Amgen Inc. v. F. Hoffmann-LaRoche LTD et al Doc. 534 Att. 7

EXHIBIT 3

Part 2 of 3

Dockets.Justia.com

Case 1:05-cv-12237-WGY Document 534-8 Filed 06/20/2007 Page 2 of 26

FIG.6B

```
ATTGGCCAAGAGGTGGCTGGGTTCAAGGACCGGCGACTTGTCAAGGACCCCGGAAGGGGGAGGGGGGTGGG

GCAGCCTCCACGTGCCGCGGGGACTTGGGGGAGTTCTTGGGGATGGCAAAAACCTGGCCTGTTGAGGGGCA

CAGTTTGGGGTTGGGGAGGAGGTTTGGGGTTCTGCTGTGCAGTTGTGTCGTTGTCAGTGTCTCG[I·S·]

TTGCACACGCACAGATCAATAAGCCAGAGGCAGCACCTGAGTGCTTGCATGGTTGGGACAGGAAGGACGAG

CTGGGGCAGAGACGTGGGGATGAAGGAAGCTGTCCTTCCACAGCCACCCTTCTCCCCCCCCGCCTGACTCT

                          -23          -20
                          Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
CAGCCTGGCTATCTGTTCTAG      AA TGT CCT GCC TGG CTG TGG CTT CTC CTG TCC CTG

    -10                                        -1  +1
Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu Ile Cys
CTG TCG CTC CCT CTG GGC CTC CCA GTC CTG GGC GCC CCA CCA CGC CTC ATC TGT

         10                                              20              *
Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Ile
GAC AGC CGA GTC CTG GAG AGG TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT ATC

 26
Thr
ACG GTGAGACCCCTTCCCCAGCACATTCCACAGAACTCACGCTCAGGGCTTCAGGGAACTCCTCCCAGAT

CCAGGAACCTGGCACTTGGTTTGGGGTGGAGTTGGGAAGCTAGACACTGCCCCCCTACATAAGAATAAGTC
```

FIG.6C

```
TGGTGGCCCCCAAACCATACCTGAAACTAGGCAAGGAGCAAAGCCAGCAGATCCTACGCCTGTGGGCCAGGG

                                  27                  30
                                  Thr Gly Cys Ala Glu
CCAGAGCCTTCAGGGACCCTTGACTCCCCGGGCTGTGTGCATTTCAG    ACG GGC TGT GCT GAA

                          *       40
His Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr
CAC TGC AGC TTG AAT GAG AAT ATC ACT GTC CCA GAC ACC AAA GTT AAT TTC TAT

                                  55
50
Ala Trp Lys Arg Met Glu
GCC TGG AAG AGG ATG GAG GTGAGTTCCTTTTTTTTTTTTTTTCCTTTCTTTTGGAGAATCTCATT

TGCGAGCCTGATTTTGGATGAAAGGGAGAATGATCGGGGGAAAGGTAAAATGGAGCAGCAGAGATGAGGCT

GCCTGGGCGCAGAGGCTCACGTCTATAATCCCAGGCTGAGATGGCCGAGATGGGAGAATTGCTTGAGCCCT

GGAGTTTCAGACCAACCTAGGCAGCATAGTGAGATCCCCCCATCTCTACAAACATTTAAAAAAATTAGTCAG

GTGAAGTGGTGCATGGTAGTCCCAGATATTTGGAAGGCTGAGGCGGGAGGATCGCTTGAGCCCAGGAA

TTTGAGGCTGCAGTGAGCTGTGATCACACCACTGCACTCCAGCCTCAGTGACAGAGTGAGGCCCTGTCTCA
```

Case 1:05-cv-12237-WGY Document 534-8 Filed 06/20/2007 Page 4 of 26

FIG. 6D

```
AAAAAGAAAAGAAAAAAGAAAAATAATGAGGGCTGTATGGAATACATTCATTATTCATTCACTCACTCACT

CACTCATTCATTCATTCATTCATTCAACAAGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGCTTGG

GGCTGCTGAGGGGCAGGAGGGAGAGGGTGACATGGGTCAGCTCGACTCCCAGAGTCCACTCCCTGTAG

 56                  60                                                  70
Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala
GTC GGG CAG CAG GCC GTA GAA GTC TGG CAG GGC CTG GCC CTG CTG TCG GAA GCT

                            80            *                             90
Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu
GTC CTG CGG GGC CAG GCC CTG TTG GTC AAC TCT TCC CAG CCG TGG GAG CCC CTG

                                    100
Gln Leu His Val Asp Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu
CAG CTG CAT GTG GAT AAA GCC GTC AGT GGC CTT CGC AGC CTC ACC ACT CTG CTT

110                     115
Arg Ala Leu Gly Ala Gln
CGG GCT CTG GGA GCC CAG GTGAGTAGGAGCGGACACTTCTGCTTGCCCTTTCTGTAAGAAGGGGA

GAAGGGTCTTGCTAAGGAGTACAGGAACTGTCCGTATTCCTTCCCTTTCTGTGGCACTGCAGCGACCTCCT

                    116                 120
                    Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
GTTTTCTCCTTGGCAG    AAG GAA GCC ATC TCC CCT CCA GAT GCG GCC TCA GCT GCT
```

FIG. 6E

```
                                                                  130
Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser
CCA CTC CGA ACA ATC ACT GCT GAC ACT TTC CGC AAA CTC TTC CGA GTC TAC TCC
                                                                  150
Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly
AAT TTC CTC CGG GGA AAG CTG AAG CTG TAC ACA GGG GAG GCC TGC AGG ACA GGG
                                                                  166
Asp Arg OP
GAC AGA TGA CCAGGTGTGTCCACCTGGGCATATCCACCACCTCCCTCACCAACATTGCTTGTGCCACA

CCCTCCCCCCGCCACTCCTGAACCCCGTCGAGGGGCTCTCAGCTCAGCGCCAGCCTGTCCCATGGACACTCC

AGTGCCAGCAATGACATCTCAGGGGCCAGAGGAACTGTCCAGAGAGCAACTCTGAGATCTAAGGATGTCAC

AGGGCCAACTTGAAGGGCCCAGAGCAGGAAGCATTCAGAGAGGCAGCTTTAAACTCAGGGACAGAGCCATGC

TGGGAAGACGCCTGAGCTCACTCGGCACCCTGCAAAATTTGATGCCAGGACACGCTTTGGAGGCGATTTAC

CTGTTTTCGCACCTACCATCAGGGACAGGATGACCTGGAGAACTTAGGTGGCAAGCTGTGACTTCTCCAGG

TCTCACGGGCATGGGCACTCCCTTGGTGGCAAGAGCCCCCTTGACACCGGGGTGGTGGAACCATGAAGAC

AXGATXGGGGCTGGCCTCTCATGGGGTCCAAGTTTTGTGTATTCTCAACCTATTGACAGACTGAA

ACACAATATGAC
```

FIG. 7

```
                                        -1  1
          XbaI                          MetAla
      CTAG AAACCATGAG GGTAATAAAA TAATGGCTCC GCCGCGTCTG
           TTTGGTACTC CCATTATTTT ATTACCGAGG CGGCGCAGAC

ATCTGCGACT CGAGAGTTCT GGAACGTTAC CTGCTGGAAG CTAAAGAAGC
TAGACGCTGA GCTCTCAAGA CCTTGCAATG GACGACCTTC GATTTCTTCG

TGAAAACATC ACCACTGGTT GTGCTGAACA CTGTTCTTTG AACGAAAACA
ACTTTTGTAG TGGTGACCAA CACGACTTGT GACAAGAAAC TTGCTTTTGT

TTACGGTACC AGACACCAAG GTTAACTTCT ACGCTTGGAA ACGTATGGAA
AATGCCATGG TCTGTGGTTC CAATTGAAGA TGCGAACCTT TGCATACCTT

GTTGGTCAAC AAGCAGTTGA AGTTTGGCAG GGTCTGGCAC TGCTGAGCGA
CAACCAGTTG TTCGTCAACT TCAAACCGTC CCAGACCGTG ACGACTCGCT

GGCTGTACTG CGTGGCCAGG CACTGCTGGT AAACTCCTCT CAGCCGTGGG
CCGACATGAC GCACCGGTCC GTGACGACCA TTTGAGGAGA GTCGGCACCC

AACCGCTGCA GCTGCATGTT GACAAAGCAG TATCTGGCCT GAGATCTCTG
TTGGCGACGT CGACGTACAA CTGTTTCGTC ATAGACCGGA CTCTAGAGAC

ACTACTCTGC TGCGTGCTCT GGGTGCACAG AAAGAGGCTA TCTCTCCGCC
TGATGAGACG ACGCACGAGA CCCACGTGTC TTTCTCCGAT AGAGAGGCGG

GGATGCTGCA TCTGCTGCAC CGCTGCGTAC CATCACTGCT GATACCTTCC
CCTACGACGT AGACGACGTG GCGACGCATG GTAGTGACGA CTATGGAAGG

GCAAACTGTT TCGTGTATAC TCTAACTTCC TGCGTGGTAA ACTGAAACTG
CGTTTGACAA AGCACATATG AGATTGAAGG ACGCACCATT TGACTTTGAC

                                                SalI
TATACTGGCG AAGCATGCCG TACTGGTGAC CGCTAATAG
ATATGACCGC TTCGTACGGC ATGACCACTG GCGATTATCA GCT
```

FIG. 8


-1 +1
HindIII
ArgAla
AGCTTGGATA AAAGAGCTCC ACCAAGATTG ATCTGTGACT CGAGAGTTTT
ACCTAT TTTCTCGAGG TGGTTCTAAC TAGACACTGA GCTCTCAAAA
GGAAAGATAC TTGTTGGAAG CTAAAGAAGC TGAAAACATC ACCACTGGTT
CCTTTCTATG AACAACCTTC GATTTCTTCG ACTTTTGTAG TGGTGACCAA
GTGCTGAACA CTGTTCTTTG AACGAAAACA TTACGGTACC AGACACCAAG
CACGACTTGT GACAAGAAAC TTGCTTTTGT AATGCCATGG TCTGTGGTTC
GTTAACTTCT ACGCTTGGAA ACGTATGGAA GTTGGTCAAC AAGCTGTTGA
CAATTGAAGA TGCGAACCTT TGCATACCTT CAACCAGTTG TTCGACAACT
AGTTTGGCAA GGTTTGGCCT TGTTATCTGA AGCTGTTTTG AGAGGTCAAG
TCAAACCGTT CCAAACCGGA ACAATAGACT TCGACAAAAC TCTCCAGTTC
CCTTGTTGGT TAACTCTTCT CAACCATGGG AACCATTGCA ATTGCACGTC
GGAACAACCA ATTGAGAAGA GTTGGTACCC TTGGTAACGT TAACGTGCAG
GATAAAGCCG TCTCTGGTTT GAGATCTTTG ACTACTTTGT TGAGAGCTTT
CTATTTCGGC AGAGACCAAA CTCTAGAAAC TGATGAAACA ACTCTCGAAA
GGGTGCTCAA AAGGAAGCCA TTTCCCCACC AGACGCTGCT TCTGCCGCTC
CCCACGAGTT TTCCTTCGGT AAAGGGGTGG TCTGCGACGA AGACGGCGAG
CATTGAGAAC CATCACTGCT GATACCTTCA GAAAGTTATT CAGAGTTTAC
GTAACTCTTG GTAGTGACGA CTATGGAAGT CTTTCAATAA GTCTCAAATG
TCCAACTTCT TGAGAGGTAA ATTGAAGTTG TACACCGGTG AAGCCTGTAG
AGGTTGAAGA ACTCTCCATT TAACTTCAAC ATGTGGCCAC TTCGGACATC
AACTGGTGAC AGATAAGCCC GACTGATAAC AACAGTGTAG
TTGACCACTG TCTATTCGGG CTGACTATTG TTGTCACATC
SalI
ATGTAACAAA G
TACATTGTTT CAGCT

FIG. 9

```
                -20       -10       +1        10        20        30        40
Human   MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLEAKEAENITTGCAEHCSLNENITVPDTK
        ********************************************* * ** * ********************
Monkey  MGVHECPAWLWLLLSLVSLPLGLPVPGAPPRLICDSRVLERYLLEAKEAENVTMGCSESCSLNENITVPDTK

                50        60        70        80        90        100       110
Human   VNFYAWKRMEVGQQAVEVWQGLALLSLEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKE
        ********************************* * ****** ******* *** ***** ********** *
Monkey  VNFYAWKRMEVGQQAVEVWQGLALLSLEAVLRGQAVLANSSQPFEPLQLHMDKAISGLRSITTLLRALGAQ-E

                120       130       140       150       160
Human   AISPPDAASAAPLRTITADTFRKLFRVYSNFLRGKLKLYTGEACRTGDR
        *** ********************* ********************** ***
Monkey  AISLPDAASAAPLRTITADTFCKLFRVYSNFLRGKLKLYTGEACRRGDR
```

FIG. 10

1. AATTCTAGAAACCATGAGGGTAATAAAATA

2. CCATTATTTTATTACCCTCATGGTTTCTAG

3. ATGGCTCCGCCGCGTCTGATCTGCGAC

4. CTCGAGTCGCAGATCAGACGCGGCGGAG

5. TCGAGAGTTCTGGAACGTTACCTGCTG

6. CTTCCAGCAGGTAACGTTCCAGAACT

7. GAAGCTAAAGAAGCTGAAAACATC

8. GTGGTGATGTTTTCAGCTTCTTTAG

9. ACCACTGGTTGTGCTGAACACTGTTC

10. CAAAGAACAGTGTTCAGCACAACCA

11. TTTGAACGAAAACATTACGGTACCG

12. GATCCGGTACCGTAATGTTTTCGTT

FIG. 11

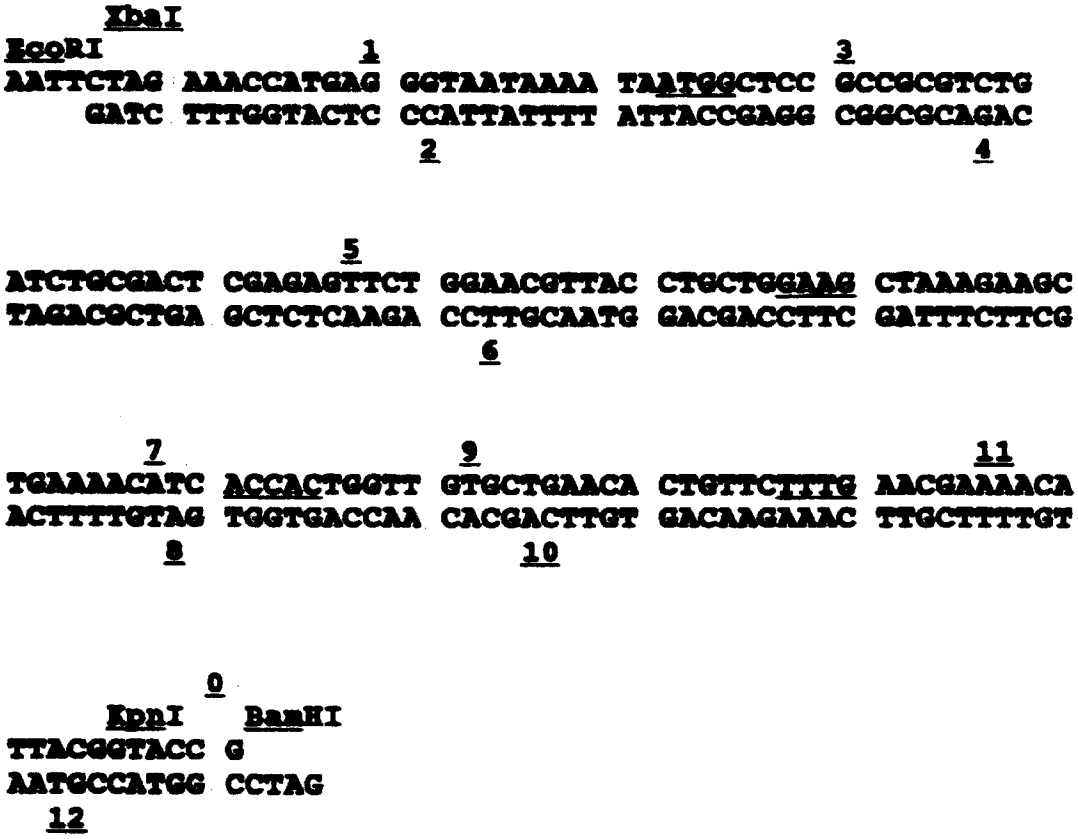

XbaI
EcoRI
1
3
AATTCTAG AAACCATGAG GGTAATAAAA TAATGGCTCC GCCGCGTCTG
GATC TTTGGTACTC CCATTATTTT ATTACCGAGG CGGCGCAGAC
2
4
5
ATCTGCGACT CGAGAGTTCT GGAACGTTAC CTGCTGGAAG CTAAAGAAGC
TAGACGCTGA GCTCTCAAGA CCTTGCAATG GACGACCTTC GATTTCTTCG
6
7
9
11
TGAAAACATC ACCACTGGTT GTGCTGAACA CTGTTCTTTG AACGAAAACA
ACTTTTGTAG TGGTGACCAA CACGACTTGT GACAAGAAAC TTGCTTTTGT
8
10
0
KpnI
BamHI
TTACGGTACC G
AATGCCATGG CCTAG
12

FIG. 12

1. AATTCGGTACCAGACACCAAGGT
2. GTTAACCTTGGTGTCTGGTACCG
3. TAACTTCTACGCTTGGAAACGTAT
4. TTCCATACGTTTCCAAGCGTAGAA
5. GGAAGTTGGTCAACAAGCAGTTGAAGT
6. CCAAACTTCAACTGCTTGTTGACCAAC
7. TTGGCAGGGTCTGGCACTGCTGAGCG
8. GCCTCGCTCAGCAGTGCCAGACCCTG
9. AGGCTGTACTGCGTGGCCAGGCA
10. GCAGTGCCTGGCCACGCAGTACA
11. CTGCTGGTAAACTCCTCTCAGCCGT
12. TTCCCACGGCTGAGAGGAGTTTACCA
13. GGGAACCGCTGCAGCTGCATGTTGAC
14. GCTTTGTCAACATGCAGCTGCAGCGG
15. AAAGCAGTATCTGGCCTGAGATCTG
16. GATCCAGATCTCAGGCCAGATACT

FIG. 13

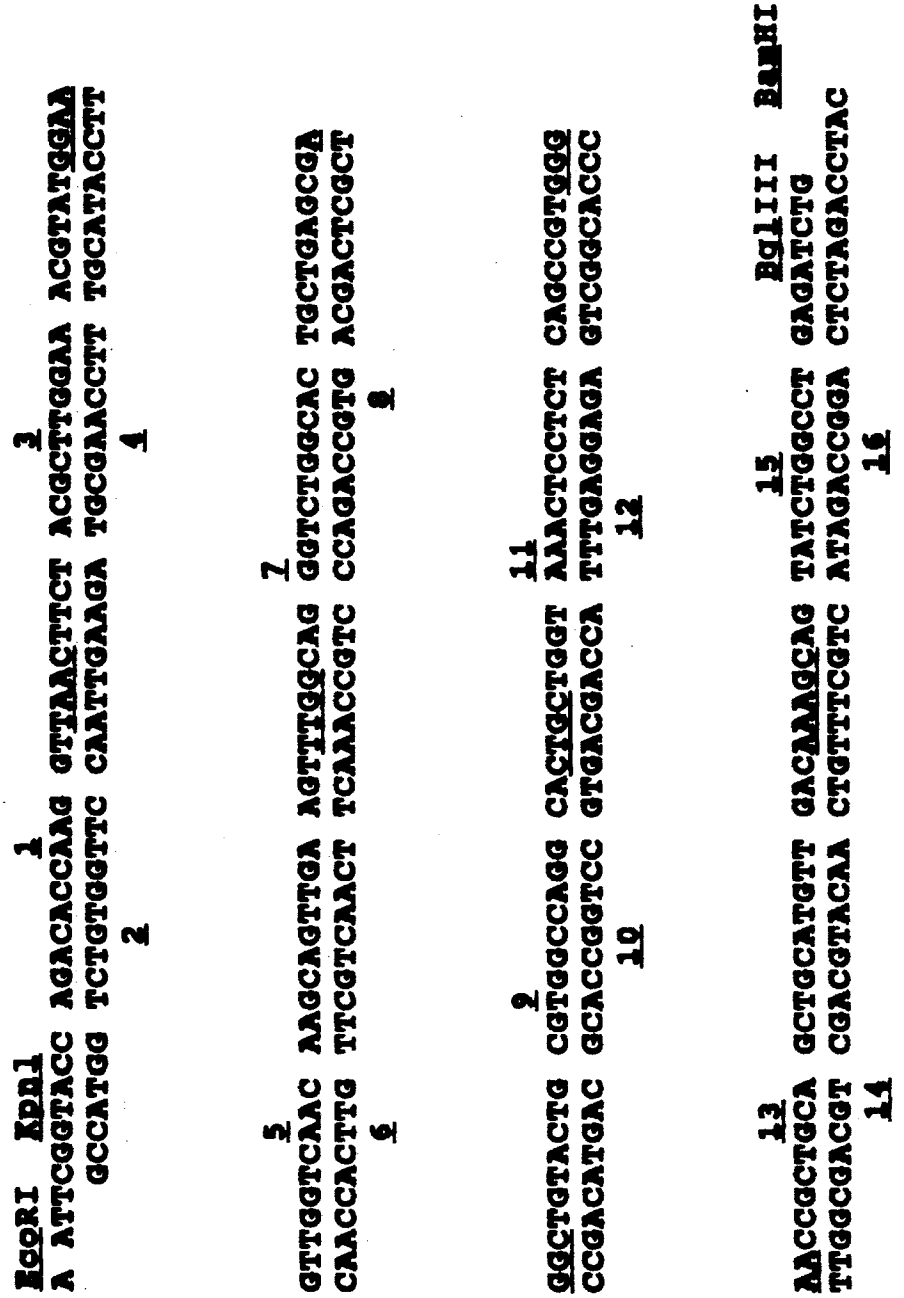

EcoRI KpnI
1
A ATTCGGTACC AGACACCAAG GTTAACTTCT ACGCTTGGAA ACGTATGGAA
GCCATGG TCTGTGGTTC CAATTGAAGA TGCGAACCTT TGCATACCTT
2
3
4
5
GTTGGTCAAC AAGCAGTTGA AGTTTGGCAG GGTCTGGCAC TGCTGAGCGA
CAACCACTTG TTCGTCAACT TCAAACCGTC CCAGACCGTG ACGACTCGCT
6
7
8
9
GGCTGTACTG CGTGGCCAGG CACTGCTGGT AAACTCCTCT CAGCCGTGGG
CCGACATGAC GCACCGGTCC GTGACGACCA TTTGAGGAGA GTCGGCACCC
10
11
12
13
AACCGCTGCA GCTGCATGTT GACAAAGCAG TATCTGGCCT GAGATCTG
TTGGCGACGT CGACGTACAA CTGTTTCGTC ATAGACCGGA CTCTAGACCTAC
14
15
16
BglIII BamHI

FIG. 14

1. GATCCAGATCTCTGACTACTCTGC

2. ACGCAGCAGAGTAGTCAGAGATCTG

3. TGCGTGCTCTGGGTGCACAGAAAGAGG

4. GATAGCCTCTTTCTGTGCACCCAGAGC

5. CTATCTCTCCGCCGGATGCTGCATCT

6. CAGCAGATGCAGCATCCGGCGGAGA

7. GCTGCACCGCTGCGTACCATCACTG

8. ATCAGCAGTGATGGTACGCAGCGGTG

9. CTGATACCTTCCGCAAACTGTTTCG

10. ATACACGAAACAGTTTGCGGAAGGT

11. TGTATACTCTAACTTCCTGCGTGGTA

12. CAGTTTACCACGCAGGAAGTTAGAGT

13. AACTGAAACTGTATACTGGCGAAGC

14. GGCATGCTTCGCCAGTATACAGTTT

15. ATGCCGTACTGGTGACCGCTAATAG

16. TCGACTATTAGCGGTCACCAGTAC

FIG. 15

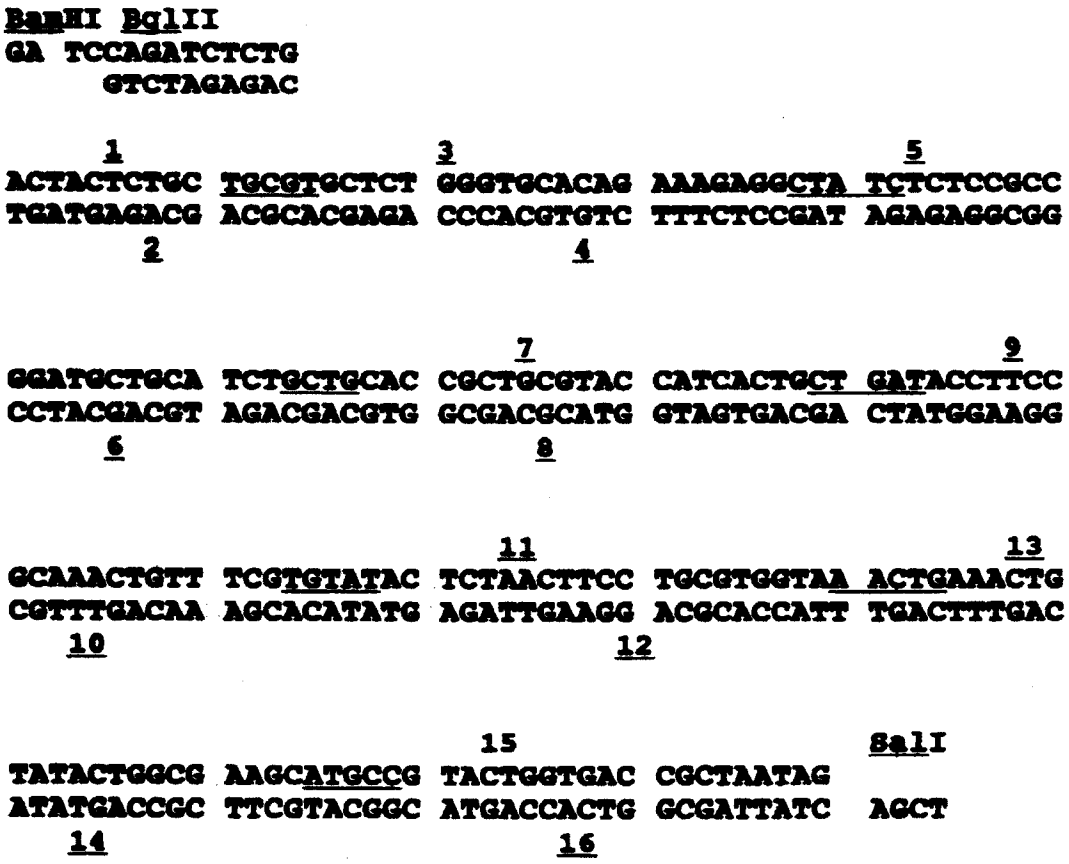

BamHI BglII
GA TCCAGATCTCTG
GTCTAGAGAC
1
3
5
ACTACTCTGC TGCGTGCTCT GGGTGCACAG AAAGAGGCTA TCTCTCCGCC
TGATGAGACG ACGCACGAGA CCCACGTGTC TTTCTCCGAT AGAGAGGCGG
2
4
7
9
GGATGCTGCA TCTGCTGCAC CGCTGCGTAC CATCACTGCT GATACCTTCC
CCTACGACGT AGACGACGTG GCGACGCATG GTAGTGACGA CTATGGAAGG
6
8
11
13
GCAAACTGTT TCGTGTATAC TCTAACTTCC TGCGTGGTAA ACTGAAACTG
CGTTTGACAA AGCACATATG AGATTGAAGG ACGCACCATT TGACTTTGAC
10
12
15
SalI
TATACTGGCG AAGCATGCCG TACTGGTGAC CGCTAATAG
ATATGACCGC TTCGTACGGC ATGACCACTG GCGATTATC AGCT
14
16

FIG. 16

1. AATTCAAGCTTGGATAAAAGAGCT

2. GTGGAGCTCTTTTATCCAAGCTTG

3. CCACCAAGATTGATCTGTGACTC

4. TCTCGAGTCACAGATCAATCTTG

5. GAGAGTTTTGGAAAGATACTTGTTG

6. CTTCCAACAAGTATCTTTCCAAAAC

7. GAAGCTAAAGAAGCTGAAAACATC

8. GTGGTGATGTTTTCAGCTTCTTTAG

9. ACCACTGGTTGTGCTGAACACTGTTC

10. CAAAGAACAGTGTTCAGCACAACCA

11. TTTGAACGAAAACATTACGGTACCG

12. GATCCGGTACCGTAATGTTTTCGTT

FIG. 17

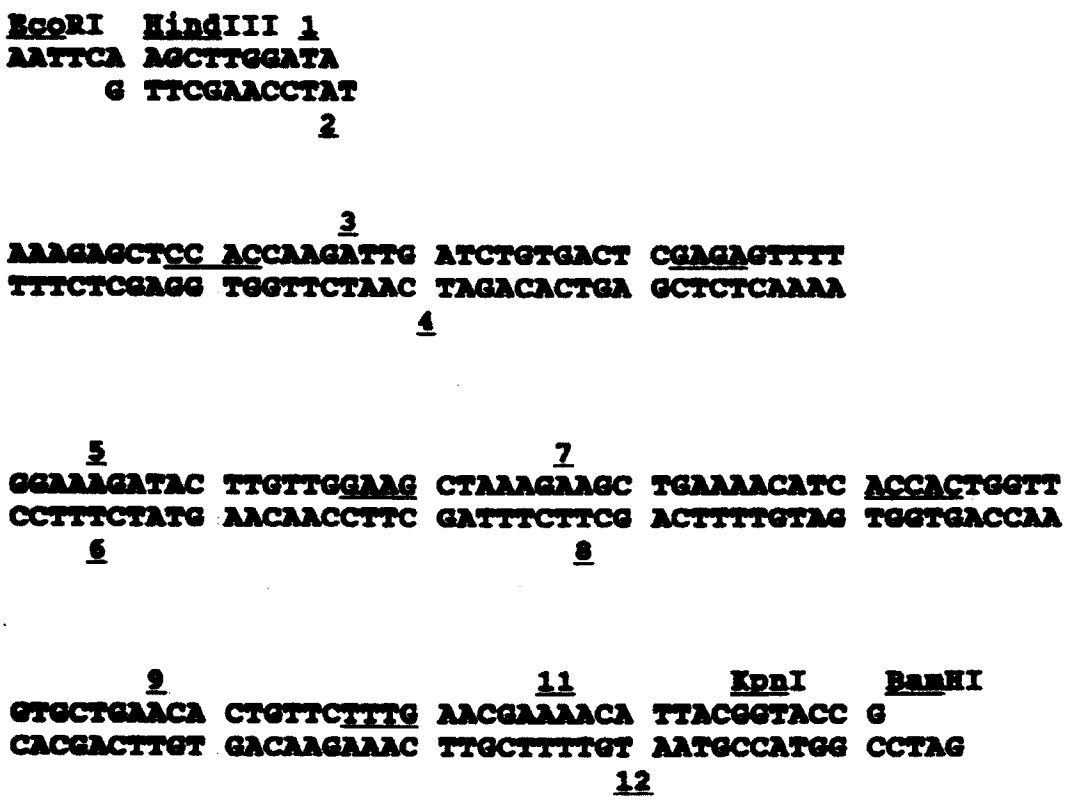

EcoRI HindIII 1
AATTCA AGCTTGGATA
G TTCGAACCTAT
2
3
AAAGAGCTCC ACCAAGATTG ATCTGTGACT CGAGAGTTTT
TTTCTCGAGG TGGTTCTAAC TAGACACTGA GCTCTCAAAA
4
5
7
GGAAAGATAC TTGTTGGAAG CTAAAGAAGC TGAAAACATC ACCACTGGTT
CCTTTCTATG AACAACCTTC GATTTCTTCG ACTTTTGTAG TGGTGACCAA
6
8
9
11
KpnI
BamHI
GTGCTGAACA CTGTTCTTTG AACGAAAACA TTACGGTACC G
CACGACTTGT GACAAGAAAC TTGCTTTTGT AATGCCATGG CCTAG
12

FIG. 18

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

FIG. 19

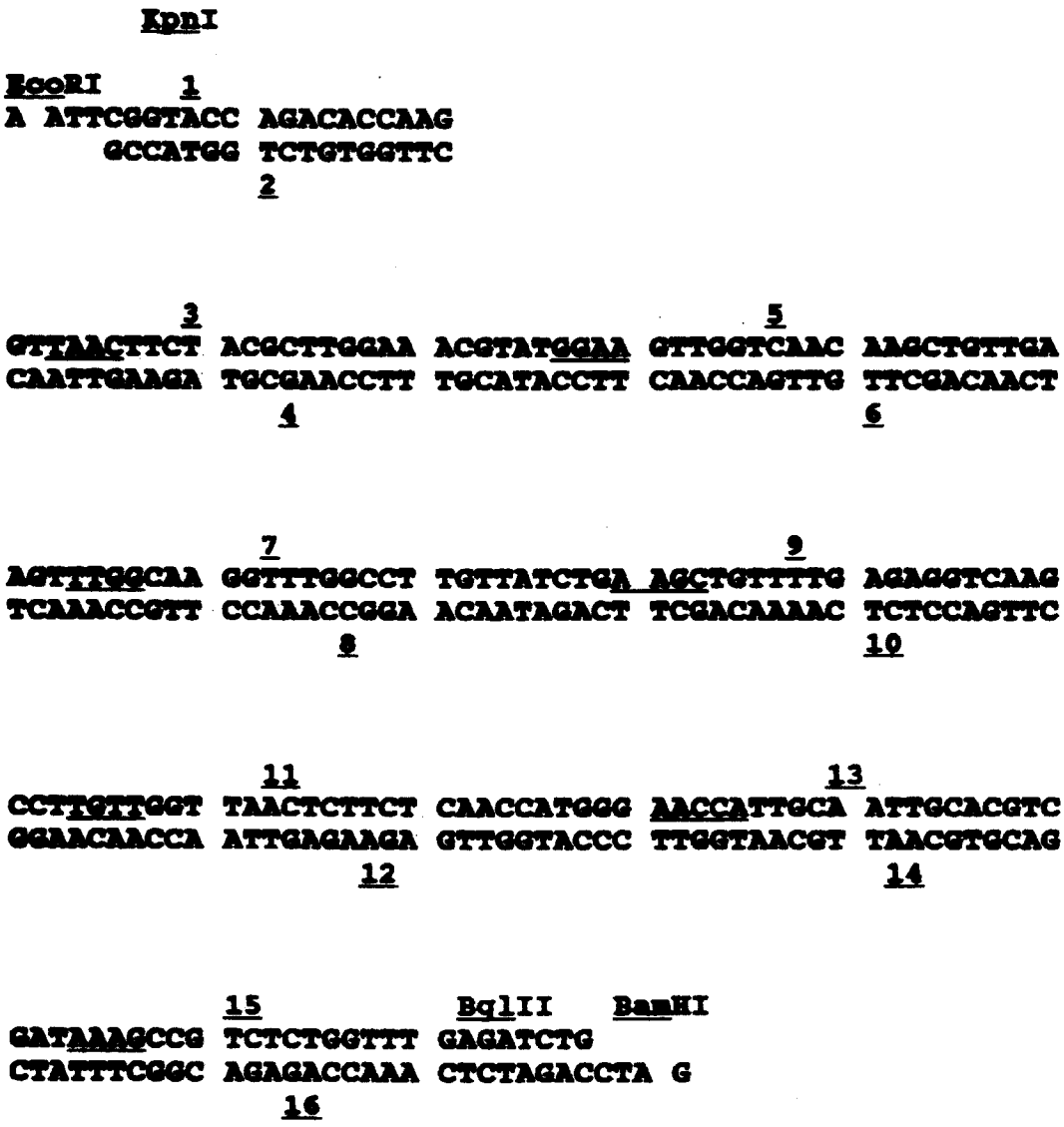

KpnI
EcoRI
1
A ATTCGGTACC AGACACCAAG
GCCATGG TCTGTGGTTC
2
3
5
GTTAACTTCT ACGCTTGGAA ACGTATGGAA GTTGGTCAAC AAGCTGTTGA
CAATTGAAGA TGCGAACCTT TGCATACCTT CAACCAGTTG TTCGACAACT
4
6
7
9
AGTTTGGCAA GGTTTGGCCT TGTTATCTGA AGCTGTTTTG AGAGGTCAAG
TCAAACCGTT CCAAACCGGA ACAATAGACT TCGACAAAAC TCTCCAGTTC
8
10
11
13
CCTTGTTGGT TAACTCTTCT CAACCATGGG AACCATTGCA ATTGCACGTC
GGAACAACCA ATTGAGAAGA GTTGGTACCC TTGGTAACGT TAACGTGCAG
12
14
15
BglII
BamHI
GATAAAGCCG TCTCTGGTTT GAGATCTG
CTATTTCGGC AGAGACCAAA CTCTAGACCTA G
16

FIG. 20

1. GATCCAGATCTTTGACTACTTTGTT

2. TCTCAACAAAGTAGTCAAAGATCTG

3. GAGAGCTTTGGGTGCTCAAAAGGAAG

4. ATGGCTTCCTTTTGAGCACCCAAAGC

5. CCATTTCCCCACCAGACGCTGCTT

6. GCAGAAGCAGCGTCTGGTGGGGAA

7. CTGCCGCTCCATTGAGAACCATC

8. CAGTGATGGTTCTCAATGGAGCG

9. ACTGCTGATACCTTCAGAAAGTT

10. GAATAACTTTCTGAAGGTATCAG

11. ATTCAGAGTTTACTCCAACTTCT

12. CTCAAGAAGTTGGAGTAAACTCT

13. TGAGAGGTAAATTGAAGTTGTACAC

14. ACCGGTGTACAACTTCAATTTACCT

15. CGGTGAAGCCTGTAGAACTGGT

16. CTGTCACCAGTTCTACAGGCTTC

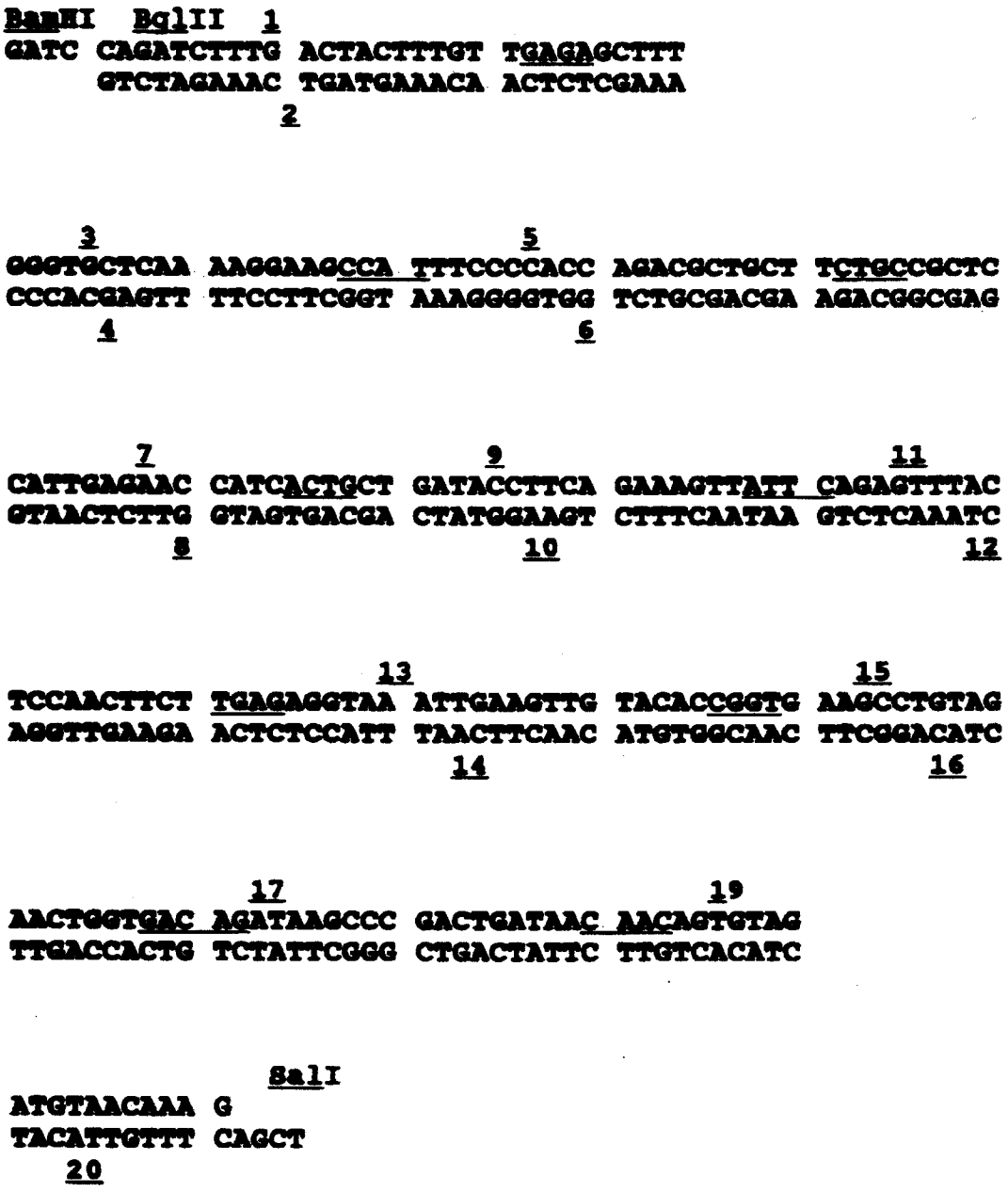
17. GACAGATAAGCCCGACTGATAA

18. GTTGTTATCAGTCGGGCTTAT

19. CAACAGTGTAGATGTAACAAAG

20. TCGACTTTGTTACATCTACACT

FIG. 21


BamHI BglII 1
GATC CAGATCTTTG ACTACTTTGT TGAGAGCTTT
GTCTAGAAAC TGATGAAACA ACTCTCGAAA
2
3
5
GGGTGCTCAA AAGGAAGCCA TTTCCCCACC AGACGCTGCT TCTGCCGCTC
CCCACGAGTT TTCCTTCGGT AAAGGGGTGG TCTGCGACGA AGACGGCGAG
4
6
7
9
11
CATTGAGAAC CATCACTGCT GATACCTTCA GAAAGTTATT CAGAGTTTAC
GTAACTCTTG GTAGTGACGA CTATGGAAGT CTTTCAATAA GTCTCAAATC
8
10
12
13
15
TCCAACTTCT TGAGAGGTAA ATTGAAGTTG TACACCGGTG AAGCCTGTAG
AGGTTGAAGA ACTCTCCATT TAACTTCAAC ATGTGGCAAC TTCGGACATC
14
16
17
19
AACTGGTGAC AGATAAGCCC GACTGATAAC AACAGTGTAG
TTGACCACTG TCTATTCGGG CTGACTATTC TTGTCACATC
SalI
ATGTAACAAA G
TACATTGTTT CAGCT
20

PRODUCTION OF ERYTHROPOIETIN

This is a continuation of application Ser. No. 08,202,874, filed Feb. 28, 1994, and now abandoned which was a continuation of U.S. application Ser. No. 07/113,178, filed Oct. 23, 1987, now abandoned, which was a continuation of U.S. application Ser. No. 06/675,298, filed Nov. 30, 1984, and issued Oct. 27, 1987 as U.S. Pat. No. 4,703,008 which was a continuation-in-part of U.S. Ser. No. 06/655,841, filed Sep. 28, 1984, and now abandoned, which was a continuation-in-part of U.S. application Ser. No. 06/582,185, filed Feb. 21, 1984, and now abandoned, which was a continuation-in-part of U.S. application Ser. No. 06/561,024, filed Dec. 13, 1983, and now abandoned.

BACKGROUND

The present invention relates generally to the manipulation of genetic materials and, more particularly, to recombinant procedures making possible the production of polypeptides possessing part or all of the primary structural conformation and/or one or more of the biological properties of naturally-occurring erythropoietin.

A. Manipulation Of Genetic Materials

Genetic materials may be broadly defined as those chemical substances which program for and guide the manufacture of constituents of cells and viruses and direct the responses of cells and viruses. A long chain polymeric substance known as deoxyribonucleic acid (DNA) comprises the genetic material of all living cells and viruses except for certain viruses which are programmed by ribonucleic acids (RNA). The repeating units in DNA polymers are four different nucleotides, each of which consists of either a purine (adenine or guanine) or a pyrimidine (thymine or cytosine) bound to a deoxyribose sugar to which a phosphate group is attached. Attachment of nucleotides in linear polymeric form is by means of fusion of the 5' phosphate of one nucleotide to the 3' hydroxyl group of another. Functional DNA occurs in the form of stable double stranded associations of single strands of nucleotides (known as deoxyoligonucleotides), which associations occur by means of hydrogen bonding between purine and pyrimidine bases [i.e., "complementary" associations existing either between adenine (A) and thymine (T) or guanine (G) and cytosine (C)]. By convention, nucleotides are referred to by the names of their constituent purine or pyrimidine bases, and the complementary associations of nucleotides in double stranded DNA (i.e., A-T and G-C) are referred to as "base pairs". Ribonucleic acid is a polynucleotide comprising adenine, guanine, cytosine and uracil rather than thymine, bound to ribose and a phosphate group.

Most briefly put, the programming function of DNA is generally effected through a process wherein specific DNA nucleotide sequences (genes) are "transcribed" into relatively unstable messenger RNA (mRNA) polymers. The mRNA, in turn, serves as a template for the formation of structural, regulatory and catalytic proteins from amino acids. This mRNA "translation" process involves the operations of small RNA strands (tRNA) which transport and align individual amino acids along the mRNA strand to allow for formation of polypeptides in proper amino acid sequences The mRNA "message" derived from DNA and providing the basis for the tRNA supply and orientation of any given one of the twenty amino acids for polypeptide "expression", is in the form of triplet "codons"—sequential groupings of three nucleotide bases. In one sense, the formation of a protein is the ultimate form of "expression" of the programmed genetic message provided by the nucleotide sequence of a gene.

"Promoter" DNA sequences usually "precede" a gene in a DNA polymer and provide a site for initiation of the transcription into mRNA. "Regulator" DNA sequences, also usually "upstream" of (i.e., preceding) a gene in a given DNA polymer, bind proteins that determine the frequency (or rate) of transcriptional initiation. Collectively referred to as "promoter/regulator" or "control" DNA sequence, these sequences which precede a selected gene (or series of genes) in a functional DNA polymer cooperate to determine whether the transcription (and eventual expression) of a gene will occur. DNA sequences which "follow" a gene in a DNA polymer and provide a signal for termination of the transcription into mRNA are referred to as transcription "terminator" sequences.

A focus of microbiological processing for the last decade has been the attempt to manufacture industrially and pharmaceutically significant substances using organisms which either do not initially have genetically coded information concerning the desired product included in their DNA, or (in the case of mammalian cells in culture) do not ordinarily express a chromosomal gene at appreciable levels. Simply put, a gene that specifies the structure of a desired polypeptide product is either isolated from a "donor" organism or chemically synthesized and then stably introduced into another organism which is preferably a self-replicating unicellular organism such as bacteria, yeast or mammalian cells in culture. Once this is done, the existing machinery for gene expression in the "transformed" or "transfected" microbial host cells operates to construct the desired product, using the exogenous DNA as a template for transcription of mRNA which is then translated into a continuous sequence of amino acid residues.

The art is rich in patent and literature publications relating to "recombinant DNA" methodologies for the isolation, synthesis, purification and amplification of genetic materials for use in the transformation of selected host organisms. U.S. Pat. No. 4,237,224 to Cohen, et al., for example, relates to transformation of unicellular host organisms with "hybrid" viral or circular plasmid DNA which includes selected exogenous DNA sequences. The procedures of the Cohen, et al. patent first involve manufacture of a transformation vector by enzymatically cleaving viral or circular plasmid DNA to form linear DNA strands. Selected foreign ("exogenous" or "heterologous") DNA strands usually including sequences coding for desired product are prepared in linear form through use of similar enzymes. The linear viral or plasmid DNA is incubated with the foreign DNA in the presence of ligating enzymes capable of effecting a restoration process and "hybrid" vectors are formed which include the selected exogenous DNA segment "spliced" into the viral or circular DNA plasmid.

Transformation of compatible unicellular host organisms with the hybrid vector results in the formation of multiple copies of the exogenous DNA in the host cell population. In some instances, the desired result is simply the amplification of the foreign DNA and the "product" harvested is DNA. More frequently, the goal of transformation is the expression by the host cells of the exogenous DNA in the form of large scale synthesis of isolatable quantities of commercially significant protein or polypeptide fragments coded for by the foreign DNA. See also, e.g., U.S. Pat. Nos. 4,264,731 (to Shine), 4,273,875 (to Manis), 4,293,652 (to Cohen), and European Patent Application 093,619, published Nov. 9, 1983.

The development of specific DNA sequences for splicing into DNA vectors is accomplished by a variety of tech-

niques, depending to a great deal on the degree of "foreignness" of the "donor" to the projected host and the size of the polypeptide to be expressed in the host. At the risk of over-simplification, it can be stated that three alternative principal methods can be employed: (1) the "isolation" of double-stranded DNA sequence from the genomic DNA of the donor; (2) the chemical manufacture of a DNA sequence providing a code for a polypeptide of interest; and (3) the in vitro synthesis of a double-stranded DNA sequence by enzymatic "reverse transcription" of mRNA isolated from donor cells. The last-mentioned methods which involve formation of a DNA "complement" of mRNA are generally referred to as "cDNA" methods.

Manufacture of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. DNA manufacturing procedures of co-owned, co-pending U.S. patent application Ser. No. 483,451, by Alton, et al., (filed Apr. 15, 1983 and corresponding to PCT U.S.83/00605, published Nov. 24, 1983 as WO83/04053), for example, provide a superior means for accomplishing such highly desirable results as: providing for the presence of alternate codons commonly found in genes which are highly expressed in the host organism selected for expression (e.g., providing yeast or *E.coli* "preference" codons); avoiding the presence of untranslated "intron" sequences (commonly present in mammalian genomic DNA sequences and mRNA transcripts thereof) which are not readily processed by procaryotic host cells; avoiding expression of undesired "leader" polypeptide sequences commonly coded for by genomic DNA and cDNA sequences but frequently not readily cleaved from the polypeptide of interest by bacterial or yeast host cells; providing for ready insertion of the DNA in convenient expression vectors in association with desired promoter/regulator and terminator sequences; and providing for ready construction of genes coding for polypeptide fragments and analogs of the desired polypeptides.

When the entire sequence of amino acid residues of the desired polypeptide is not known, direct manufacture of DNA sequences is not possible and isolation of DNA sequences coding for the polypeptide by a cDNA method becomes the method of choice despite the potential drawbacks in ease of assembly of expression vectors capable of providing high levels of microbial expression referred to above. Among the standard procedures for isolating cDNA sequences of interest is the Preparation of plasmid-borne cDNA "libraries" derived from reverse transcription of mRNA abundant in donor cells selected as responsible for high level expression of genes (e.g., libraries of cDNA derived from pituitary cells which express relatively large quantities of growth hormone products). Where substantial portions of the polypeptide's amino acid sequence are known, labelled, single-stranded DNA probe sequences duplicating a sequence putatively present in the "target" cDNA may be employed in DNA/DNA hybridization procedures carried out on cloned copies of the cDNA which have been denatured to single stranded form. [See, generally, the disclosure and discussions of the art provided in U.S. Pat. No. 4,394,443 to Weissman, et al. and the recent demonstrations of the use of long oligonucleotide hybridization probes reported in Wallace, et al., *Nuc.Acids Res.*, 6, pp. 3543–3557 (1979), and Reyes, et al., *P.N.A.S.* (*U.S.A.*), 79, pp. 3270–3274 (1982), and Jaye, et al., *Nuc.Acids Res.*, 11, pp. 2325–2335 (1983). See also, U.S. Pat. No. 4,358,535 to Falkow, et al., relating to DNA/DNA hybridization procedures in effecting diagnosis; published European Patent Application Nos. 0070685 and 0070687 relating to light-emitting labels on single stranded polynucleotide probes; Davis, et al., "A Manual for Genetic Engineering, Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1980) at pp. 55–58 and 174–176, relating to colony and plaque hybridization techniques; and, New England Nuclear (Boston, Mass.) brochures for "Gene Screen" Hybridization Transfer Membrane materials providing instruction manuals for the transfer and hybridization of DNA and RNA, Catalog No. NEF-972.]

Among the more signficant recent advances in hybridization procedures for the screening of recombinant clones is the use of labelled mixed synthetic oligonucleotide probes, each of which is potentially the complete complement of a specific DNA sequence in the hybridization sample including a heterogenous mixture of single stranded DnAs or RNAs. These procedures are acknowledged to be especially useful in the detection of cDNA clones derived from sources which provide extremely low amounts of mRNA sequences for the polypeptide of interest. Briefly put, use of stringent hybridization conditions directed toward avoidance of non-specific binding can allow, e.g., for the autoradiographic visualization of a specific cDNA clone upon the event of hybridization of the target DNA to that single probe within the mixture which is its complete complement. See generally, Wallase, et al., *Nuc.Acids Res.*, 9, pp. 879–897 (1981; Suggs, et al. *P.N.A.S.* (*U.S.A.*), 78, pp. 6613–6617 (1981); Choo, et al., *Nature*, 229, p. 178–180 (1982); Kurachi, et al., *P.N.A.S.* (*U.S.A.*), 79, pp. 6461–6464 (1982); Ohkubo, et al., *P.N.A.S.* (*U.S.A.*), 80, pp. 2196–2200 (1983); and Kornblihtt, et al. *P.N.A.S.* (*U.S.A.*), 80, pp. 3218–3222 (1983). In general, the mixed probe procedures of wallace, et al. (1981), supra, have been expanded upon by various workers to the point where reliable results have reportedly been obtained in a cDNA clone isolation using a 32-member mixed "pool" of 16-base-long (16-mer) oligonucleotide probes of uniformly, varying DNA sequences together with a single 11-mer to effect a two-site "positive" confirmation of the presence of cDnA of interest. See, Singer-Sam, et al., *P.N.A.S.* (*U.S.A.*), 80, pp. 802–806 (1983).

The use of genomic DNA isolates is the least common of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures. This is especially true in the area of recombinant procedures directed to securing microbial expression of mammalian polypeptides and is due, principally to the complexity of mammalian genomic DNA. Thus, while reliable procedures exist for developing phage-borne libraries of genomic DNA of human and other mammalian species origins [see, e.g., Lawn, et al. *Cell*, 15, pp. 1157–1174 (1978) relating to procedures for generating a human genomic library commonly referred to as the "Maniatis Library"; Karn, et al., *P.N.A.S.* (*U.S.A.*), 77, pp. 5172–5176 (1980) relating to a human genomic library based on alternative restriction endonuclease fragmentation procedure; and Blattner, et al., *Science*, 196, pp. 161–169 (1977) describing construction of a bovine genomic library] there have been relatively few successful attempts at use of hybridization procedures in isolating genomic DNA in the absence of extensive foreknowledge of amino acid or DNA sequences. As one example, Fiddes, et al., *J.Mol. and App.Genetics*, 1, pp. 3–18 (1981) report the successful isolation off a gene coding for the alpha subunit of human pituitary glycoprotein hormones from the Maniatis Library through use of a "full length" probe including a complete 621 base pair fragment of a previously-isolated cDNA sequence for the alpha subunit. As another example, Das, et al., *P.N.A.S.* (*U.S.A.*), 80, pp. 1531–1535 (1983) report isolation of human genomic clones

for human HLA-DR using a 175 base pair synthetic oligonucleotide. Finally, Anderson, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 6838–6842 (1983) report the isolation of genomic clone for bovine pancreatic trypsin inhibitor (BPTI) using a single probe 86 base pairs in length and constructed according to the known amino acid sequence of BPTI. The authors note a determination of poor prospects for isolating mRNA suitable for synthesis of a cDNA library due to apparent low levels of mRNA in initially targeted parotid gland and lung tissue sources and then address the prospects of success in probing a genomic library using a mixture of labelled probes, stating: "More generally, mixed-sequence oligodeoxynucleotide probes have been used to isolate protein genes of unknown sequence from cDNA libraries. Such probes are typically mixtures of 8–32 oligonucleotides, 14–17 nucleotides in length, representing every possible codon combination for a small stretch (5–6 residues) of amine acid sequence. Under stringent hybridization conditions that discriminate against incorrectly base-paired probes, these mixtures are capable of locating specific gene sequences in clone libraries of low-to-moderate complexity. Nevertheless, because of their short length and heterogeneity, mixed probes often lack the specificity required for probing sequences as complex as a mammalian genome. This makes such a method impractical for the isolation of mammalian protein genes when the corresponding mRNAs are unavailable." (Citations omitted).

There thus continues to exist a need in the art for improved methods for effecting the rapid and efficient isolation of cDNA clones in instances where little is known of the amine acid sequence of the polypeptide coded for and where "enriched" tissue sources of mRNA are not readily available for use in constructing cDNA libraries. Such improved methods would be especially useful if they were applicable to isolating mammalian genomic clones where sparse information is available concerning amine acid sequences of the polypeptide coded for by the gene sought.

B. Erythropoietin As A Polypeptide Of Interest

Erythropoiesis, the production of red blood cells, occurs continuously throughout the human life span to offset cell destruction. Erythropoiesis is a very precisely controlled physiological mechanism enabling sufficient numbers of red blood cells to be available in the blood for proper tissue oxygenation, but not so many that the cells would impede circulation. The formation of red blood cells occurs in the bone marrow and is under the control of the hormone, erythropoietin.

Erythropoietin, an acidic glycoprotein of approximately 34,000 dalton molecular weight, may occur in three forms: α, βand asialo. The α and β forms differ slightly in carbohydrate components, but have the same potency, biological activity and molecular weight. The asialo form is an α or β form with the terminal carbohydrate (sialic acid) removed. Erythropoietin is present in very low concentrations in plasma when the body is in a healthy state wherein tissues receive sufficient oxygenation from the existing number of erythrocytes. This normal low concentration is enough to stimulate replacement of red blood cells which are lost normally through aging.

The amount of erythropoietin in the circulation is increased under conditions of hypoxia when oxygen transport by blood cells in the circulation is reduced. Hypoxia may be caused by loss of large amounts of blood through hemorrhage, destruction of red blood cells by over-exposure to radiation, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or various forms of anemia. In response to tissues undergoing hypoxic stress, erythropoietin will increase red blood cell production by stimulating the conversion of primitive precursor cells in the bone marrow into proerythroblasts which subsequently mature, synthesize hemoglobin and are released into the circulation as red blood cells. When the number of red blood cells in circulation is greater than needed for normal tissue oxygen requirements, erythropoietin in circulation is decreased.

See generally, Testa, et al., *Exp.Hematol.*, 8(Supp. 8), 144–152 (1980); Tong, et al., *J.Biol.Chem.*, 256(24), 12666–12672 (1981); Goldwasser, *J.Cell.Physiol.*, 110(*Supp* 1), 133–135 (1982); Finch, *Blood*, 60(6), 1241–1246 (1982); Sytowski, et al., *Exp.Hematol.*, 8(*Supp* 8), 52–64 (1980): Naughton, *Ann.Clin.Lab.Sci.*, 13(5), 432–438 (1983); Weiss, et al., *Am.J.Vet.Res.*, 44(10), 1832–1835 (1983); Lappin, et al., *Exp.Hematol.*, 11(7), 661–666 (1983); Baciu, et al., *Ann.N.Y.Acad. Sci.*, 414, 66–72 (1983); Murphy, et al., *Acta.Haematologica Japonica*, 46(7), 1380–1396 (1983); Dessypris, et al., *Brit.J.Haematol*, 56, 295–306 (1984) ; and, Emmanouel, et al., *Am.J.Physiol.*, 247 (1 Pt 2), F168–76 (1984).

Because erythropoietin is essential in the process of red blood cell formation, the hormone has potential useful application in both the diagnosis and the treatment of blood disorders characterized by low or defective red blood cell production. See, generally, Pennathur-Das, et al., *Blood*, 63(5), 1168–71 (1984) and Haddy, *Am.Jour.Ped.Hematol./Oncol.*, 4, 191–196, (1982) relating to erythropoietin in possible therapies for sickle cell disease, and Eschbach, et al. *J.Clin.Invest.*, 74(2), pp. 434–441, (1984), describing a therapeutic regimen for uremic sheep based on in vivo response to erythropoietin-rich plasma infusions and proposing a dosage of 10 U EPO/kg per day for 15–40 days as corrective of anemia of the type associated with chronic renal failure. See also, Krane, *Henry Ford Hosp.Med.J.*, 31(3), 177–181 (1983).

It has recently been estimated that the availability of erythropoietin in quantity would allow for treatment each year of anemias of 1,600,000 persons in the United States alone. See, e.g., Morrison, "Bioprocessing in Space—an Overview", pp. 557–571 in The World Biotech Report 1984, Volume 2:USA, (Online Publications, New York, N.Y. 1984). Recent studies have provided a basis for projection of efficacy of erythropoietin therapy in a variety of disease states, disorders and states of hematologic irregularity: Vedovato, et al., *Acta.Haematol*, 71, 211–213 (1984) (beta-thalassemia); Vichinsky, et al., *J.Pediatr.*, 105(1), 15–21 (1984) (cystic fibrosis); Cotes, et al., *Brit.J.Obstet.Gyneacol.*, 90(4), 304–311 (1983) (pregnancy, menstrual disorders); Haga, et al., *Acta.Pediatr.Scand.*, 72, 827–831 (1983) (early anemia of prematurity); Claus-Walker, et al., *Arch.Phys.Med.Rehabil.*, 65, 370–374 (1984) (spinal cord injury); Dunn, et al., *Eur.J.Appl.Physiol.*, 52, 178–182 (1984) (space flight); Miller, et al., *Brit.J.Haematol.*, 52, 545–590 (1982) (acute blood loss); Udupa, et al., *J.Lab.Clin.Med.*, 103(4), 574–580 and 581–588 (1984); and Lipschitz, et al., *Blood*, 63(3), 502–509 (1983) (aging); and Dainiak, et al., *Cancer*, 51(6), 1101–1106 (1983) and Schwartz, et al., *Otolaryngol.*, 109, 269–272 (1983) (various neoplastic disease states accompanied by abnormal erythropoiesis).

Prior attempts to obtain erythropoietin in good yield from plasma or urine have proven relatively unsuccessful. Complicated and sophisticated laboratory techniques are necessary and generally result in the collection of very small amounts of impure and unstable extracts containing erythropoietin.

U.S. Pat. No. 3,033,753 describes a method for partially purifying erythropoietin from sheep blood plasma which

provides low yields of a crude solid extract containing erythropoietin.

Initial attempts to isolate erythropoietin from urine yielded unstable, biologically inactive preparations of the hormone. U.S. Pat. No. 3,865,801 describes a method of stabilizing the biological activity of a crude substance containing erythropoietin recovered from urine. The resulting crude preparation containing erythropoietin purportedly retains 90% of erythropoietin activity, and is stable.

Another method of purifying human erythropoietin from urine of patients with aplastic anemia is described in Miyake, et al., *J.Biol.Chem.*, Vol. 252, No. 15 (Aug. 10, 1977), pp. 5558–5564. This seven-step procedure includes ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography, and yields a pure erythropoietin preparation with a potency of 70,400 units/mg of protein in 21% yield.

U.S. Pat. No. 4,397,840 to Takezawa, et al. describes methods for preparing "an erythropoietin product" from healthy human urine specimens with weakly basic ion exchangers and proposes that the low molecular weight products obtained "have no inhibitory effects" against erythropoietin.

U.K. Patent Application No. 2,085,887 by Sugimoto, et al., published May 6, 1982, describes a process for the production of hybrid human lymphoblastoid cells, reporting production levels ranging from 3 to 420 Units of erythropoietin per ml of suspension of cells (distributed into the cultures after mammalian host propagation) containing up to 10^7 cells per ml. At the highest production levels asserted to have been obtained, the rate of erythropoietin production could be calculated to be from 40 to about 4,000 Units/10^6 cells/48 hours in in vitro culture following transfer of cells from in vivo propagation systems. (See also the equivalent U.S. Pat. No. 4,377,513.) Numerous proposals have been made for isolation of erythropoietin from tissue sources, including neoplastic cells, but the yields have been quite low. See, e.g., Jelkman, et al., *Expt.Hematol.*, 11(7), 581–588 (1983); Tambourin, et al., *P.N.A.S.* (*U.S.A.*), 80, 6269–6273 (1983); Katsuoka, et al., *Gann*, 74, 534–541 (1983); Hagiwara, et al., *Blood*, 63(4), 828–835 (1984); and Choppin, et al., *Blood*, *64*(2), 341–347 (1984).

Other isolation techniques utilized to obtain purified erythropoietin involve immunological procedures. A polyclonal, serum-derived antibody directed against erythropoietin is developed by injecting an animal, preferably a rat or rabbit, with human erythropoietin. The injected human erythropoietin is recognized as a foreign antigenic substance by the immune system of the animal and elicits production of antibodies against the antigen. Differing cells responding to stimulation by the antigenic substance produce and release into circulation antibodies slightly different from those produced by other responding cells. The antibody activity remains in the serum of the animal when its blood is extracted. While unpurified serum or antibody preparations purified as a serum immunoglobulin G fraction may then be used in assays to detect and complex with human erythropoietin, the materials suffer from a major disadvantage. This serum antibody, composed of all the different antibodies produced by individual cells, is polyclonal in nature and will complex with components in crude extracts other than erythropoietin alone.

Of interest to the background of the present invention are recent advances in the art of developing continuous cultures of cells capable of producing a single species of antibody which is specifically immunologically reactive with a single antigenic determinant of a selected antigen. See, generally, Chisholm, *High Technology*, Vol. 3, No. 1, 57–63 (1983). Attempts have been made to employ cell fusion and hybridization techniques to develop "monoclonal" antibodies to erythropoietin and to employ these antibodies in the isolation and quantitative detection of human erythropoietin. As one example, a report of the successful development of mouse-mouse hybridoma cell lines secreting monoclonal antibodies to human erythropoietin appeared in abstract form in Lee-Huang, Abstract No. 1463 of *Fed.Proc.*, 41, 520 (1982). As another example, a detailed description of the preparation and use of a monoclonal, anti-erythropoietin antibody appears in Weiss, et al., *P.N.A.S.* (*U.S.A.*), 79, 5465–5469 (1982). See also, Sasaki, *Biomed.Biochim.Acta.*, 42(11/12), S202–S206 (1983); Yanagawa, et al., *Blood*, 64(2), 357–364 (1984); Yanagawa, et al., *J.Biol. Chem.*, 259(5), 2707–2710 (1984); and U.S. Pat. No. 4,465,624.

Also of interest to the background of the invention are reports of the immunological activity of synthetic peptides which substantially duplicate the amino acid sequence extant in naturally-occurring proteins, glycoproteins and nucleoproteins. More specifically, relatively low molecular weight polypeptides have been shown to participate in immune reactions which are similar in duration and extent to the immune reactions of physiologically significant proteins such as viral antigens, polypeptide hormones, and the like. Included among the immune reactions of such polypeptides is the provocation of the formation of specific anti bodies in immunologically active animals. See, e.g., Lerner, et al., *Cell*, 23, 309–310 (1981); Ross, e t al., *Nature*, 294, 654–656 (1981); Walter, et al., *P.N.A.S.* (*U.S.A.*), 77, 5197–5200 (1980); Lerner, et al., *P.N.A.S.* (*U.S.A.*), 78, 3403–3407 (1981); Walter, et al., *P.N.A.S.* (*U.S.A.*), 78, 4882–4886 (1981); Wong, et al., *P.N.A.S.* (*U.S.A.*), 78, 7412–7416 (1981); Green, et al. *Cell*, 28, 477–487 (1982); Nigg, et al., *P.N.A.S.* (*U.S.A.*), 79, 5322–5326 (1982); Baron, et al., *Cell*, 28, 395–404 (1982); Dreesman, et al., *Nature*, 295, 158–160 (1982); and Lerner, *Scientific American*, 248, No. 2, 66–74 (1983). See, also, Kaiser, et al., *Science*, 223, pp. 249–255 (1984) relating to biological and immunological activities of synthetic peptides which approximately share secondary structures of peptide hormones but may not share their primary structural conformation. The above studies relate, of course, to amino acid sequences of proteins other than erythropoietin, a substance for which no substantial amino acid sequence information has been published. In co-owned, co-pending U.S. patent application Ser. No. 463,724, filed Feb. 4, 1983, by J. Egrie, published Aug. 22, 1984 as European Patent Application No. 0 116 446, there is described a mouse–mouse hybridoma cell line (A.T.C.C. No. HB8209) which produces a highly specific monoclonal, anti–erythropoietin antibody which is also specifically immunoreactive with a polypeptide comprising the following sequence of amino acids: NH_2-Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-COOH. The polypeptide sequence is one assigned to the first twenty amino acid residues of mature human erythropoietin isolated according to the method of Miyake, et al., *J.Biol. Chem.*, 252, 5558–5564 (1977) and upon which amino acid analysis was performed by the gas phase sequencer (Applied Biosystems, Inc.) according to the procedure of Hewick, M., et al., *J.Biol. Chem.*, 256, 7990–7997 (1981). See, also, Sue, et al., *Proc. Nat. Acad. Sci.* (*USA*), 80, pp. 3651–3655 (1983) relating to development of polyclonal antibodies against a synthetic 26-mer based on a differing amino acid sequence, and Sytowski, et al., *J.Immunol. Methods*, 69, pp.181–186 (1984).

While polyclonal and monoclonal antibodies as described above provide highly useful materials for use in immunoas-

says for detection and quantification of erythropoietin and can be useful in the affinity purification of erythropoietin, it appears unlikely that these materials can readily provide for the large scale isolation of quantities of erythropoietin from mammalian sources sufficient for further analysis, clinical testing and potential wide-ranging therapeutic use of the substance in treatment of, e.g., chronic kidney disease wherein diseased tissues fail to sustain production of erythropoietin. It is consequently projected in the art that the best prospects for fully characterizing mammalian erythropoietin and providing large quantities of it for potential diagnostic and clinical use involve successful application of recombinant procedures to effect large scale microbial synthesis of the compound.

While substantial efforts appear to have been made in attempted isolation of DNA sequences coding for human and other mammalian species erythropoietin, none appear to have been successful. This is due principally to the scarcity of tissue sources, especially human tissue sources, enriched in mRNA such as would allow for construction of a cDNA library from which a DNA sequence coding for erythropoietin might be isolated by conventional techniques. Further, so little is known of the continuous sequence of amino acid residues of erythropoietin that it is not possible to construct, e.g., long polynucleotide probes readily capable of reliable use in DNA/DNA hybridization screening of cDNA and especially genomic DNA libraries. Illustratively, the twenty amino acid sequence employed to generate the above-named monoclonal antibody produced by A.T.C.C. No. HB8209 does not admit to the construction of an unambiguous, 60 base oligonucleotide probe in the manner described by Anderson, et al., supra. It is estimated that the human gene for erythropoietin may appear as a "single copy gene" within the human genome and, in any event, the genetic material coding for human erythropoietin is likely to constitute less than 0.00005% of total human genomic DNA which would be present in a genomic library.

To date, the most successful of known reported attempts at recombinant-related methods to provide DNA sequences suitable for use in microbial expression of isolatable quantities of mammalian erythropoietin have fallen far short of the goal. As an example, Farber, et al. *Exp.Hematol.*, 11. Supp. 14, Abstract 101 (1983) report the extraction of mRNA from kidney tissues of phenylhydrazine-treated baboons and the injection of the mRNA into *Xenopus laevis* oocytes with the rather transitory result of in vitro production of a mixture of "translation products" which included among them displaying biological properties of erythropoietin. More recently, Farber, et al., *Blood*, 62, No. 5, Supp. No. 1, Abstract 392, at page 122a (1983) reported the in vitro translation of human kidney mRNA by frog oocytes. The resultant translation product mixture was estimated to include on the order of 220 mU of a translation product having the activity of erythropoietin per microgram of injected mRNA. While such levels of in vitro translation of exogenous mRNA coding for erythropoietin were acknowledged to be quite low (compared even to the prior reported levels of baboon mRNA translation into the sought-for product) it was held that the results confirm the human kidney as a site of erythropoietin expression, allowing for the construction of an enriched human kidney cDNA library from which the desired gene might be isolated. [See also, Farber, *Clin.Res.*, 31(4), 769A (1983).]

Since the filing of U.S. patent application Ser. Nos. 561,024 and 582,185, there has appeared a single report of the cloning and expression of what is asserted to have been human erythropoietin cDNA in *E.coli*. Briefly put, a number of cDNA clones were inserted into *E.coli* plasmids and β-lactamase fusion products were noted to be immunoreactive with a monoclonal antibody to an unspecified "epitope" of human erythropoietin. See, Lee-Huang, *Proc. Nat. Acad. Sci. (USA)*, 81, pp. 2708–2712 (1984).

BRIEF SUMMARY

The present invention provides, for the first time, novel purified and isolated polypeptide products having part or all of the primary structural conformation (i.e., continuous sequence of amino acid residues) and one or more of the biological properties (e.g., immunological properties and in vivo and in vitro biological activity) of naturally-occurring erythropoietin, including allelic variants thereof. These polypeptides are also uniquely characterized by being the product of procaryotic or eucaryotic host expression (e.g., by bacterial, yeast and mammalian cells in culture) of exogenous DNA sequences obtained by genomic or cDNA cloning or by gene synthesis. Products of microbial expression in vertebrate (e.g., mammalian and arian) cells may be further characterized by freedom from association with human proteins or other contaminants which may be associated with erythropoietin in its natural mammalian cellular environment or in extracellular fluids such as plasma or urine. The products of typical yeast (e.g., *Saccaromyces cerevisiae*) or procaryote (e.g., *E.coli*) host cells are free of association with any mammalian proteins. Depending upon the host employed, polypeptides of the invention may be glycosylated with mammalian or other eucaryotic carbohydrates or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue (at position −1).

Novel glycoprotein products of the invention include those having a primary structural conformation sufficiently duplicative of that of a naturally-occurring (e.g., human) erythropoietin to allow possession of one or more of the biological properties thereof and having an average carbohydrate composition which differs from that of naturally-occurring (e.g., human) erythropoietin.

Vertebrate (e.g., COS-1 and CHO) cells provided by the present invention comprise the first cells ever available which can be propagated in vitro continuously and which upon growth in culture are capable of producing in the medium of their growth in excess of 100 U (preferably in excess of 500 U and most preferably in excess of 1,000 to 5,000U) of erythropoietin per 10^6 cells in 48 hours as determined by radioimmunoassay.

Also provided by the present invention are synthetic polypeptides wholly or partially duplicative of continuous sequences of erythropoietin amino acid residues which are herein for the first time elucidated. These sequences, by virtue of sharing primary, secondary or tertiary structural and conformational characteristics with naturally-occurring erythropoietin may possess biological activity and/or immunological properties in common with the naturally-occurring product such that they may be employed as biologically active or immunological substitutes for erythropoietin in therapeutic and immunological processes. Correspondingly provided are monoclonal and polyclonal antibodies generated by standard means which are immunoreactive with such polypeptides and, preferably, also immunoreactive with naturally-occurring erythropoietin.

Illustrating the present invention are cloned DNA sequences of monkey and human species origins and polypeptide sequences suitably deduced therefrom which

represent, respectively, the primary structural conformation of erythropoietins of monkey and human species origins.

Also provided by the present invention are novel biologically functional viral and circular plasmid DNA vectors incorporating DNA sequences of the invention and microbial (e.g., bacterial, yeast and mammalian cell) host organisms stably transformed or transfected with such vectors. Correspondingly provided by the invention are novel methods for the production of useful polypeptides comprising cultured growth of such transformed or transfected microbial hosts under conditions facilitative of large scale expression of the exogenous, vector-borne DNA sequences and isolation of the desired polypeptides from the growth medium, cellular lysates or cellular membrane fractions.

Isolation and purification of microbially expressed polypeptides provided by the invention may be by conventional means including, e.g., preparative chromatographic separations and immunological separations involving monoclonal and/or polyclonal antibody preparations.

Having herein elucidated the sequence of amino acid residues of erythropoietin, the present invention provides for the total and/or partial manfucture of DNA sequences coding for erythropoietin and including such advantageous characteristics as incorporation of codons "preferred" for expression by selected non-mammalian hosts, provision of sites for cleavage by restriction endonuclease enzymes and provision of additional initial, terminal or intermediate DNA sequences which facilitate construction of readily expressed vectors. Correspondingly, the present invention provides for manufacture (and development by site specific mutagenesis of cDNA and genomic DNA) of DNA sequences coding for microbial expression of polypeptide analogs or derivatives of erythropoietin which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (i.e., deletion analogs containing less than all of the residues specified for EPO and/or substitution analogs wherein one or more residues specified are replaced by other residues and/or addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptide); and which share some or all the properties of naturally-occurring forms.

Novel DN sequences of the invention include all sequences useful in securing expression in procaryotic or eucaryotic host cells of polypeptide products having at least a part of the primary structural conformation and one or more of the biological properties of erythropoietin which are comprehended by: (a) the DNA sequences set out in FIGS. **5** and **6** herein or their complementary strands; (b) DNA sequences which hybridize (under hybridization conditions such as illustrated herein or more stringent conditions) to DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to DNA sequences defined in (a) and (b) above. Specifically comprehended in part (b) are genomic DNA sequences encoding allelic variant forms of monkey and human erythropoietin and/or encoding other mammalian species of erythropoietin. Specifically comprehended by part (c) are manufactured DNA sequences encoding EPO, EPO fragments and EPO analogs which DNA sequences may incorporate codons facilitating translation of messenger RNA in non-vertebrate hosts.

Comprehended by the present invention is that class of polypeptides coded for by portions of the DNA complement to the top strand human genomic DNA sequence of FIG. **6** herein, i.e., "complementary inverted proteins" as described by Tramontano, et al., *Nucleic Acids Research*, 12, pp. 5049–5059 (1984).

Also comprehended by the invention are pharmaceutical compositions comprising effective amounts of polypeptide products of the invention together with suitable diluents, adjuvants and/or carriers which allow for provision of erythropoietin therapy, especially in the treatment of anemic disease states and most especially such anemic states as attend chronic renal failure.

Polypeptide products of the invention may be "labelled" by covalent association with a detectable marker substance (e.g., radiolabelled with ^{125}I) to provide reagents useful in detection and quantification of erythropoietin in solid tissue and fluid samples such as blood or urine. DNA products of the invention may also be labelled with detectable markers (such as radiolabels and non-isotopic labels such as biotin) and employed in DNA hybridization processes to locate the erythropoietin gene position and/or the position of any related gene family in the human, monkey and other mammalian species chromosomal map. They can also be used for identifying the erythropoietin gene disorders at the DNA level and used as gene markers for identifying neighboring genes and their disorders.

As hereinafter described in detail, the present invention further provides significant improvements in methods for detection of a specific single stranded polynucleotide of unknown sequence in a heterogeneous cellular or viral sample including multiple single-stranded polynucleotides where

(a) a mixture of labelled single-stranded polynucleotide probes is prepared having uniformly varying sequences of bases, each of said probes being potentially specifically complementary to a sequence of bases which is putatively unique to the polynucleotide to be detected,

(b) the sample is fixed to a solid substrate,

(c) the substrate having the sample fixed thereto is treated to diminish further binding of polynucleotides thereto except by way of hybridization to polynucleotides in said sample,

(d) the treated substrate having the sample fixed thereto is transitorily contacted with said mixture of labelled probes under conditions facilitative of hybridization only between totally complementary polynucleotides, and,

(e) the specific polynucleotide is detected by monitoring for the presence of a hybridization reaction between it and a totally complementary probe within said mixture of labelled probes, as evidenced by the presence of a higher density of labelled material on the substrate at the locus of the specific polynucleotide in comparison to a background density of labelled material resulting from non-specific binding of labelled probes to the substrate.

The procedures are especially effective in situations dictating use of 64, 128, 256, 512, 1024 or more mixed polynucleotide probes having a length of 17 to 20 bases in DNA/DNA or RNA/RNA or DNA/RNA hybridizations.

As described infra, the above-noted improved procedures have illustratively allowed for the identification of cDNA clones coding for erythropoietin of monkey species origins within a library prepared from anemic monkey kidney cell mRNA. More specifically, a mixture of 128 uniformly varying 20-mer probes based on amino acid sequence information derived from sequencing fractions of human erythropoietin was employed in colony hybridization procedures to identify seven "positive" erythropoietin cDNA clones within a total of 200,000 colonies. Even more remarkably, practice of the improved procedures of the invention have