downstream" of the EcoRI sticky end is a XbaI restriction enzyme recognition site; and that "upstream" of the BamHI sticky end is a KpnI recognition site. Section 1 could readily be amplified using the M13 phage vector employed for verification of sequence of the section. Some difficulties were encountered in isolating the section as an XbaI/KpnI fragment from RF DNA generated in E.coli, likely due to methylation of the KpnI recognition site bases within the host. Single-stranded phage DNA was therefore isolated and rendered into double-stranded form in vitro by primer extension and the desired double-stranded fragment was thereafter readily isolated.

ECEPO gene Sections 2 and 3 (Figures 13 and 15) were constructed in a similar manner from the oligonucleotides of Figures 12 and 14, respectively. Each section was amplified in the M13 vector employed for sequence verification and was isolated from phage DNA. As is apparent from Figure 13, ECEPO Section 2 was constructed with EcoRI and BamHI sticky ends and could be isolated as a K[illegible]/BglII fragment. Similarly, ECEPO Section 3 was prepared with BamHI and SalI sticky ends and could be isolated from phage RF DNA as a BglII/SalI fragment. The three sections thus prepared can readily be assembled into a continuous DNA sequence (Figure 7) encoding the entire human species EPO polypeptide with an amino terminal methionine codon (ATG) for E.coli translation initiation. Note also that "upstream" of the initial ATG is a series of base pairs substantially

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duplicating the ribosome binding site sequence of the highly expressed OMP-f gene of E.coli.

Any suitable expression vector may be employed to carry the ECEPO. The particular vector chosen for expression of the ECEPO gene as the "temperature sensitive" plasmid pCFM536 -- a derivative of plasmid pCFM414 (A.T.C.C. 40076) -- as described in co-pending U.S. Patent Application Serial No. 636,727, filed August 6, 1984, (published EPO application No 130,990) by Charles F. Morris. More specifically, pCFM536 was digested with XbaI and HindIII; the large fragment was isolated and employed in a two-part ligation with the ECEPO gene. Sections 1 (XbaI/KpnI), 2 (KpnI/BglII) and 3 (BglII/SalI) had previously been assembled in the correct order in M13 and the EPO gene was isolated therefrom as a single XbaI/HindIII fragment. This fragment included a portion of the polylinker from M13 mp9 phage spanning the SalI to HindIII sites therein. Control of expression in the resulting expression plasmid, p536, was by means of a lambda P_L promoter, which itself may be under control of the C_{I857} repressor gene (such as provided in E.coli strain K12ΔHtrp).

The manufactured ECEPO gene above may be variously modified to encode erythropoietin analogs such as [Asn^2, des-Pro^2 through Ile^6]hEPO and [His^7]hEPO, as described below.

A. [Asn^2, des-Pro^2 through Ile^6]hEPO

Plasmid 536 carrying the ECEPO manufactured gene of ~~Table XIV~~ Figure 7 as a XbaI to HindIII insert was digested with HindIII and XhoI. The latter endonuclease cuts the ECEPO gene at a unique, 6 base pair recognition site spanning the last base of the codon encoding Asp^8 through the second base of the Arg^{10} codon. A XbaI/XhoI "linker" sequence was manufactured having the following sequence:

T0610X

```
          XbaI          +1  2   7   8   9
                Met Ala Asn Cys Asp    , XhoI
5'-CTAG ATG GCT AAT TGC GAC-3'
3'-TAC CGA TTA ACG CTG AGCT-5'
```

The XbaI/XhoI linker and the XhoI/HindIII ECEPO gene sequence fragment were inserted into the large fragment resulting from XbaI and HindIII digestion of plasmid pCFM526 -- a derivative of plasmid pCFM414 (A.T.C.C. 40076) -- as described in co-pending U.S. Patent Application Serial No. 636,727, filed August 6, 1984, by Charles F. Morris, to generate a plasmid-borne DNA sequence encoding E.coli expression of the Met^{-1} form of the desired analog.

B. [His^{7}]hEPO

Plasmid 536 was digested with HindIII and XhoI as in part A above. A XbaI/XhoI linker was manufactured having the following sequence:

T0611X

```
          XbaI          +1  2   3   4   5   6   7   8   9      XhoI
                Met Ala Pro Pro Arg Leu Ile His Asp
5'-CTAG ATG GCT CCG CCA CGT CTG ATC CAT GAC-3'
3'-TAC CGA GGC GGT GCA GAC TAG GTA CTG  AGCT-5'
```

The linker and the XhoI/HindIII ECEPO sequence fragment were then inserted into pCFM526 to generate a plasmid-borne DNA sequence encoding E.coli expression of the Met^{-1} form of the desired analog.

Construction of a manufactured gene ("SCEPO") incorporating yeast preference codons is as described in the following ~~Tables XV through XXI~~ Figures 16 through 21 and 28. As was the case with the ECEPO gene, the entire construction involved formation of three sets of oligonucleotides (~~Tables XV, XVI and XIX~~ Figures 16, 18 and 28) which were formed into duplexes and assembled into sections (~~Tables XVI, XVIII and XX~~ Figures 17, 19 and 21). Note that synthesis was facilitated in part by use of some sub-optimal codons in both the SCEPO and ECEPO construc-

tions, i.e., oligonucleotides 7-12 of Section 1 of both genes were identical, as were oligonucleotides 1-6 of Section 2 in each gene.

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TABLE XV

SCEPO SECTION 1 OLIGONUCLEOTIDES

1. AATTCAAGCTTGGATAAAAGAGCT
2. GTGGAGCTCTTTTATCCAAGCTTG
3. CCACCAAGATTGATCTGTGACTC
4. TCTCGAGTCACAGATCAATCTTG
5. GAGAGTTTTGGAAAGATACTTGTTG
6. CTTCCAACAAGTATCTTTCCAAAAC
7. GAAGCTAAAGAAGCTGAAAACATC
8. GTGGTGATGTTTTCAGCTTCTTTAG
9. ACCACTGGTTGTGCTGAACACTGTTC
10. CAAAGAACAGTGTTCAGCACAACCA
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTTCGTT

TABLE XVI

SCEPO SECTION 1

EcoRI HindIII 1
AATTCA AGCTTGGATA
GT TCGAACCTAT
2

3
AAAGAGCTCC ACCAAGATTG ATCTGTGACT CGAGAGTTTT
TTTCTCGAGG TGGTTCTAAC TAGACACTGA GCTCTCAAAA
4

5 7
GGAAAGATAC TTGTTGGAAG CTAAAGAAGC TGAAAACATC ACCACTGGTT
CCTTTCTATG AACAACCTTC GATTTCTTCG ACTTTTGTAG TGGTGACCAA
6 8

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

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TABLE XVII

SCEPO SECTION 2 OLIGONUCLEOTIDES

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

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