Recombinant plasmids that carry the structural gene for human proinsulin in expressible form. The human proinsulin structural gene is fused to a functional transport sequence. Host organisms transformed by the recombinant plasmids express a preproinsulin product that is correctly processed to human proinsulin \textit{in vivo}, and is transported across the host organism's cell wall. Following its recovery, \textit{in vitro} methods are used to convert the human proinsulin to human insulin.
FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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PLASMID FOR PRODUCING HUMAN INSULIN

This invention relates to molecular biology and
more particularly to the so-called art of recombinant DNA.
Specifically the invention relates to recombinant plasmids
for producing human insulin.

The invention describes novel genetically
engineered plasmids that carry a gene coding for human
proinsulin. Representative plasmid, pJW2172, illustrates
the invention and has been deposited with the American
Type Culture Collection, Rockville, Maryland 20852; it has
been awarded ATCC number 31891.

As is well known, insulin is a peptide hormone
that exquisitely regulates a variety of vital metabolic
events. In most animals insulin is synthesized as a
single large precursor polypeptide, preproinsulin. A
transport sequence and an internal peptide sequence are
excised from preproinsulin during biosynthesis, yielding
a biologically active molecule consisting of two peptide
chains linked together by disulfide bonds. Excision of
only the transport sequence yields a peptide molecule
known as proinsulin. Proinsulins have now been isolated
and characterized from a number of animals. See Steiner,
D., "Peptide Hormone Precursors" in Peptide Hormones,
Parsons, J.A. Editor, McMillan Press Limited, London
(1976) at pages 49-64.

The beta-cells of the islets of Langerhans
produce insulin in vivo. Initially insulin is express-
ed as a fused polypeptide comprised of the proinsulin
sequence plus a pre-sequence known as the transport or
signal sequence. This transport sequence facilitates
passage of the insulin molecule through the cellular
membranes. In the higher eukaryotes, the preproinsulin
is synthesized on ribosomes that are associated with
the rough endoplasmic reticulum. See Permutt, M. and
(1972). When it produced in vivo, the newly translated
peptide is first transported intracellularly to the Golgi apparatus where it becomes incorporated within newly forming secretion granules. Exactly how the cell converts proinsulin to insulin is not known, but, conversion is believed to be initiated in either the Golgi apparatus or in the newly formed secretion granules. The secretion granules contain zinc and membrane bound proteases which are believed to participate in the conversion mechanism. The types of proteases can vary from species to species, perhaps to accomodate species specific amino acid substitutions in the insulin peptide. See Steiner, supra at 54.

The in vivo production of insulin ultimately results in the secretion of mature insulin molecules from which both the transport sequence and the C chains have been removed. Although it is known that insulin is liberated from the proinsulin in the secretion granules, and that the insulin transport sequence is involved in transport of the peptide across the cell membranes, neither the transport mechanism nor the processing mechanism are well understood.

Because some people fail to produce insulin at all, or fail to produce it in amounts sufficient to meet their own individual needs, it has long been necessary to find supplemental sources of insulin to administer to these people. Most of the supplemental insulin used to treat insulin deficient individuals has come from bovine and porcine pancreata obtained from animals killed at the slaughterhouses. Unfortunately, this animal insulin is immunogenic in some patients. Therefore it would be preferable to have human insulin to administer to those human patients who need it. However, until very recently there was no practical way to obtain enough human insulin to supply the needs of those patients who require it. Although it is possible
to chemically synthesize human insulin, on a commercial scale the cost of such production is prohibitive.

Fortunately, recent advances in recombinant DNA technology now make it possible to genetically engineer bacteria so that they can produce eukaryotic proteins such as insulin. For example, Goeddel, D. V., et al., Proc. Nat. Acad. Sci., USA 76:106-110 (1979) chemically synthesized the DNA sequences coding for the human A and B insulin chains. Recombinant techniques were then used to separately ligate each sequence to the 3' end of an *E. coli* beta-galactosidase gene. As expected, two hybrid proteins were obtained, each consisting of either the human A or B chain fused to the bacterial beta-galactosidase. The A and B chains were released from the fusion peptides by chemical degradation. The chains were purified individually and then recombined to form biologically active insulin. See Chance, R. E. et al, *Diabetes Care* 4:147-154 (1981).

Although the Goedell et al technique provides one method of producing human insulin, a more natural approach involves the biosynthesis of proinsulin, which is the immediate precursor of insulin. An advantage of producing insulin from proinsulin is the economy of fermenting and processing only one recombinant organism. The Goedell et al technique requires the use of two recombinant organisms. Numerous researches have shown that proinsulin will oxidize spontaneously to form the correct disulfide bonds and can be quantitatively converted to insulin by controlled digestion with trypsin and carboxypeptidase B. See Steiner, D. and Clark, J. Proc. Nat. Acad. Sci., USA 60:622-629 (1968); and Kemmler, W. et al, J. Biol. Chem., 246:6786-6791 (1971). Gilbert and his co-workers demonstrated the feasibility of the proinsulin approach when they constructed plasmids in which most
of the coding sequences of rat preproinsulin were fused
to portions of the prepeptide region of penicillinase.
When transformed into E. coli, some of these constructs
produced fused preproteins that were correctly cleaved
to rat proinsulin and segregated into the periplasmic
space of the host organism. Analysis of the Gilbert
plasmids shows that they contained fusions involving
portions of both the penicillinase and the preproin-
sulin leader sequences. These were usually connected
by short interposed sequences derived from the con-
struction process and not related to either prese-
USA 77:3988-3992 (1980).

Although the Gilbert et al experiments
demonstrate the feasibility of using the preproinsulin
approach to achieve expression of eukaryotic rat in-
sulin, they do not teach how to create a recombinant
plasmid carrying the human proinsulin gene in expressible
successfully cloned and sequenced the human preproin-
sulin gene. However, their work does not demonstrate
successful expression of the human gene. European
Patent Application 80303195.4, filed by the Regents of
the University of California, incorporates some of the
work disclosed in the Bell et al publication, supra.
The application itself suggests ways of constructing
plasmids carrying the human insulin gene so that the
human gene should be expressed. However, the applica-
tion does not demonstrate actual expression of the
human gene and does not indicate how to obtain samples
of the plasmid. Furthermore, it does not demonstrate
that the human preproinsulin gene product is properly
processed by the transformed bacterial host.

Therefore it is an object of the present
invention to create a recombinant plasmid carrying the
gene for human proinsulin in expressible form.
A further object of the present invention is to create a recombinant plasmid capable of transforming a host organism so that the host will express human proinsulin as a preproinsulin product that will be processed to human proinsulin by the host and then transported across the host's cell membrane.

A further object of the present invention is to demonstrate actual expression of the preproinsulin product by a host organism transformed by a recombinant plasmid carrying the gene for human proinsulin.

Another object of the present invention is to demonstrate that the preproinsulin product expressed by a host organism transformed by a recombinant plasmid carrying the gene for human proinsulin is processed to human proinsulin within the host and then transported across the host's cell membrane.

Other objects of the invention will become apparent to those skilled in the art from the following description, taken in connection with the accompanying drawings wherein:

FIGURE 1 shows the distribution of immunoreactivity after gel filtration of osmotic shockates from bacterial cultures containing plasmid pJW2172. Elution positions of standard proteins are also indicated.

FIGURE 2 shows the distribution of radioactivity measured in slices obtained from tube gel SDS electrophoresis of an aliquot of $^{125}$I-labeled immunoprecipitated plasmid pJW2172 product. The vertical bar indicates the position of the stained band of bovine proinsulin.

FIGURE 3 shows the distribution of radioactivity obtained from automated sequential Edman degradation of $^{125}$I-labeled proinsulin product of plasmid pJW2172.

FIGURE 4 shows the distribution of radio-
activity obtained from automated sequential Edman
degradation of $^{35}$S-labeled proinsulin product of
plasmid pJW2172.

FIGURE 5 shows the binding of $^{125}$I-labeled
plasmid pJW2172 immunoprecipitated proinsulin to a
human C-peptide antiserum. Samples were tested with
normal rabbit serum (N) and human C-peptide antiserum
(C).

Very generally, the invention involves
construction of novel recombinant plasmids carrying the
human proinsulin structural gene in expressible form.
Immediately preceeding the eukaryotic proinsulin
structural gene on the plasmid is a functional transport
presequence. The presequence can be comprised entirely
of a prokaryotic bacterial transport presequence,
entirely of a eukaryotic transport presequence, or of
any functional combination of the two presequence
types. The eukaryotic proinsulin structural gene
sequence is oriented in the plasmid so that it is in
correct translational reading frame with respect to
the initiation codon of the functional transport pre-
sequence. Such an orientation allows a preproinsulin
product to be synthesized by actively metabolizing
transformed host organisms. The preproinsulin product
is correctly processed to human proinsulin by the
transformed host organisms, which then transport the
human peptide across the cell membrane. In a trans-
formed E. coli host, the human proinsulin accumulates
in the bacterial periplasmic space. Following recovery,
in vitro methods can be used to convert the human
proinsulin to human insulin. See Steiner and Clark,
supra, and Kemmler et al, supra. Alternatively, the
recombinant plasmids could be used to transform host
organisms that are not capable of correctly processing
preproinsulin to human proinsulin in vivo. In that
case the preproinsulin peptide would be recovered by
disrupting the transformed hosts' cell walls. The transport sequence portion of the preproinsulin peptide could be removed enzymatically. See generally, Zwizinski, C. and Wickner, W., *J. Biol. Chem.*, 255:7973-7977 (1980). In vitro methods could then be used to convert human proinsulin to human insulin. See Steiner and Clark, supra and Kemmler et al, supra.

More specifically, to illustrate the invention, construction of a representative recombinant plasmid is disclosed. This plasmid, designated as plasmid pJW2172, has been deposited with the American Type Culture Collection, Rockville, Maryland, 20852, USA. It has been awarded ATCC number 31891. Plasmid pJW2172 was constructed by modifying a parental plasmid, plasmid pHn677, which in turn was derived from the well-characterized plasmid pBR322 (ATCC 37017). Parental plasmid pHn677, which contains human preproinsulin cDNA cloned into the Pst I site of the ampicillinase gene of plasmid pBR322, was modified with a restriction endonuclease and a double stranded exonuclease. Large portions of the ampicillinase coding region were excised by these enzymes, resulting in the production of a variety of fused gene segments. Many of these generated proteins were detectable with insulin or C-peptide antiserum. One such fused gene segment generating these detectable proteins is carried by plasmid pJW2172. Plasmid pJW2172 contains a perfect fused hybrid gene segment consisting of the aminoterminal coding half of the leader sequence of the prokaryotic ampicillinase (residues -23 to -12) fused to the eukaryotic human preproinsulin prepeptide beginning at residue -13. Expression of the fused segment results in the synthesis of a preproinsulin product, in vivo processing of the preproinsulin product and finally, and secretion of human proinsulin into the periplasmic space of *E. coli* host organisms transformed by plasmid...
pJW2172. Characterization of the recovered human proinsulin product shows that it contains the A and B chain regions of insulin as well as specific C-peptide immunodeterminants. Tryptic digestion can be used in vitro to convert proinsulin to insulin. See Steiner and Clark, supra, and Kemmler et al, supra.

Parental plasmid, pHn677, was obtained originally by cDNA cloning of mRNA isolated from human insulinoma. The cDNA was inserted into the Pst site of pBR322 by means of dC-dG tailing. Plasmid pHn677 contains all of the proinsulin sequence plus the coding sequence for 16 amino acids of the presequence. Although the DNA sequence in the parental plasmid was in the right orientation with respect to the beta-lactamase promoter, it was in the wrong reading frame with respect to the initiation codon of the beta-lactamase gene. As a result there was no expression of the DNA sequence coding for the human proinsulin gene.

Therefore, in order to construct expression plasmids having the cDNA sequence in the correct reading frame, a restriction endonuclease and a double stranded exonuclease had to be used to remove most of the ampicillinase gene upstream from the preproinsulin cDNA sequence. To accomplish this, both parental plasmid pHn677 and progenitor plasmid pBR322 had to be subjected to enzymatic digestion.

Plasmid pHn677 was digested with restriction endonuclease Pvu I. The DNA was then treated with double stranded exonuclease Bal 31 to digest approximately 150 nucleotides from each end. This DNA was further treated with restriction endonuclease Hind III. After subjecting the resulting DNA to gel electrophoresis on agarose gels, the larger set of DNA fragments was isolated from the gels by electroelution.

In a parallel procedure, Hind II digested plasmid pBR322 DNA was subjected to Bal 31 to digest
approximately 180 nucleotides from each end of the linearized plasmid. This DNA was then further digested with Hind III and Bam HI. Finally the fragment containing the beta-lactamase promoter region was isolated from an acrylamide gel. This fragment was ligated to the Pvu I/Bal 3I/Hind III treated large fragment. The ligation mixture was used to transform E. coli K12 strain CS412.

Transformants were first screened for tetracycline resistance. Some 350 clones exhibiting such resistance were further examined for insulin expression using the in situ radioimmunoassay procedure described by Broom, S. and Gilbert W., Proc. Nat. Acad. Sci., USA 75:2749 (1978). This procedure showed that 118 of the 350 clones might be expressing insulin. Therefore, these 118 clones were further assayed by means of a radioimmunoassay that utilized both the anti-insulin and the anti-C-peptide antibodies. The radioimmunoassays showed that at least 23 of these clones showed significant, i.e. greater than 1 unit per ml of insulin activity.

From the total series of plasmids containing a variety of hybrid bacterial-eukaryotic transport sequences attached to the human proinsulin sequence, one plasmid, designated as plasmid pJW2172, was selected for further study. As described in Chan, S. et al Proc. Nat. Acad. Sci., USA 78:5401-5405 (1981), and illustrated here in FIGURE 1, gel filtration of a concentrated asmotic shockate from cultures of transformed bacteria containing plasmid pJW2172 gave rise to a single homogeneous peak of C-peptide and insulin immunoreactivity which coeluted at the position of the proinsulin standard. Moreover, the ratio of C-peptide to insulin immunoreactivity of this component, i.e. approximately 1:15, corresponded well to the known cross-reactivity of human proinsulin in the

These findings are readily explained by DNA sequence analysis of the fused region of plasmid pJW2172. As described more fully in Chan et al, *supra*, these studies revealed that fusion had occurred between residue -12 of the bacterial ampicillinase leader peptide sequence and residue -13 of the human presequence, thus creating a perfect hybrid leader sequence containing roughly half of each presequence without any substitution or modifications. This fused leader sequence preserves all of the structural features known to be required for export and cleavage. See Faber, et al, *supra*.

The experiments outlined in the examples that follow demonstrate that plasmid pJW2172 generates a correctly cleaved secreted insulin product.

Example I - Characterization of the Protein Product Secreted by Plasmid pJW2172

As described in Chan et al, *supra*, the proinsulin material generated by plasmid pJW2172 was characterized in the following matter. A sample of an osmotic shockate, from cultures of bacteria transformed by plasmid pJW2172, containing about 0.05 nM of the peptide was extracted with acid ethanol. The extract was then subjected to gel filtration on a column of Biogel P60 eluted with 2.5 M propionic acid. See generally, Steiner, D., et al, in *Cell Biology: A Comprehensive Treatise* 4:175-201 (1980). The tube containing the peak of insulin immunoreactivity, i.e., approximately 0.01 nM, was dried *in vacuo* and divided into two equal aliquots.

One aliquot was labeled by odination and then immunoprecipitated with insulin anti-serum. The resultant immunoprecipitate gave a single peak at the position of proinsulin when examined on a Biogel P30
column eluted with 3 M acetic acid. As shown in FIGURE 2, when run on tube gel SDS electrophoresis, the immunoprecipitate migrated as a single component having the same mobility as a bovine proinsulin standard.

The second aliquot was used to determine whether cleavage of the presequence had occurred at the first residue of human proinsulin. The 0.5 pM aliquot of the immunoprecipitate was first reduced and carboxymethylated, and then subjected to automated Edman degradation. See Chan et al, supra. The results are shown in FIGURE 3. They show that significant amounts of radioactively labeled tyrosine were found only at positions 16 and 26, as expected for human proinsulin. There was no indication of heterogeneity at the N-terminus. Further corroboration of this point was obtained when the osmotic shockate from a culture grown in the presence of $^{35}$SO$_4$ was similarly immunoprecipitated, gel filtered, reduced, carboxymethylated and then sequenced. These results are shown in FIGURE 4; they demonstrate the presence of the sulphur labeled B7 and B19 S-carboxymethyl-cysteine residues in correct register.

Example II - Characterization of the Proinsulin Like Peptide

Again, as described more fully in Chan et al, supra, to further characterize the proinsulin like peptide, three additional experiments were performed. In the first, an aliquot of the reduced and carboxymethylated iodinated immunoprecipitate was digested with trypsin and then submitted to automated Edman degradation. These results are shown in FIGURE 3. They demonstrate unequivocally that the protein contained the normal human insulin A chain, having tyrosines at position 14 and 19. In addition they show that trypsin had cleaved the B chain region at the arginine

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located at amino acid position 22, thus generating a heptapeptide that now contained the B26 tyrosine residue at position 4.

In the second experiment, the presence of C-peptide immunodeterminants in the aliquot material, which had originally been obtained by immunoprecipitation with an insulin antiserum, was assayed by binding to an antiserum against human C-peptide. These results are shown in FIGURE 5. They demonstrate that the carboxymethylated protein reacted as well as authentic iodinated human C-peptide. Porcine proinsulin did not bind significantly to this antiserum.

In a third experiment, the plasmid pJW2172 proinsulin product was treated with trypsin and carboxypeptidase B. This treatment converted the product to a component eluting at the position of insulin on gel filtration. There was no indication of the release of free A or B chain material.

On the basis of the evidence discussed in Examples I and II, it is clear that the final protein product generated by host bacteria transformed by plasmid pJW2172 is intact human proinsulin. Using techniques well known to those skilled in the art, it is possible to convert human proinsulin to human insulin in vitro. See Steiner and Clark, supra and Kemmler et al, supra.

It may be seen therefore, that the invention involves construction of novel recombinant plasmids carrying the human proinsulin structural gene in expressible form. Immediately preceding the eukaryotic structural gene on each plasmid is a functional transport presequence. The presequence can be comprised entirely of a prokaryotic bacterial transport presequence, entirely of a eukaryotic transport presequence, or of any functional combination of the two. Representative plasmid pJW2172 carries the
human proinsulin structural gene attached to a functional fused hybrid transport presequence. The hybrid transport presequence is comprised of roughly half of the prokaryotic bacterial presequence fused to roughly half of the eukaryotic human presequence. The bacterial presequence codes for the amino-terminal end of the hybrid transport peptide; the human presequence codes for the carboxy-terminal end. E. coli host bacteria transformed by plasmid pJW2172 express a preproinsulin peptide product that is correctly processed to human proinsulin by the bacteria. This human proinsulin is transported by the bacteria across the cell membrane to the periplasmic space, where it accumulates. Following its recovery, in vitro methods such as tryptic digestion can be used to convert the human proinsulin to human insulin.

Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.
REFERENCES


CLAIMS:

1. A cloning vector for producing human pro-insulin through expression of DNA by a transformed host organism comprising a plasmid capable of transforming a host organism and having a preproinsulin coding DNA sequence therein, said preproinsulin coding DNA sequence further comprising a functional transport coding DNA presequence fused to a structural gene coding for human proinsulin, said preproinsulin coding DNA sequence being under the control of an operator, promoter and ribosome binding site sequence and further being positioned in the vector in correct reading frame with respect to the initiation codon for the functional transport coding DNA presequence so that a preproinsulin product is expressed by a host organism transformed by said cloning vector.

2. A cloning vector for producing human pro-insulin through expression of DNA by a transformed host organism comprising plasmid pJW2172 (ATCC 31891) and its progeny.

3. A host organism transformed by the cloning vector of Claim 1 or 2.

4. A host organism according to Claim 3 wherein the host organism is *Escherichia coli*.

5. A host organism according to Claim 4 wherein the host organism is *E. coli K12*.

6. Preproinsulin produced by a host organism according to Claim 3.

7. A transformant culture cloned from one or more host organisms according to Claim 3.

8. Preproinsulin produced by a transformant culture according to Claim 3.

9. A host organism according to Claim 3 wherein said host further comprises an organism capable of correctly processing expressed preproinsulin to human
proinsulin in vivo and transporting the human proinsulin across the host's cell membrane.

10. Human proinsulin produced by a host organism according to Claim 9.

11. Human proinsulin produced according to Claim 10 wherein said human proinsulin is converted to human insulin in vitro.

12. A transformant culture cloned from one or more host organisms according to Claim 9.

13. Human proinsulin produced by a transformant culture according to Claim 12.

14. Human proinsulin produced according to Claim 13 wherein said human proinsulin is converted to human insulin in vitro.

15. A process for producing human insulin comprising: providing a cloning vector having a preproinsulin sequence therein, said preproinsulin sequence further comprising a functional transport presequence fused to a structural gene coding for human proinsulin, said preproinsulin sequence being under the control of an operator, promoter and ribosome binding site sequence and further being positioned in the vector in correct reading frame with respect to the initiation codon for the functional transport presequence so that a preproinsulin product is expressed by a host organism transformed by said cloning vector; transforming, with said cloning vector, a host organism capable of correctly processing expressed preproinsulin to human proinsulin in vivo and transporting the human proinsulin across the host's cell membrane; growing said transformed host organism under conditions suitable for expression of a DNA sequence coding for a preproinsulin peptide product; allowing said transformed host organism to process said expressed preproinsulin peptide to human proinsulin in vivo and allowing said human proinsulin to accumulate outside the host's cell membrane; recovering said accumulated
- 18 -
human proinsulin; converting said recovered human pro-
insulin to human insulin in vitro; and purifying said
human insulin produced in step 6.

16. Human insulin produced according to the
5 process of Claim 15.
FIG. 3
AUTOMATED EDMAN DEGRADATION

INTACT RCM-PROTEIN

TRYPsin-DIGESTED

CYCLE NO.

$\text{IC}_{125}^1\text{CPM} \times 10^{-3}$
**INTERNATIONAL SEARCH REPORT**

**Classification of Subject Matter**

According to International Patent Classification (IPC) or to both National Classification and IPC

- **INT. CLP C12P 21/00** and **A61K 37/26**
- **U.S. CL. 435/68** and **424/178**

**Fields Searched**

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**Chemical Abstracts 1961-1972**

**Computer Search, BIOSIS Index Medicus**

**Documents Considered to be Relevant**

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<td>NATURE, VOLUME 282 ISSUED 1979, &quot;NUCLEOTIDE SEQUENCE OF A cDNA CLONE ENCODING HUMAN PREPRO-INSULIN&quot;. G.I. BELL ET AL, SEE PP. 525-527.</td>
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<td>PROC. NATL. ACAD. SCI. USA, VOLUME 77, ISSUED 1980, &quot;BACTERIA MATURE PREPROINSULIN TO PROINSULIN&quot;. K. TALMADGE ET AL, SEE PP. 3988-3992.</td>
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<td>PROC. NATL. ACAD. SCI. USA, VOLUME 76, ISSUED 1979, &quot;EXPRESSION IN ESCHERICHIA COLI OF CHEMICALLY SYNTHESIZED GENES FOR HUMAN INSULIN&quot;. D.V. GOEDDE, SEE PP. 106-110.</td>
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<td>USA, 4,082,613, PUBLISHED 04 APRIL 1978, THIRUMALACHAR ET AL.</td>
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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 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.

**IV. Certification**

- **Date of Actual Completion of the International Search**
  27 OCTOBER 1982

- **Date of Mailing of this International Search Report**
  02 NOV 1982

- **International Searching Authority**
  ISA/US

- **Signature of Authority Officer**
  KATHLEEN S. McCOWIN
V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers............., because they relate to subject matter 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 out, specifically:

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

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

I. Genetic Engineering: Claims 1-5, 7, 9, 12 and 15; and
II. Proteins: Claims 6, 8, 11, 13, 14 and 16.

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.