

U.S. Patent

Apr. 15, 1997

Sheet 11 of 27

5,621,080

EXHIBIT B, Part 2

FIG. 6D

AAAAAGAAAAGAAAAGAAAATAATGAGGGCTGTATGGAATACATTCAATTATTCATTCACTCACTCACT
 CACTCATTCAATTCATTCATTCAACAAGTCTTATTGCATACCTTCTGTTTGCTCAGCTTGGTGTCTTGG
 GGCTGTGAGGGCAGGAGGGAGGGGTGACATGGGTGAGCTCGACTCCCAGAGTCCACTCCCCTGTAG
 56 Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Ser Glu Ala
 GTC GGG CAG CAG GCC GTA GAA GTC TGG CAG GGC CTG GCC CTG TCG TCG GAA GCT
 70
 Val Leu Arg Gly Gln Ala Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu
 GTC CTG CCG GGC CAG GCC CTG TTG GTC AAC TCT TCC CAG CCG TGG GAG CCC CTG
 80 * 90
 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
 100
 110 Arg Ala Leu Gly Ala Gln
 CGG GCT CTG GGA GCC CAG GTGAGTAGGAGCGGACACTTCTGCTTGCCCTTCTGTAAAGAGGGGA
 GAAGGGTCTTGCTAAGGAGTACAGGAACTGTCCGTATTCCCTTCTGTTGGCACTGCAGCGACCTCCT
 115
 116 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
 120

U.S. Patent

Apr. 15, 1997

Sheet 12 of 27

5,621,080

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
 140
 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 AAG CTG TAC ACA GGG GAG GCC TGC AGG ACA GGG
 160
 166 Asp Arg OP
 GAC AGA TGA CCAGGTGTCCACCTGGGCATATCCACCACCTCCCTCACCACCAACATGCTTGTCACACA
 CCCTCCCCGACACTCCTGAACCCCGTCGAGGGGCTCTCAGCTCAGCGCCAGCCCTGTCCCATGGACACTCC
 AGTGCCAGCAATGACATCTCAGGGGCCAGAGGAACCTGTCCAGAGAGCAACTCTGAGATCTAAGGATGTCCAC
 AGGCCAACTTGAAGGCCAGAGCAGGAAGCAATTCAGAGAGCAGCTTTAAACTCAGGGACAGAGCCATGC
 TGGGAAGACGCCGTGAGCTCACTCGGCACCCTGTCAAAAATTGTATGCCAGGACACGCTTTGGAGGCCGATTAC
 CTGTTTTCGCACCTACCATCAGGGACAGGATGACCCTGGAGAACTTAGGTGGCAAGCTGTGACTTCTCCAGG
 TCTCACGGGCATGGGCATCCCTTGGTGGCAAGAGCCCTTGACACCGGGGTGGGAAACCATGAAGAC
 AXGATXGGGGCTGGCCCTCTGCTCATGGGGTCCAAAGTTTGTGATTTCTCAACCCTATTGACAGACTGAA
 ACACAATATGAC

U.S. Patent

Apr. 15, 1997

Sheet 13 of 27

5,621,080

FIG. 7

<u>XbaI</u>	-1	1	<u>MetAla</u>
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 ACGCTTGGAAC ACGTATGGAA			
AATGCCATGG TCTGTGGTTC CAATTGAAGA TGCGAACCTT TGCATACCTT			
GTTGGTCAAC AAGCAGTTGA AGTTTGGCAG GGTCTGGCAC TGCTGAGCGA			
CAACCAGTTG TTCGTCAACT TCAAACCGTC CCAGACCGTG ACGACTCGCT			
GGCTGTACTG CGTGGCCAGG CACTGCTGGT AACTCCTCT 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			
GCAAAGTGT TCGTGATATC TCTAACTTCC TGCGTGGTAA ACTGAAACTG			
CGTTTGACAA AGCACATATG AGATTGAAGG ACGCACCATT TGACTTTGAC			
TATACTGGCG AAGCATGCCG TACTGGTGAC CGCTAATAG			<u>SalI</u>
ATATGACCGC TTCGTACGGC ATGACCACTG GCGATTATCA GCT			

U.S. Patent

Apr. 15, 1997

Sheet 14 of 27

5,621,080

FIG. 8

<u>HindIII</u>	-1	+1			
	Arg	Ala			
AGCTTGGATA	AAAGAGCTCC	ACCAAGATTG	ATCTGTGACT	CGAGAGTTTT	
ACCTAT	TTTCTCGAGG	TGGTTCTAAC	TAGACACTGA	GCTCTCAAAA	
GGAAAGATAC	TTGTTGGAAG	CTAAAGAAGC	TGAAAACATC	ACCACTGGTT	
CCTTTCATATG	AACAACCTTC	GATTTCTTCG	ACTTTTGTAG	TGGTGACCAA	
GTGCTGAACA	CTGTTCTTTG	AACGAAAACA	TTACGGTACC	AGACACCAAG	
CACGACTTGT	GACAAGAAAC	TTGCTTTTGT	AATGCCATGG	TCTGTGGTTC	
GTTAACTTCT	ACGCTTGGAA	ACGTATGGAA	GTTGGTCAAC	AAGCTGTTGA	
CAATTGAAGA	TGCGAACCTT	TGCATACCTT	CAACCAGTTG	TTCGACAAC	
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	
CTATTTCCGGC	AGAGACCAAA	CTCTAGAAAC	TGATGAAACA	ACTCTCGAAA	
GGGTGCTCAA	AAGGAAGCCA	TTTCCCACC	AGACGCTGCT	TCTGCCGCTC	
CCCACGAGTT	TTCTTCGGT	AAAGGGTGG	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		

	<u>SalI</u>
ATGTAACAAA	G
TACATTGTTT	CAGCT

FIG. 9

	-20	-10	+1	10	20	30	40
Human	MGVHECPAWLWLLSLLSPLGLPVLGAPPRLLICDSRVLERYLLEAKEAENITGCAEHCSLNENITVPDTK						
	*****	*****	*****	*****	*****	*****	*****
Monkey	MGVHECPAWLWLLSLLSPLGLPVLGAPPRLLICDSRVLERYLLEAKEAENVTMGCSSECSLNENITVPDTK						

	50	60	70	80	90	100	110
Human	VNFYAWKRMEVGGQAVEVWQGLALLSEAVLRGQALLVNSSQPWEPLQLHVVDKAVSGLRSITLLRALGAQKE						
	*****	*****	*****	*****	*****	*****	*****
Monkey	VNFYAWKRMEVGGQAVEVWQGLALLSEAVLRGQAVLANSSQPFEPQLHMDKAISGLRSITLLRALGAQ-E						

	120	130	140	150	160
Human	AISLPPDAASAAPLRTITADTFRKLFRVYSNFIKGLKLYTGEACRTGDR				
	***	*****	*****	*****	*****
Monkey	AISLPPDAASAAPLRTITADTFCKLFRVYSNFIKGLKLYTGEACRRGDR				

FIG. 10

1. AATTCTAGAAACCATGAGGGTAATAAAATA
2. CCATTATTTTATTACCCTCATGGTTTCTAG
3. ATGGCTCCGCCGCGTCTGATCTGCGAC
4. CTCGAGTCGCAGATCAGACGCGGCGGAG
5. TCGAGAGTTCTGGAACGTTACCTGCTG
6. CTTCCAGCAGGTAACGTTCCAGAACT
7. GAAGCTAAAGAAGCTGAAAACATC
8. GTGGTGATGTTTTTCAGCTTCTTTAG
9. ACCACTGGTTGTGCTGAACACTGTTC
10. CAAAGAACAGTGTTTCAGCACAACCA
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTTTCGTT

FIG. 12

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

FIG. 13

EcoRI KpnI 1
 A ATTCGGTACC AGACACCAAG GTTAACTTCT ACGTTGGAA ACGTATGGAA
GCCATGG TCTGTGGTTC CAATTGAAGA TGCGAACCTT TGCATACCTT
2 3 4

5
GTTGGTCAAC AAGCAGTTGA AGTTTGGCAG GGTCTGGCAC TGCTGAGCGA
CAACCACTTG TTCGTCAACT TCAAACCGTC CCAGACCCGTG ACGACTCGCT
6 7 8

9
GGCTGTACTG CGTGGCCAGG CACTGCTGGT AAACTCCTCT CAGCCGTGGG
CCGACATGAC GCACCCGGTCC 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. TCGGTGCTCTGGGTGCACAGAAAGAGG
4. GATAGCCTCTTTCTGTGCACCCAGAGC
5. CTATCTCTCCGCCGGATGCTGCATCT
6. CAGCAGATGCAGCATCCGGCGGAGA
7. GCTGCACCGCTGCGTACCATCACTG
8. ATCAGCAGTGATGGTACGCAGCGGTG
9. CTGATACCTTCCGCAAACCTGTTTCG
10. ATACACGAAACAGTTTGCGGAAGGT
11. TGTATACTCTAACTTCCTGCGTGGTA
12. CAGTTTACCACGCAGGAAGTTAGAGT
13. AACTGAAACTGTATACTGGCGAAGC
14. GGCATGCTTCGCCAGTATACAGTTT
15. ATGCCGTACTGGTGACCGCTAATAG
16. TCGACTATTAGCGGTCACCAGTAC

U.S. Patent

Apr. 15, 1997

Sheet 21 of 27

5,621,080

FIG. 15

BamHI BglIIGA TCCAGATCTCTG
GTCTAGAGAC

1 ACTACTCTGC TGCGTGCTCT 3 GGGTGCACAG AAAGAGGCTA 5 TCTCTCCGCC
 TGATGAGACG 2 ACGCACGAGA CCCACGTGTC 4 TTTCTCCGAT AGAGAGGCGG

GGATGCTGCA TCTGCTGCAC 7 CGCTGCGTAC CATCACTGCT 9 GATACCTTCC
 CCTACGACGT 6 AGACGACGTG 8 GCGACGCATG GTAGTGACGA CTATGGAAGG

GCAAACTGTT TCGTGTATAC 11 TCTAACTTCC TGCGTGGTAA 13 ACTGAAACTG
 CGTTTGACAA 10 AGCACATATG AGATTGAAGG 12 ACGCACCATT TGACTTTGAC

TATACTGGCG 14 AAGCATGCCG 15 TACTGGTGAC CGCTAATAG SalI
 ATATGACCGC TTCGTACGGC ATGACCACTG GCGATTATC AGCT 16

FIG. 16

1. AATCAAGCTTGGATAAAAGAGCT
2. GTGGAGCTCTTTTATCCAAGCTTG
3. CCACCAAGATTGATCTGTGACTC
4. TCTCGAGTCACAGATCAATCTTG
5. GAGAGTTTTGGAAAGATACTTGTTG
6. CTTCCAACAAGTATCTTTCCAAAAC
7. GAAGCTAAAGAAGCTGAAAACATC
8. GTGGTGATGTTTTTCAGCTTCTTTAG
9. ACCACTGGTTGTGCTGAACACTGTTT
10. CAAAGAACAGTGTTTCAGCACAACCA
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTTTCGTT

FIG. 17

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 AACAACTTC 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. CCAAACTTCAACTGCTTGTGACCAAC
7. TTGGCAAGGTTTGGCCTTGTTATCTG
8. GCTTCAGATAACAAGGCCAAACCTTG
9. AAGCTGTTTTGAGAGGTGAAGCCT
10. AACAAGGCTTGACCTCTCAAACA
11. TGTTGGTTAACTCTTCTCAACCATGGG
12. TGGTTCCCATGGTTGAGAAGAGTTAACC
13. AACCATTGCAATTGCACGTCGAT
14. CTTTATCGACGTGCAATTGCAA
15. AAAGCCGTCTCTGGTTTGAGATCTG
16. GATCCAGATCTCAAACCAGAGACGG

FIG. 20

1. GATCCAGATCTTTGACTACTTTGTT
2. TCTCAACAAAGTAGTCAAAGATCTG
3. GAGAGCTTTGGGTGCTCAAAGGAAG
4. ATGGCTTCCTTTTGAGCACCCAAAGC
5. CCATTTCCCACCAGACGCTGCTT
6. GCAGAAGCAGCGTCTGGTGGGGAA
7. CTGCCGCTCCATTGAGAACCATC
8. CAGTGATGGTTCTCAATGGAGCG
9. ACTGCTGATACCTTCAGAAAGTT
10. GAATAACTTTCTGAAGGTATCAG
11. ATTCAGAGTTTACTCCAACCTTCT
12. CTCAAGAAGTTGGAGTAAACTCT
13. TGAGAGGTAAATTGAAGTTGTACAC
14. ACCGGTGTACAACCTTCAATTTACCT
15. CGGTGAAGCCTGTAGAACTGGT
16. CTGTCACCAGTTCTACAGGCTTC
17. GACAGATAAGCCCGACTGATAA
18. GTTGTTATCAGTCGGGCTTAT
19. CAACAGTGTAGATGTAACAAAG
20. TCGACTTTGTTACATCTACACT

U.S. Patent

Apr. 15, 1997

Sheet 27 of 27

5,621,080

FIG. 21

BamHI BglII 1GATC CAGATCTTTG ACTACTTTGT TGAGAGCCTT

GTCTAGAAAC TGATGAAACA ACTCTCGAAA

2

3 5
GGGTGCTCAA AAGGAAGCCA TTTCCCACC AGACGCTGCT TCTGCCGCTC
CCCACGAGTT TTCCTTCGGT AAAGGGGTGG TCTGCGACGA AGACGGCGAG

46

7 9 11
CATTGAGAAC CATCACTGCT GATACCTTCA GAAAGTTATT CAGAGTTTAC
GTA ACTCTTG GTAGTGACGA CTATGGAAGT CTTTCAATAA GTCTCAAATG

81012

13 15
TCCA ACTTCT TGAGAGGTA ATTGAAGTTG TACACCGGTG AAGCCTGTAG
AGGTTGAAGA ACTCTCCATT TAACTTCAAC ATGTGGCCAC TTCGACATC

1416

17 19
AACTGGTGAC AGATAAGCCC GACTGATAAC AACAGTGTAG
TTGACCACTG TCTATTCGGG CTGACTATTG TTGTCACATC

SalIATGTAACAAA G
TACATTGTTT CAGCT20

5,621,080

1

PRODUCTION OF ERYTHROPOIETIN

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

2

"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

5,621,080

3

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 techniques, 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 US83/00605, published Nov. 24, 1983 as W083/04053), 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 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.*,

4

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 significant 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, Wallace, 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*, 299, pp. 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 shove-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 of a gene coding for the alpha subunit of human pituitary glycoprotein hormones from the Maniatis Library through use of a "full length"

5,621,080

5

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 amino 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 amino 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 amino 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 , β and 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 trans-

6

port 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.*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.Gynaecol.*, 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. Com-