(54) Title: INTERLEUKIN-2 PRODUCTION USING CLONED GENES FOR INTERLEUKIN-2 AND YEAST ALPHA-FACTOR

3'...5' IL2-6 IL2-7 IL2-8 IL2-9 IL2-10 IL2-11 IL2-12 IL2-13 -3'
  IL2-19 IL2-20 IL2-21 IL2-22 IL2-23 IL2-24 IL2-25 IL2-26 IL2-27 -5'

(57) Abstract

Methods and compositions for efficient production of human interleukin-2. A synthetic interleukin-2 gene is joined to yeast alpha-factor secretory leader and processing signals to provide for expression and secretion of mature gene product in yeast. Enhanced yields of the product may be recovered from the nutrient medium.
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INTERLEUKIN–2 PRODUCTION USING GLOINED GENES FOR INTERLEUKIN–2
AND YEAST ALPHA–FACTOR

Background of the Invention

1. Field of the Invention

Lymphokines are naturally-occurring polypeptides, produced by normal lymphocytes, which mediate the host's immune response to antigenic challenge. A particular lymphokine, interleukin-2, appears to promote the host's immune response and has potential value in the treatment of tumors, immuno-deficiency diseases and several other clinical conditions, and as an adjuvant for vaccine administration. Interleukin-2 appears to act as a potent mitogen for T lymphocytes. At present, only limited quantities of interleukin-2 are obtained by separation from human serum or from certain human cell tissue culture media. It is therefore of great scientific and clinical importance to be able to produce sufficiently large quantities of interleukin-2. Economic, efficient methods for producing a product having the properties of mature human interleukin-2 have therefore become significant goals.

2. Description of the Prior Art

Repeats of Mature Alpha-Factor," describe the sequence encoding for the α-factor and spacers between two of such sequences.


Summary of the Invention

Novel methods and DNA constructs are provided for the production of polypeptides having biological activity analogous to interleukin-2 (IL2). Enhanced efficiency in production of the polypeptide is achieved, in part, by the complete synthesis of the structural gene employing codons preferentially utilized by yeast, the intended host. Desirably, at least about 50%, usually, at least about 60% of the codons are modified such that most of the codons of the structural gene are those preferentially utilized by yeast. The construct includes a replication system for yeast and the structural gene joined in reading frame to secretory and processing signals recognized by yeast. The yeast host transformant provides for efficient and economic production of a product useful as interleukin-2.
Brief Description of the Drawings

Fig. 1 illustrates the order of assembly of synthetic ssDNA segments used in preparing the 5'-half of the synthetic IL2 gene.

Fig. 2 illustrates the scheme utilized to clone the synthetic DNA fragment of Fig. 1.

Fig. 3 illustrates the order of assembly of ssDNA segments used in preparing the 3'-half of the synthetic IL2 gene.

Fig. 4 illustrates the scheme utilized to clone the synthetic gene fragment of Fig. 3.

Fig. 5 illustrates the scheme utilized to join the two halves of the synthetic IL2 gene.

Description of the Specific Embodiments

DNA constructs capable of expressing mammalian, particularly human interleukin-2 (IL2) in a eukaryotic microorganism host are provided. (Unless otherwise indicated when referring to interleukin-2 (IL2) polypeptide(s), it is intended to include not only the naturally occurring mammalian factors, but also fragments or analogs thereof exhibiting analogous biological activity.) These DNA fragments can be incorporated into vectors, and the resulting plasmids used to transform susceptible hosts. Transformation of a susceptible host with such recombinant plasmids results in expression of the IL2 gene and production of a mature polypeptide product having the physiological and immunological activity of IL2. That is, it acts in the same manner as IL2 isolated from either a rat or a human host in recognized bioassays.

Extrachromosomal constructs are provided having as essential elements for expression of mature polypeptides, a replication system recognized by yeast, a synthetic structural gene having a plurality of
codons preferentially utilized by yeast, the structural
gene being in reading frame with efficient secretory
leader and processing signals to provide for the
efficient secretion and processing of the polypeptide
in a yeast host, and production of a product in high
yield which has biological, particularly immunological
and physiological activity commensurate with human
interleukin-2. The construct provides for the initial
formation of "pre"-IL2.

By "pre"-IL2, it is meant that the DNA
sequence encoding the mature polypeptide is joined to
and in reading frame with a leader sequence including
processing signals efficiently recognized by the yeast
host. Thus, "pre" denotes the inclusion of secretory
leader and processing signals sequences (on the
precursor polypeptide) recognized by yeast and does not
refer to any processing signals associated with the
natural human IL2 gene.

The IL2 structural gene of this invention is
synthetic DNA, which is prepared using codons preferred
by yeast as evidenced by the codon frequency in
structural genes encoding for yeast glycolytic enzymes.
The secretory leader and processing signals are
conveniently derived from naturally-occurring DNA
sequences in yeast, which sequences provide for the
secretion and processing of polypeptide(s). Such
polypeptides which are naturally secreted by yeast
include a-factor, a-factor, acid phosphatase, and the
like. The remaining sequences in the construct,
including the replication system, promoter, and
terminator, are well known and amply described in the
literature.

In preparing the DNA constructs of the
present invention, it is necessary to bring the
individual DNA sequences embodying the structural gene,
the secretory leader and processing signals,
replication system, promoter, and terminator together
in a predetermined order to assure that they are able to properly function in the resulting plasmid. Since the various DNA sequences which are joined to form the DNA construct of the present invention will be derived from diverse sources, in some instances it will be convenient or necessary to join the sequences by means of connecting or adaptor molecules, which in the subject invention are incorporated into the synthetic gene.

In developing the subject invention, advantage is taken of a pre-existing vector, which includes a multicopy number yeast replication system, a bacterial replication system, appropriate markers for selection, as well as a promoter, transcriptional terminator and modified leader sequence associated with the secretion of α-factor. See copending application Serial No. 522,909, filed August 12, 1983.

The structural gene encoding for IL2 is prepared by first preparing a series of single stranded fragments ranging in number of from about 10 to 40, conveniently 14 to 34 bases, which provide for overlapping of other fragments and overhangs, so that upon bringing together the fragments under ligating conditions, dsDNA is produced having appropriate cohesive ends. In view of the large size of the IL2 structural gene and to provide for future flexibility in potential manipulations, a 5'- and 3'-fragment were prepared and then combined to provide for a structural gene encoding the entire amino acid sequence of IL2 and providing for appropriate termini for linking to restriction sites in the vector, which provide for the structural gene being in frame with the secretory leader sequence.

Each of the fragments encoding for the gene - 5'-fragment and 3'-fragment with flanking regions as appropriate - may be cloned and amplified in an appropriate vector to expand the amount of the fragment
and to ensure its integrity. The 5'-fragment is inserted downstream from transcriptional regulatory signals for transcription initiation and a secretory leader sequence which includes a convenient restriction site at or about the processing signal.

By employing a synthetic fragment, the termini of the fragment can be tailored to fulfill the requirements necessary for subsequent processing steps. For the 5'-fragment, its 5'-terminus is designed to join to the secretory leader sequence and processing signal in reading frame and to replace any nucleotides which have been lost due to restriction or other processing of the nucleotide sequence encoding for the secretory leader and processing signal. Thus, in restricting the vector, a restriction site can be chosen which is internal to the coding region for the secretory leader and processing signal.

In addition, it may be useful to extend the 5'-fragment in the 3'-direction beyond the site where the 5'-fragment and 3'-fragment are to be linked. In this way, a convenient 3'-terminus of the 5'-fragment is present for joining to the vector 5'-terminus. The extension is then removed by restriction to provide a 3'-terminus of the 5'-fragment which is complementary to the 5'-terminus of the 3'-fragment.

Parallel manipulations may be employed with the 3'-fragment to provide for appropriate restriction sites and termini for joining to a vector and to the 5'-fragment, as well as supplying nucleotides in the 3'-non-coding region associated with termination of transcription and translation.

After cloning the synthetic 5'-fragment in the vector containing the secretory leader sequence, a new extended 5'-fragment is excised. The new 5'-fragment has its 5'-end beginning with the transcriptional regulatory signals controlling the transcription of the secretory leader sequence and
processing signal, followed by the synthetic 5'-fragment in reading frame with the secretory leader sequence. Thus, this new fragment now includes the promoter and other associated transcriptional regulatory sequences, such as the TATA box and capping sequence, as well as any other sequences involved in the efficient transcription of α-factor. By employment of appropriate restriction enzymes, the fragment is obtained which has all the necessary transcriptional regulatory functions, the secretory leader and processing signal, the 5'-end of the IL2 structural gene and a cohesive end or blunt end for ligating to the 5'-terminus of the 3'-fragment.

The 3'-half of the IL2 is inserted into an appropriate vector for cloning. The resulting plasmid has a unique restriction site at the 5'-end of the 3'-fragment resulting in cohesive termini when cut, and appropriate transcriptional termination sequences downstream, such as a terminator and polyadenylation signal. The fragment to be inserted conveniently has the same cohesive termini. In order to prevent circularization of the plasmid without insertion of the 5'-end of the IL2, the plasmid is treated with phosphatase. The fragment containing the transcriptional regulatory signals and 5'-end of the IL2 structural gene is then inserted into the phosphatase-treated plasmid for bacterial transformation and subsequently excised and ligated to provide for a plasmid capable of transforming a yeast host efficiently, being multicopy, and providing for the efficient secretion of the polypeptide encoded by the IL2 gene.

While the homologous promoter associated with the secretory leader sequence may be used, it may also be replaced with other promoters or may be used in tandem with other promoters.

A wide variety of promoters are available or can be obtained from yeast genes. Promoters of
particular interest include acid phosphatase and those promoters involved with enzymes in the glycolytic pathway, such as promoters for alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, triose phosphate isomerase, phosphoglucoisomerase, phosphofructokinase, etc. By employing these promoters with regulatory sequences, such as enhancers, operators, etc., and using a host having an intact regulatory system, one can regulate the expression of the "pre"-IL2, and various small organic molecules, e.g., glucose, may be employed for the regulation of production of the desired polypeptide.

One may also employ temperature controlled systems, e.g., temperature-sensitive regulatory mutants which allow for modulation of transcription by varying the temperature. Thus, by growing the cells at either the non-permissive or permissive temperature, as appropriate, one can grow the cells to high density, before changing the temperature in order to provide for expression of the "pre"-polypeptide for IL2.

Other capabilities may also be introduced into the construct. For example, some genes provide for amplification, where upon stress to the host, not only is the gene which responds to the stress amplified, but also flanking regions. By placing such a gene upstream from the promoter, coding region and the other regulatory signals providing transcriptional control of the "pre"-polypeptide, and stressing the yeast host, plasmids may be obtained which have a plurality of repeating sequences, which sequences include the "pre"-polypeptide gene with its regulatory sequences. Illustrative genes include metallothioneins and dihydrofolate reductase.

The construct may include, in addition to the secretory leader and processing signal sequence, other DNA homologous to the host genome. If it is desired that there be integration of the IL2 gene into the
chromosome(s), integration can be enhanced by providing for sequences flanking the IL2 gene construct which are homologous to host chromosomal DNA.

The replication system which is employed will be recognized by the yeast host. Therefore, it is desirable that the replication system be native to the yeast host. A number of yeast vectors are reported by Botstein et al., Gene (1979) 8:17-24. Of particular interest are the YEp plasmids, which contain the 2μm plasmid replication system. These plasmids are stably maintained at multiple copy number. Alternatively, or in addition, one may use a combination of ARS1 and CEN4, to provide for stable maintenance.

The plasmids may be introduced into the yeast host by any convenient means, employing yeast host cells or spheroplasts and using DNA for transformation, or liposomes, or calcium precipitated DNA or other conventional technique. The modified hosts may be selected in accordance with the genetic markers which are usually provided in a vector used to construct the expression plasmid. An auxotrophic host may be employed, where the plasmid has a gene which complements the host and provides prototrophy. Alternatively, resistance to an appropriate biocide, e.g., antibiotic, heavy metal, toxin, or the like, may be included as a marker in the plasmid. Selection may then be achieved by employing a nutrient medium which stresses the parent cells, so as to select for the cells containing the plasmid. The plasmid containing cells may then be grown in an appropriate nutrient medium, and the desired secreted polypeptide isolated in accordance with conventional techniques. The polypeptide may be purified by chromatography, filtration, extraction, etc. Since the polypeptide will be present in mature form in the nutrient medium, one can cycle the nutrient medium, continuously removing the desired polypeptide.
The following examples are offered by way of example and not by way of limitation.

**EXPERIMENTAL**

A nucleotide sequence for interleukin-2 comprising preferentially utilized yeast codons was devised. The sequence included a portion of a modified a-factor secretory leader and processing signal at its 5' end. The three glu-ala pairs were deleted and a base pair was changed internal to the secretory leader sequence to promote a KpnI site and change the codon from ser to gln. The sequence with the coding strand shown 5' to 3', was as follows:

Processing Site

5'-AGCTGGATACGAGCTCTCAAGCTTCTCTACCAAGAGCAGCTGCAATTGG

3'-CATGTGACCTATTTTCTGCAGGTGGGAGAGAGATGTTTCTGCTGAGTTAAC (KpnI)

GluHisLeuLeuAspLeuGlnMetIleLeuAsnGlyIleAsnAsnTryLysAsnPro
GAACACTTTGTTGTTGACTTGCAAAGAACATCTGAGGATGATCAACACACTCAAGAGAACC
CTTGTGAAACAAACACCTGAAAGCTTTACTGAACATTGCGATAGTTGATGTTCTGAGG

LysLeuThrArgMetLeuThrPheLysPheTyrMetProLysLysAlaThrGluLeuLys
AAGTGGACGAGAAATGCTGACCTTCAAGCTCTACAGCTCAAAGAGCAGCTTCAAGAGAACC
TTCAACTCGTCTTCAACTGGAGATCTGCTCAAGAGCAGCTTCTGAGGCTGTACGTTCTC

HisLeuGlnCysLeuGluGluLeuLysProLeuGluGluValLeuAsnLeuAlaGln
CACCTGCAGCTCTAGCAAGGAGTTGGAACGCAAGCTGCTGACCTGCTCAGA
GTTGACGCTGACGACATCGTCTCTCCTCAACCTTCTGAAAGACTGTAACCTTCTCCTGAAG

XbaI

SerLysAsnPheHisLeuArgProArgAspLeuIleSerAsnIleAsnValIleValLeu
TCTAAGAATTCTCGAGACCAAGACACTGATCTTCAACATCAAGCTGTTATCGTTC
AGAATCTTGGAAGTTGAACTCTGTTCTCGTGAAGCTGAGATTGTGTTGCAATAGGCAAA

GluLeuLysGlySerGluThrGluGluThrPheMetCysGluTyrAlaAspGluThrAlaThrIle
GAATTGAAAGTTGTTGCAAGACACTCTCAGATGAACTACCGAGAAGACGGCTACATC
CTTAACCTCCTCAAGACTTTTGTTGCAACTACATGATTGAGATGCTG

ValGluPheLeuAsnArgTrpIleThrPheCysGlnSerIleIleSerThrLeuThrOP
GCTGAACTCTGAGACAGATGACACCTTCAGTTCACTACTATGCTCTTACGATCCTGAG
CAACCTAAGAACTTTGCTACTACCATAGATTGAGATGCTGAACTTGACT
AM
TAGGGTGCATACT-3'
ATGGAGGACGAGCT-5'

SalI
The sequence is provided with a KpnI cohesive end at the 5'-end and a SalI cohesive end at the 3'-end. The coding sequence for the mature polypeptide begins after the processing site.

A synthetic DNA fragment for interleukin-2 having the above sequence was prepared by synthesizing and cloning two halves of the fragment separately. Each half fragment was prepared by synthesizing overlapping ssDNA segments using the phosphoramidite method, as described by Beaucage and Caruthers (1981) Tetrahedron Lett. 22:1859-1862. The ssDNA segments were as follows:

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<th>Designation</th>
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<td>Linker</td>
<td>AGCTGGGATAAAAGA</td>
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<td>GCTCCCAACTCCCTTCTTCCACCAAGAGACCCAGG</td>
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<td>IL2-1</td>
<td>CTGCAATTGGAACACTTTGTTGTGACTT</td>
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<td>IL2-2</td>
<td>GCAAATGTCTTGAACGGTATCACAACAACCTACA</td>
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<td>IL2-5</td>
<td>GAAGGACCTGAGTGCAGTGAGAGGAAGAGTG</td>
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<td>IL2-6</td>
<td>AAGCCATTGGAAGAAGCTCTGAACCTTGCTCAAT</td>
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<td>CTAAGAACTTCCACTTGGAGACCAAGACCTT</td>
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<td>TCTTGAAACAGATGGATCACCCTCTGTCAATC</td>
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<td>TATCATTCTCCTACCTGCTGATGGCCGTCG</td>
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<td>IL2-13</td>
<td>GAAGAGTTGGGAGCTTTTATACCGACTGTAC</td>
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<td>IL2-14</td>
<td>GTTCCAATTTGGCTGGTCTCTGTGTTAGAG</td>
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<td>GTCAACCTTTGGTGTTTGTAGTTGTTGATACC</td>
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<td>TTGGCATGTAAGACTTGGTGAAGTCAACATTCTG</td>
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<td>IL2-18</td>
<td>ACACTGCGAGGTGCTCAATTGGTAGCTTCCTTT</td>
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<tr>
<td>IL2-19</td>
<td>TTCTTCCAATGCGTTCTCAACCTTCCTTCTCTAG</td>
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IL2-20' TCGACAACTCTCTCCTCTAG
IL2-21 AGTGGAAAGTCTTTAGATAGCAGGCTAGGCTAGG
IL2-22 GTTGTGTTAGGATGATCGCTCTGGTCTC
IL2-23 GTTTGCAAACCTCTTCAATTCCAAACAGGATAAC
IL2-24 GGTTCGTCAGCGTTATCCACACGATGAGG
IL2-25 TCCATCTGTTCAAGAATTCAACGATGGTAGC
IL2-26 AAGGTAGAGATGATAGATTGACGAAGGTA
IL2-27 TCGAGCGCGCTATCAAGGTC

The 5'-half of the sequence was assembled as

illustrated in Fig. 1. Fifty pmoles of each ssDNA segment (except Linker and IL2-20') were
5'-phosphorylated with T4 polynucleotide kinase. The segments were then annealed in a single step by
combining and cooling from 95°C to 25°C over 1.5 hours.

Ligation was performed in a reaction volume of 30μl
containing 1mM ATP, 10mM DTT, 100mM tris-HCl, pH 7.8,
10mM MgCl₂, 1μg/ml spermidine and T4 ligase. The
resulting double stranded fragment was purified on a 7%
native polyacrylamide electrophoresis gel. The dsDNA
fragment included a KpnI cohesive end at the 5'-end and
a SalI cohesive end at the 3'-end.

After assembly, the 5'-half of the sequence
was inserted into pαEGF-24 downstream and in frame with
the modified α-factor secretory leader and processing
signal. The plasmid pαEGF-24 is described in
application serial no. 522,909, filed August 12, 1983,
which pertinent portion is incorporated by reference
and reproduced in pertinent part as follows.

A synthetic sequence for human epidermal
growth factor (EGF) based on the amino acid sequence of
EGF reported by H. Gregory and B. M. Preston Int. J.
Peptide Protein Res. 9, 107-118 (1977) was prepared.
The sequence was inserted into the EcoRI site of pBR328
to produce a plasmid p328EGF-1 and cloned.

Approximately 30μg of p328EGF-1 was digested
with EcoRI and approximately 1μg of the expected 190
base pair EcoRI fragment was isolated. This was followed by digestion with the restriction enzyme Hgae. Two synthetic oligonucleotide connectors HindIII-Hgae and Hgae-SalI were then ligated to the 159 base pair Hgae fragment. The Hgae-HindIII linker had the following sequence:

AGCTGAAGCT
CTTCGATTGAG

This linker restores the α-factor processing signals interrupted by the HindIII digestion and joins the Hgae end at the 5'-end of the EGF gene to the HindIII end of pAB112.

The Hgae-SalI linker had the following sequence:

TGAGATGATAAG
ACTATTCAGCT

This linker has two stop codons and joins the Hgae end at the 3'-end of the EGF gene to the SalI end of pAB112.

The resulting 181 base pair fragment was purified by preparative gel electrophoresis and ligated to 100ng of pAB112 which had been previously completely digested with the enzymes HindIII and SalI. Surprisingly, a deletion occurred where the codon for the 3rd and 4th amino acids of EGF, asp and ser, were deleted, with the remainder of the EGF being retained. pAB112 is a plasmid containing a 1.75 kb EcoRI fragment with the yeast α-factor gene cloned in the EcoRI site of pBR322 in which the HindIII and SalI sites had been deleted (pAB11). pAB112 was derived from plasmid pAB101 which contains the yeast α-factor gene as a partial Sau3A fragment cloned in the BamHI site of plasmid YEp24. pAB101 was obtained by screening a yeast genomic library in YEp24 using a synthetic 20-mer oligonucleotide probe (3'-GGCCGGTTGGTACATGATT0-5') homologous to the published α-factor coding region (Kurjan and

The resulting mixture was used to transform E. coli HB101 cells and plasmid pAB201 obtained.

Plasmid pAB201 (5 μg) was digested to completion with the enzyme EcoRI and the resulting fragments were:

a) filled in with DNA polymerase I Klenow fragment;
b) ligated to an excess of BamHI linkers; and
c) digested with BamHI. The 1.75 kbp EcoRI fragment was isolated by preparative gel electrophoresis and approximately 100 ng of the fragment was ligated to 100 ng of pCl/1, which had been previously digested to completion with the restriction enzyme BamHI and treated with alkaline phosphatase.

Plasmid pCl/1 is a derivative of pJDB219, Beggs, Nature (1978) 275:104, in which the region corresponding to bacterial plasmid pMB9 in pJDB219 has been replaced by pBR322 in pCl/1. This mixture was used to transform E. coli HB101 cells. Transformants were selected by ampicillin resistance and their plasmids analyzed by restriction endonucleases. DNA from one selected clone (pYEGF-8) was prepared and used to transform yeast AB103 cells. Transformants were selected by their leu+ phenotype.

The above described construction was modified using different sequences for joining structural genes to the α-factor secretory leader sequence and/or site specific mutagenesis, thus providing for different processing signals. In the following table, a. through e. show the sequence of the fusions at the N-terminal region of the structural gene hEGF, as exemplary, which sequences differ among the several constructions.
80 85 90 5
Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Glu Ala Asn Ser Asp Ser Glu

Linker-1
GGG GTA TCT TTG GAT AAA AGA GAC GCT GAA GCT GAA GCT TAC GAC TCC GAA (pYEGF-21)
CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CTA CTT CGA TTG AGG CTG AGG CTT
80 85 90 5
Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu Linker-2
GGG GTA TCT TTG GAT AAA AGA GAC GCT GAA GCT TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA (pYEGF-22)
CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CTA CTT CGA AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
80 85 5
Gly Val Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu

GGG GTA TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA (pYEGF-23)
CCC CAT AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
80 85 5
Gly Val Pro Leu Asp Lys Arg Asn Ser Asp Ser Glu

KpnI
GGG GTA CCT TTG GAT AAA AGA AAC TCC GAC TCC GAA (pYEGF-24)
CCC CAT GGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
80 85 5
Gly Val Gin Leu Asp Lys Arg Asn Ser Asp Ser Glu

FnuII Linker-3
GGG GTA CGG CTG GAT AAA AGA AAC TCC GAC TCC GAA (pYEGF-25)
CCC CAT GTC GAC CTA TTT TCT TTG AGG CTG AGG CTT
50 53
Trp Trp Glu Leu Arg

Linker-4
TGG TGG GAA TGG AGA TGA TAA GTC GAC CGA TG
ACC ACC CTT AAC TCT ACT ATT CAC CTG GCC AC
f. shows the sequences at the C-terminal region of hEGF, which is the same for all constructions. Synthetic oligonucleotide linkers used in these constructions are boxed.

These fusions were made as follows. Construction (a) was made as described above. Construction (b) was made in a similar way except that linker 2 was used instead of linker 1. Linker 2 modifies the α-factor processing signal by inserting an additional processing site (ser-leu-asp-lys-arg) immediately preceding the hEGF gene. The resulting yeast plasmid is named pYaEGF-22. Construction (c), in which the dipeptidyl aminopeptidase maturation site (glu-ala) has been removed, was obtained by in vitro mutagenesis of construction (a). A PstI-SalI fragment containing the α-factor leader-hEGF fusion was cloned in phage M13 and isolated in a single-stranded form. A synthetic 31-mer of sequence

5'-TCTTTGGATAAAGAAACTCCGACTCCCCG-3'

was synthesized and 70 picomoles were used as a primer for the synthesis of the second strand from 1 picomole of the above template by the Klenow fragment of DNA polymerase. After fill-in and ligation at 14° for 18 hrs., the mixture was treated with S1 nuclease (5 units for 15 minutes) and used to transfect E. coli JM101 cells. Bacteriophage containing DNA sequences in which the region coding for (glu-ala) was removed were located by filter plaque hybridization using the 32P-labelled primer as probe. RF DNA from positive plaques was isolated, digested with PstI and SalI and the resulting fragment inserted in pAB114 which had been previously digested to completion with SalI and partially with PstI and treated with alkaline phosphatase.

The plasmid pAB114 was derived as follows: plasmid pAB112 was digested to completion with HindIII and then religated at low (4μg/ml) DNA concentration
and plasmid pAB113 was obtained in which three 63bp HindIII fragments have been deleted from the α-factor structural gene, leaving only a single copy of mature α-factor coding region. A BamHI site was added to plasmid pAB11 by cleavage with EcoRI, filling in of the overhanging ends by the Klenow fragment of DNA polymerase, ligation of BamHI linkers, cleavage with BamHI and religation to obtain pAB12. Plasmid pAB113 was digested with EcoRI, the overhanging ends filled in, and ligated to BamHI linkers. After digestion with BamHI the 1500bp fragment was gel-purified and ligated to pAB12 which had been digested with BamHI and treated with alkaline phosphatase. Plasmid pAB114, which contains a 1500bp BamHI fragment carrying the α-factor gene, was obtained. The resulting plasmid (pAB114 containing the above described construct) is then digested with BamHI and ligated into plasmid pCl/1.

The resulting yeast plasmid is named pYaEGF-23. Construction (d), in which a new KpnI site was generated, was made as described for construction (c) except that the 36-mer oligonucleotide primer of sequence 5'-GGGTACCTTGGATATATAGAACTCCGACTCCGAAT-3' was used. The resulting yeast plasmid is named pYaEGF-24. Construction (e) was derived by digestion of the plasmid containing construction (d) with KpnI and SalI instead of linker 1 and 2. The resulting yeast plasmid is named pYaEGF25.

The 5'-half of the IL2 sequence was inserted according to the scheme illustrated in Fig. 2. Plasmid pαEGF-24 was restricted with a mixture of restriction endonucleases KpnI and SalI to remove a KpnI/SalI fragment. The 5'-synthetic fragment of IL2 was inserted into the resulting cut vector to produce plasmid pαIL2-5' which was then cloned in E. coli HB101.

The 3'-half of the sequence was assembled as illustrated in Fig. 3. Fifty pmoles of each ssDNA
segment (except IL2-6 and IL2-27) were 5'-phosphorylated with T4 polynucleotide kinase. The segments were then annealed in a single step by combining and cooling from 95°C to 25°C over 1.5 hours. Ligation was performed in a reaction volume of 30μl containing 1mM ATP, 10mM DTT, 100mM tris-HCl, pH 7.8, 10mM MgCl₂, 1μg/ml spermidine and T4 ligase. The resulting double stranded fragment was purified on a 7% native polyacrylamide electrophoresis gel. The dsDNA fragment included a XbaI cohesive end at the 5'-end and a SalI cohesive end at the 3'-end.

After assembly, the 3'-half of the sequence was inserted in XbaI/SalI digested pAB114. Plasmid pAB114 is described in application serial no. 522,909 which has in part been reproduced above.

The 3'-half of the sequence was cloned according to the scheme illustrated in Fig. 4. Plasmid pAB114 was restricted with a mixture of restriction endonucleases XbaI and SalI to remove a XbaI/SalI fragment. The 3'-synthetic fragment was inserted into the resulting cut vector to produce pAIL2-3' which was then cloned in E. coli HB101.

After amplification, the two halves of the synthetic IL2 sequence in frame with yeast secretory and processing signals, as well as joined at the 5'-end to yeast transcriptional regulatory signals, were joined together in the pAIL2-3' plasmid according to the scheme illustrated in Fig. 5. Plasmid pAIL2-5' was restricted with restriction endonuclease XbaI to generate an XbaI/XbaI fragment carrying the a-factor transcriptional regulatory sequences and modified secretory leader and processing signals derived from plasmid pÆGF-24 and the 5'-half of the IL2 sequence. The 3'-proximal XbaI site in the 5'-segment is located interior of the synthetic sequence so that a 15 bp segment at the 3'-end is removed. Plasmid pAIL2-3' was
also restricted with XbaI and treated with alkaline phosphatase to prevent recircularization. The XbaI fragment from pAIL2-5' was then inserted into the XbaI site on pAIL2-3' to form plasmid pAIL2 which had the two fragments in frame in the correct orientation. The latter was determined by restriction analysis after plasmid pAIL2 was cloned in E. coli HB101.

Plasmid pAIL2 was then digested to completion with BamHI, and the resulting fragment carrying the IL2 construct was isolated by preparative gel electrophoresis. Approximately 100 ng of the fragment was inserted into the BamHI site of PC1/1, which had been previously digested to completion with BamHI and treated with alkaline phosphatase. The resulting plasmids were designated pYaIL-2/3.6 and pYaIL-2/18.7, representing the opposite orientations of the inserted sequence. Plasmid PC1/1 was described above.

One-liter cultures of yeast strain AB103 (genotype: MATa, pep 4-3, leu 2-3, leu 2-112, ura 3-52, his 4-580) were transformed with either plasmid pYaIL-2/3.6 or pYaIL-2/18.7. The cultures were grown overnight at 30°C in leu- medium to saturation (optical density of 5 at 600 nm) and left shaking at 30°C for an additional 12 hr. period. The cells were then removed by centrifugation, and the supernatant medium dialyzed overnight at 5°C against 10 mM HEPES, pH 7.3.

Microtiter dishes (96-well) were prepared with 2x10^4 HT-2 cells in 100 µl of RPMI-1640 mammalian cell growth medium with L-glutamine (300 µg/ml) supplemented with 10% fetal calf serum, 50µM 2-mercaptoethanol and antibiotics (50 U/ml penicillin, 50µg/ml streptomycin) per well. HT-2 cells are a subclone of the HTL-1 line of murine T lymphocytes described by Watson et al. (1979) J. Exp. Med. 150:849 and 1510, dependent upon the presence of interleukin-2 for viability and/or growth. Since these mouse cells respond to human material, their use provides a
bioassay for human interleukin-2. A semi-quantitative assay was employed by visually estimating cell survival and growth employing a microscope and comparing test results with standards employing known amounts of IL2.

Serial 2-fold dilutions of the yeast medium dialysate above were then prepared in mammalian cell growth medium (RPMI-1640 with supplements, as above) and 100 µl of each diluted sample added to individual wells containing HT-2 cells.

Reference standards known to contain interleukin-2 (concanavalin A-free, conditioned rat splenocyte medium) were either obtained commercially (Monoclone®, Collaborative Research, Inc.) or prepared by stimulation of rat spleen cell cultures (in RPMI-1640 medium with supplements, as above) with concanavalin A (1 µg/10⁶ cells) for 48 hours at 37°C in 7% CO₂/air, followed by recovery of the medium, absorption with Sephadex® G-25 to remove concanavalin A and filter sterilization. A 2-fold dilution series of each reference standard was prepared as described for the yeast medium dialysate.

The HT-2 cell microplate cultures were incubated at 37°C in 7% CO₂/air for 48 hours, scored for viability and/or growth and the approximate interleukin-2 content of the yeast preparation determined by reference to the standards. This comparison indicated activities equivalent to or greater than the commercial material, i.e., estimated to be in the range of 20-100 ng/ml, probably about 50 ng/ml.

In accordance with the subject invention, novel DNA constructs are provided which may be inserted into vectors to provide for expression of "pre"-interleukin-2 and intracellular processing and secretion of the mature polypeptide in good yield to promote a polypeptide product having high IL2 biological activity in a recognized bioassay based on
murine cellular growth. Thus, it is possible to obtain a polypeptide having the physiological activity of the naturally-occurring human interleukin-2. By providing for secretion, greatly enhanced yields can be obtained and subsequent isolation and purification of the polypeptides simplified.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.
WHAT IS CLAIMED IS:

1. A method for producing a biologically active polypeptide having the biological activity of human IL2 in good yield, said method comprising:
   - growing yeast transformants having a multicopy extrachromosomal element including a DNA construct which comprises in the direction of transcription, yeast recognized transcriptional regulatory sequences, and secretory leader and processing signals, in frame with said secretory leader and processing signals a synthetic gene encoding at least substantially for the polypeptide sequence of human IL2, stop codon(s) and a terminator, whereby a mature polypeptide having at least substantially the same amino acid sequence of human IL2 is secreted; and
   - isolating said mature polypeptide from the medium having the biological activity of human IL2.

2. A method according to Claim 1, wherein the coding strand of said synthesized sequence has the following nucleotide sequence:

   GCTCAACCTCTTCTCTACCAAAAGAAAGCAGCTGCAATTG
   CACTTGTGTGTGGAATGATTGTTGATCAACCTCTCTACATAGCAAGAAGCCCA
   AAGTTGACCAGGATTTGGAAGGTTCCACTTCACTTCAAAAAGAAGGCTACGGAAATTGAA
   CACCCTGACGATTGCTTAGAGGACAGTGGAAAGGCTTTGAAAGGAGCTTGATCTCACAGATCAGTTGTTGCTCAA
   TCTAAAGAAGCTCTCAGATGAGACAGAAGACTTTGATCTCTCAACAGTCAAGATCGTTATCGTTTTG
   GAATTGAAGGGTTCTGAAACCCCCAACCTTCTAGTCTTGAGATACCGCTGACGAAAGCCTACACCAC
   GTTGAATTTCTGGACAAGATGATGATGATGATTGGTCTGCAATCTATATCGTCTCTACGCTTACC

3. A method according to Claim 2, wherein said secretory leader and processing signal is derived from α-factor.

4. A method according to Claim 3, wherein said α-factor secretory leader and processing signal
are modified at least by removal of the glu-alal dipeptide codons.

5. A method according to Claim 3, wherein said DNA sequence is synthesized by producing two or more dsDNA fragments with flanking regions for linking to other sequences, which fragments are joined together to produce the entire sequence.

6. A DNA construct comprising in the direction of transcription yeast recognized transcriptional regulatory signals and a secretory leader sequence and processing signal; in reading frame with said secretory leader and processing signals, a synthetic DNA sequence encoding for the human IL2 gene having at least a plurality of codons preferentially utilized by yeast; stop codon(s); and, a transcriptional terminator.

7. A DNA construct according to Claim 6, wherein said DNA sequence is as follows:

GCTCCAACCTCTTCTCTACACAAGAAGACCCAGCTGCAATTG

GAACACTTTTGTGTGACTCGGATATCGACGTATCAAAACTCAGAAGACCC
AAGTGGACAGAATTGTGGACCTCTCAACGTCATGCGCAAAAAGGCTACCGAAATTGAG
GACCTGACCTGTCGAGAGGAGGATTTGAAGCCATTGGAAGAAGTCTGGAATCTCTGCTCAA
TCTAAAGAATCTTCCACTTGAGACCAAGAGAGCTTTGATCTCTAAACTCAGTTATTCCTTTTG
GAATTGAAAGGTTCTGAAACCACTCCCACGATGTTGAATACGGGCTGAAGAAACCGCTACCATC

25

GAATTCATTTGAAAGAGATGCTACAGTCTCTGCAAATCCTATCATCCTCTACCTGACC

8. A DNA construct according to Claim 7, wherein said secretory leader and processing signal is derived from the g-factor secretory leader and processing signal.
9. A DNA construct according to Claim 8, wherein said $\alpha$-factor secretory leader and processing signal are modified at least by removal of the glu-ala dipeptide codons.
INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/01853

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3
According to International Patent Classification (IPC) or to both National Classification and IPC

IPC: C12P21/00; C12P21/02; C07H21/04

II. FIELDS SEARCHED

<table>
<thead>
<tr>
<th>Classification System</th>
<th>Classification Symbols</th>
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<tr>
<td>U.S.</td>
<td>435/68, 70, 71, 172, 3, 253, 317; 536/27; 935/11, 13, 28, 47, 69</td>
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Documentation Search other than Minimum Documentation to the extent that such Documents are included in the FIELDS SEARCHED 4

CA SEARCH DATABASE: 1972-84

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

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<tr>
<th>Category</th>
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* Special categories of cited documents: 15
  *"A"* document defining the general state of the art which is not considered to be of particular relevance
  *"E"* earlier document but published on or after the international filing date
  *"L"* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  *"O"* document referring to an oral disclosure, use, exhibition or other means
  *"P"* document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 3 | Date of Mailing of this International Search Report 3
11 January 1985 | 29 JAN 1985

International Searching Authority 3 | Signature of Authorized Officer 10
ISA/US | James Martinell

Form PCT/ISA/210 (second sheet) (October 1981)
### FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

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<th>N, Kurjan et al, Cell, Volume 30, 1982, Pages 933-943</th>
<th>1-9</th>
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### VI. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁸

This international search report has not been established in respect of certain claims under Article 17(3) (a) for the following reasons:

1. Claim numbers............., because they relate to subject matter¹⁵ not required to be searched by this Authority, namely:

2. Claim numbers............., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹⁵, specifically:

### VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

**Remark on Protest**

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.
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