EXHIBIT H-12

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

Application of:	)	"PRODUCTION OF
	)	
FU-KUEN LIN	)	ERYTHROPOIETIN"
	)	
Serial No: 675,298	)	Group No. 127
	)	
Filed: November 30, 1984	)	Examiners - J. M. Giesser
	)	T. G. Wiseman

Applicant's Amendment and Reply
Under 35 U.S.C. §§1.111 and 1.115

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Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

This is in response to the Office Action dated July 3, 1986 in the above-identified application wherein all provisionally elected claims (14, 15, 17-36, 58 and 61-72) were variously rejected under one or more of the provisions of 35 U.S.C. §§101, 112 (paragraphs 1 and 2), 102 and 103 and non-elected claims (1-13, 16, 37-57 and 59-60) were withdrawn from consideration.

Reconsideration and allowance of all pending claims is respectfully requested in view of the following amendments and remarks.

IN THE SPECIFICATION

Please enter into the application the attached new Figures 5 through 8 which duplicate original Tables V, VI, XIV and XXI.

At page 25, line 5, please insert the following sentences after the period.

--Reference is made to Figures 1 through 8, wherein: FIG. 1 is a graphic representation of a

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radioimmunoassay analysis of products of the invention; FIGS 2 through 4 illustrate vector constructions according to the invention; and, FIGS. 5 through 8 are DNA sequences according to the invention.--

At page 37, line 6, after the term "Table V", please insert --, duplicated as FIGURE 5 comprising portions 5A, 5B and 5C--.

At page 42, lines 25, after the term "Table VI", please insert --, duplicated as FIGURE 6 comprising portions 6A, 6B, 6C, 6D and 6E--.

At page 73, line 33, after the designation "XIV", please insert --, duplicated as FIGURE 7--.

At page 75, line 28, after the designation "XXI", please insert --(the last-mentioned Table being duplicated as FIGURE 8).

IN THE CLAIMS

Please amend claim 14 as follows:

--14. (Amended) A purified and isolated DNA sequence for use in securing expression in a procaryotic or eucaryotic host cell of a polypeptide product having at least a part of the primary structural conformation and one or more of the biological [properties] activities of naturally-occurring erythropoietin, said DNA sequence selected from [among] the group consisting of:

(a) the DNA sequences set out in [Tables V and VI] Figures 5 and 6 or their complementary strands;

(b) DNA sequences which hybridize to the DNA sequences defined in (a) or fragments thereof; and

B5 Cont'd

(c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).--

In claim 17, line 4, please delete "properties" and insert --activities-- in place thereof.

In claim 20, line 2, please delete "Table V" and insert --Figure 5-- in place thereof.

In claim 23, line 2, please delete "Table VI" and insert --Figure 6-- in place thereof.

In claim 24, line 2, please delete "14" and insert --17-- in place thereof.

In claim 27, line 3, please delete "Table XIV" and insert --Figure 7-- in place thereof.

In claim 30, line 3, please delete "Table XXI" and insert --Figure 8-- in place thereof.

In claim 34, line 1, please insert --purified and isolated-- before the term, "DNA".

In claim 58, line 2, please delete "Table V or VI" and insert --Figure 5 or 6-- in place thereof.

In claim 69, line 3, please delete "properties" and insert --activities-- in place thereof.

In claim 69, line 7, please insert --62-- after the word "claim".

In claim 70, line 3, please delete "properties" and insert --activities-- in place thereof.

In claim 71, line 3, please delete "properties" and insert --activities-- in place thereof.

In claim 72, line 3, please delete "properties" and insert --activities-- in place thereof.

REMARKS

Upon entry of the above-requested amendments, claims 14 (Amended), 15, 16-36, 58 and 61-72 will remain in the application.

Applicant acknowledges with thanks the interview kindly granted by Examiners Wisemen and Giesser to Applicant's counsel, Mr. Borun and Mr. Odre, on July 30, 1986. Attached hereto as Exhibit No. 1 are copies of the documents referred to as Exhibits "A" and "B" in the Examiner Interview Summary Record prepared by Examiner Giesser.

A. The Claimed Subject Matter

The present invention reflects Applicant's discovery of DNA sequences encoding erythropoietin. This discovery, in turn, has allowed the first determination ever made of the entire primary structural conformation of erythropoietin. Significantly, this discovery has allowed recombinant methods to be brought to bear in the development of DNA vectors and transformed and transfected host cells useful to secure large scale production of polypeptide products sharing in the biological activities of erythropoietin.

The present claims are accordingly directed to DNA sequences, DNA vectors, transformed and transfected host cells and processes for the use of these materials in the preparation of erythropoietin products including, e.g., polypeptide fragments and polypeptide analogs of erythropoietin. Independent claim 14 is thus generally directed to purified and isolated DNA sequence defined by reference to the DNA sequences revealed in Figures 4 and 6. Dependent claims 15, 16, 62 and 69 respectively relate to host cells transformed or transfected with DNA of claim 14, vectors

including the DNA of claim 14, hosts transformed with such vectors, and production processes employing such hosts. Independent claim 17 is directed generally to DNA sequences which code for procaryotic or eucaryotic host polypeptides having erythropoietin amino acid sequences and having one or more of erythropoietin's biological activities. Dependent claims 18-33, 63-64 and 70 are directed to presently preferred forms of DNA sequences, vectors, transformed or transfected hosts and production processes based on the claim 17 DNA sequences. Independent claim 34 is generally directed to DNA sequences of the invention which encode polypeptide fragments and analogs of erythropoietin and dependent claims 35, 36, 65-68, 71 and 72 are likewise directed to preferred forms of sequences, vectors, transformed and transfected hosts and production processes. Finally, independent claim 58 is directed to the specific human and monkey erythropoietin-encoding purified and isolated DNA sequences as revealed in Figures 5 and 6 (previously Tables V and VI).

B. The Outstanding Office Action, The Rejections of the Claims and Applicant's Responses Thereto

In the Action dated July 3, 1986, the Examiner noted that the full text of the Chirgwin, et al. reference (Ref. C8) did not accompany Applicant's Information Disclosure Statement filed April 24, 1986. Attached hereto as Exhibit No. 2 is a full text copy. Applicant respectfully solicits the Examiner's consideration of the same. and notation of such consideration on the previously submitted Form PTO-1449.

Due to the number and variety of objections and rejections set forth in the Action dated July 3, 1986,

Applicant submits that the issues raised therein are best treated by responses which precisely "track" the order of their appearance in the Action.

1. The Rejection of Claims 14, 15, 17-36, 58 and 61-72 Under The First Paragraph of 35 U.S.C. §112 May Property Be Withdrawn

At page 4 of the Action, the Examiner lodged a rejection of all claims under 35 U.S.C. §112 (first paragraph) based on a corresponding objection to the specification wherein the absence of an "assurance" of potential replacement of A.T.C.C. Budapest Treaty microorganism deposits was noted. While Applicant specifically disagrees with the Examiner's assertion to the effect that the "invention depends on certain specific plasmids/microorganisms", he has attached hereto as Exhibit No. 3 a Declaration by an officer of his Assignee, Kirin-Amgen, Inc., assuring replacement of deposited cultures if lost or destroyed during the 30-year Budapest Treaty deposit period. This Declaration is of the general form presented in Wiseman, T. "Biotechnology Patents", pp. 33-42 appearing in "Biotechnology Patent Conference Workbook" (American Type Culture Collection, Rockville, MD., 1986).

Applicant respectfully submits that all requirements of the first paragraph of Section 112 are met, that the objection to the specification should be withdrawn, and that the corresponding rejection of claims 14, 15 17-36, 58 and 61-72 may properly be withdrawn.

2. The Rejection of Claims 14, 15, 17-36, 58 and 61-72 Under The Second Paragraph of 35 U.S.C. §112 May Properly Be Withdrawn

Bridging pages 4 and 5 of the Action, the Examiner lodged a rejection of all claims under 35 U.S.C. §112 (second paragraph) based on multiple assertions of indefiniteness of claim terminology. Each specific objection, designated (a) through (f), is discussed below.

(a) Applicant respectfully disagrees with the Examiner's assertion of indefiniteness for the term "procaryotic or eucaryotic" as employed to describe host cells in claims 14, 15 62, 64, 66, 68 and in claims dependent thereon. While Applicant agrees in general that unduly alternative language may not be in conformity with Section 112 requirements and that wholly non-equivalent terms ought not to be presented as equivalents in claims, it is respectfully submitted that the claim term "procaryotic or eucaryotic" quite accurately (i.e., "duly") specifies well known alternatives in selection of available host cell types for the application of recombinant DNA methods in polypeptide production. The Examiner's attention is directed to M.P.E.P. §706.03(d) wherein it is noted that:

> "Generally speaking, the inclusion of (1) negative limitations and (2) alternative expressions, provided that the alternatively expressed elements are basically equivalents for purposes of the invention, are permitted if there is no uncertainty or ambiguity with respect to the question of scope or breadth of claim is presented."

It is thus the case that the kind of invention claimed, together with the "purposes of the invention" are

[redacted]h properly considered in determining the propriety of alternative language within any given claim.*

In this instance, support for the conclusion that "procaryotic or eucaryotic" is duly alternative and unambiguous may be found upon consideration of the nature of the "expression" process by which cells produce a polypeptide based on a DNA sequence as claimed, together with the context of the teachings of the present specification with regard to production of erythropoietin polypeptides. Whether a host cell is procaryotic or eucaryotic, the general cellular process by which any given (DNA) codon gives rise to the disposition of a given amino acid residue within a polypeptide is the same. The ATG codon, for example, codes, via mRNA and tRNA, for disposition of a methionine residue whether it is within a procaryotic or eucaryotic host, and no DNA codon directs a different amino acid residue simply depending on the procaryotic or eucaryotic nature of the host it is in. This concept is clearly reflected in the present specification wherein, at page 19, lines 6-11, it is noted that:

> "These polypeptides are also uniquely characterized by being the product of procaryotic or eucaryotic host expression (e.g., by bacterial, yeast and mammalian cells in culture) of exogenous DNA sequences obtained by genomic or cDNA cloning or by gene synthesis".

Specific examples within the specification describe the actual results of genomic, cDNA and synthetic DNA expression in mammalian, E.coli, and yeast systems. Thereafter, the

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* As an example, while "black or white" might appear unduly alternative or ambiguous, in vacuo, the term is quite properly employed when describing an invention related to squares of a chess board.

Specification goes on to state, at page 92, line 33 through page 93, line 5:

> "Put another way, DNA sequences provided by the invention are useful in generating new and useful viral and circular plasmid DNA vectors, new and useful transformed and transfected microbial procaryotic and eucaryotic host cells (including bacterial and yeast cells and mammalian cells grown in culture), and new and useful methods for cultured growth of such microbial host cells capable of expression of EPO and EPO products".

Applicant respectfully submits that the term, "procaryotic or eucaryotic" is completely in keeping with the nature and purposes of the present invention as fully described in the specification and that the outstanding rejection of claims 14, 15, 62, 64, 66, 68 and claims dependent thereon may properly be withdrawn.

(b) The Examiner has alleged that claims 14, 17, 34, 58, 69-72 and claims dependent thereon are indefinite for failure to specify a "fragment" size and are thus "so vague as to read on single base pairs". Applicant respectfully disagrees. Whether the Examiner is referring to a DNA or polypeptide "fragment" is unclear, but it is clear from the context of the claims under consideration that a polypeptide is encoded (necessitating the presence of multiple 3-base pair codons) and that the polypeptide encoded must possess at least one of the biological properties of naturally-occurring erythropoietin. Within this context Applicant's claims certainly do not read on single base pairs (which "encode" nothing). Rather, they include specific and readily understood structural and functional limitations as to the length of the DNA sequences claimed which in turn allows for production of useful, biologically active mate-

rials. The outstanding rejection of claims 14, 17, 34, 58, 69-72 and claims dependent thereon may thus properly be withdrawn.

(c) The Examiner also objected to claims 14, 17, 69-72 and claims dependent thereon for their recitation of "biological properties". This term is alleged to be "so indefinite as to be meaningless". Applicant disagrees. While the "biological properties" of erythropoietin may be varied, they are not indefinite. The term, as used in reference to erythropoietin, is essentially defined at specification page 19, lines 3-5, by the recitation:

> "...one or more of the biological properties (e.g., immunological properties and in vivo and in vitro biological activity) of naturally-occurring erythropoietin...".

The presently known in vivo and in vitro activities of erythropoietin are well described in the prior art cited in the specification's "Background", beginning at page 9, line 33 and continuing through page 12, line 19. Moreover, at page 86, lines 21-32, certain of the major reported in vivo biological activities of erythropoietin are again recited:

> "...stimulation of reticulocyte response, development of ferrokinetic effects (such as plasma iron turnover effects and marrow transit time effects), erythrocyte mass changes, stimulation of hemoglobin C synthesis...increasing hematocrit levels in mammals".

Applicant thus respectfully submits that the term "biological properties", as used in the specification, is definite and meaningful and that its use in the claims is fully in keeping with the requirements of Section 112. For purposes of advancing prosecution of this application, however, and without waiver of any right to pursue claims of

same or similar scope in a duly filed continuing application, claims 14, 17 and 69-72 have been amended to refer to biological activities of polypeptides encoded rather than biological properties. This term is widely employed in the literature of recombinant technologies and is fully supported in the present specification by, e.g., the above-cited disclosures of the biological activities of erythropoietin. Applicant thus respectfully submits that the outstanding objection to, and rejection of, claims 14, 17 and 69-72 is mooted and may properly be withdrawn.

(d) Claims 14, 20, 23, 27, 30, 58 and claims dependent thereon were rejected on grounds of reference to figures, with the assertion that the DNA sequences of the figures "can be adequately expressed in words". Applicant respectfully disagrees. Applicant first notes that while the DNA sequences set forth are alphabetical in nature, they are not "words" in the ordinary sense. Rather, they are "diagrams" reciting the relationship of many nucleic acids and the amino acids encoded thereby. It has always been the case that the requirements of Section 112 could be satisfied by a diagramatic, rather than verbal, presentation in the claims where, as here, prolixity is avoided and clarity of description is preserved. See, e.g., In re Faust, 86 U.S.P.Q. 114, 115 (1943) and Ex parte Squires, 133 U.S.P.Q. 598, 600 (Bd. App. 1961).* In issued U.S. Patents relating to inventions in biotechnology, it has been found appropriate to identify novel microorgansims and cell lines in the claims through reference to a deposit accession numbers. As an example, in recently issued U.S. Patent No.

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* In the last-mentioned decision, the claim on appeal was "1. A font of numerals as shown in Fig. 1".

530,901, specific DNA sequences encoding interferon polypeptides were also claimed by reference to unspecified (but presumably "knowable") DNA sequences present as plasmid inserts contained in deposited microorganisms.

Reference to figures of the drawing herein is in full conformity with the "particularity" and "distinctness" requirements of the second paragraph of Section 112 and such reference clearly avoids prolixity without introducing ambiguity. It is thus respectfully submitted that the outstanding rejection of claims 14, 20, 23, 27, 30, 58 and claims dependent thereon may properly be withdrawn.

(e) The above-requested amendment of the language of claim 14 (to specify selection "from the group consisting of") is believed to moot the outstanding rejection of the claim on grounds of "improper Markush language".

(f) The requested amendment of claim 69 to reflect dependence on claim 62 is believed to moot the outstanding rejection thereof.

3. The "Provisional" Rejections of Claims 14, 15, 17-36, 58 and 61-72 Under 35 U.S.C. §101 Based on Applicant's Co-pending Applications May Properly Be Withdrawn

Applicant acknowledges with thanks the Examiner's provisional notation of the possibility of "double patenting" grounds for rejection should the present claims issue and all original claims also issue in "parent" U.S. Patent Application Serial Nos. 561,024, 582,185 and 655,841. This notation will be kept in mind in any subsequent prosecution of said applications. Applicant submits, however, that the provisional notation does not provide a basis for present rejection of the claims or otherwise constitute a bar to allowance of the claims.

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4. The Rejection of Claims 14, 24, 34 and 36 Under 35 U.S.C. §101 May Properly Be Withdrawn

Amendment of claims 14 and 34 to include the recitation of "purified and isolated" with reference to the claimed DNA sequences is believed to moot the outstanding rejection of claims 14, 24, 34 and 26 wherein the Examiner suggested that non-statutory subject matter (non-isolated erythropoietin genes in cells naturally producing erythropoietin) might be embraced by the claims.

5. The Rejection of Claims 14, 24, 34 and 36 Under 35 U.S.C. §§102(b) or 103 Over the Sugimoto et al. Reference May Properly Be Withdrawn

Amendment of claims 14 and 34 to include the recitation of "purified and isolated" with reference to the claimed DNA sequences is believed to render moot the outstanding rejection of claims 14, 24, 34 and 36. Under no circumstance can the claims be urged to "read on" non-isolated DNA present in the erythropoietin-producing lymphoblastoid cells of the Sugimoto et al. reference.

6. The Rejection of Claims 14, 15, 17, 18, 20, 24-27, 33, 34, 61-66, 69, 70 and 71 Under 35 U.S.C. §§102(a)/103 Based on the Lee-Huang (PNAS) Reference May Properly Be Withdrawn

It was the Examiner's position that the DNA sequences described in claims 14, 15 17, 18, 20, 24-27, 33, 34, 61-66, 69, 70 and 71 "appear to be the same as those made by Lee-Huang et al." [referring to PTO Reference "R"; Applicant's Reference C-68; Lee-Huang, P.N.A.S., 81, 2708-12 (1984)] and that these claims should therefore be rejected as anticipated or obvious under 35 U.S.C. §§102(a)/103. Applicant respectfully disagrees.

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Applicant notes at the outset that the Lee-Huang article was published in May, 1984, a date which is well after the filing dates of "parent" U.S. Patent Application Serial Nos. 561,024 (December 12, 1983) and 582,185 (February 21, 1984).*

Applicant maintains that, irrespective of the publication date of the Lee-Huang P.N.A.S. article, it is not "legally" available as a reference under 35 U.S.C. §102(a) or (b). As set out in detail below, this is so because the publication's disclosure is conspicuously insufficient to allow a person ordinarily skilled in the art, armed with the publication and his own knowledge, to duplicate the alleged cloning and expression of a human erythropoietin gene.

(a) The Legal Requirements for a Publication to Qualify As a Reference Bar Under 35 U.S.C. §102

Applicant submits that no publication may serve as a bar to the patentability of a discovery under Section 102 if the reference does not itself substantially "enable" the duplication of the claimed discovery. For over a century the courts have maintained that for a publication to be such a bar, the account published must be of a complete and operable invention capable of being put into practical operation. See, Seymour v. Osborne, 78 U.S. 516, 555 (U.S. 1870). This position was uniformly adopted and applied by the former Court of Customs and Patent Appeals. The Court's decision in In re LeGrice, 301 F.2d 929 (CCPA, 1962) is directly in point.

* Note, also, that the December 13, 1983 filing date of Serial No. 561,024 precedes the January 11, 1984 filing date of U.S. Serial No. 570,040, referred to on the face of Reference B-13, the Lee-Huang PCT published PCT Application.

> "We think it is sound law, consistent with public policy underlying our patent law, that before any publication can amount to a statutory bar to the grant of a patent, its disclosure must be such that a skilled artisan could take its teachings in combination with his own knowledge of the particular art and be in possession of the invention" Id, at page 936.
>
> * * * * *
>
> "...the proper test of description in a bar to a patent as the clause is used in section 102(b) requires a determination of whether one skilled in the art to which the invention pertains could take the description of the invention in the printed publication and combine it with his own knowledge of the particular art and from this combination be put in possession of the invention on which the patent is sought." Id, at page 939.

See also, In re Sasse, 629 F.2d 675, 681 (CCPA, 1980).

This position concerning the "enablement" requirement of a reference has been carried forward by the Patent Office Board of Appeals with respect to a variety of technologies, including those involving microbiology. Thus, while the CCPA decision in In re Argoudelis et al., 434 F.2d 1390, (1970) is frequently cited for its holding concerning an applicant's "enablement" requirements, the Board of Appeals decision which gave rise to the CCPA decision clearly applied the ruling of In re LeGrice to eliminate from consideration under Section 102 two Japanese "prior art" references disclosing the same antibiotic as claimed, but disclosing the means for obtaining it in a manner which could not be duplicated by an ordinarily skilled worker. See, Ex parte Argoudelis et al., 157 U.S.P.Q. 437, 443-4 (Bd. App., 1967).

(b) The Lee-Huang (P.N.A.S.) Reference Is Not Susceptible to Duplication By Exercise of Ordinary Skill

Applicant submits that it is manifest from the four corners of the Lee-Huang publication that a person of ordinary skill in the art could not duplicate its disclosures to obtain cDNA encoding human erythropoietin -- principally because the monoclonal antibody designated "7A7" which was used in the work reported was not publicly available at the time of the publication and could not have been obtained by an ordinarily skilled artisan at that time. Moreover, the highly purified immunogen used by Lee-Huang to generate the 7A7-producing hybridoma cell line could not be obtained by "non-inventive" means.

The work represented in the Lee-Huang publication can be fairly summarized by reference to page 2708 and the paragraph bridging its two columns, which states:

> "Recently, we have been engaged in the purification of human EP. Progress has been made in the development of effective techniques for improved Ep purification (10). This has enabled us to prepare sufficient quantities of purified materials for the production of monoclonal antibodies to human Ep. (11) These antibodies provide a specific means for identifying Ep mRNA and for screening recombinant plasmids containing Ep gene [DNA] sequences." (Emphasis supplied)

In the "Materials and Methods" section on page 2708 of the publication reveals that:

> "Monoclonal antibody to human Ep was prepared according to the hybridoma technique of Kohler and Milstein (17). Purified IgG was used for all experiments described in the present work. The particular antibody used in all these studies was designated monoclonal 7A7. It reacts specifically with our purified Ep. Our purified Ep gives a single polypeptide band of Mr 34,000 by silver stain on NaDodSO$_4$/polyacrylamide gel (see Fig. 2A, lane 3). It has a specific activity of 66,000 units/mg." (Emphasis supplied.)

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Commencing with the second full paragraph of the "Results" section on page 2709, it is stated that:

> "To determine whether the poly(A)+ RNA isolated from the Ep-rich human renal carcinomas indeed contained Ep message, in vitro translation was carried out. The labelled translation products were immunoprecipitated with monoclonal antibody to human Ep." (Emphasis supplied.)

In the description of isolation of messenger RNA from renal carcinoma cells* and of use of the messenger to generate cDNA, the publication states, at page 2710:

> "The majority of Ep mRNA was resolved in fraction 11 (Fig. 3, lane 11) as detected by immunoprecipitation with anti-Ep$_3$7A7... This fraction was used to synthesize ^{32}P-labelled single-stranded cDNA..."
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> * * * * *
>
> "Positive recombinants from colony hybridization were picked and grown on gridded nitrocellulose filters in a registered fashion for immunological screening. This procedure relies on expression of the cDNA inserted in the pBR322 ß-lactamase operon to produce a fused polypeptide containing the appropriate epitope for the anti-Ep recognition. From 1.4 x 10^5 screened transformants, three positive clones were identified that reacted consistently with 7A7. These were designated pEp1, -2, and -3." (Emphasis added.)

In the penultimate paragraph of the "Discussion on page 2712, it is stated that:

> "Several monoclonal antibodies to human Ep have been isolated; only one (7A7) was chosen for routine screening of the cDNA

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* There is at least a serious question concerning the public availability of the "Ep-rich human renal carcinomas" used by Lee-Huang. In the first paragraph of the "Results" section on page 2709, Lee-Huang describes an "extensive search" for such carcinomas. Only 2 of 36 renal carcinoma extracts tested qualified as "Ep-rich" sources for messenger RNA.

> library, because it has the highest antigen binding affinity. Since these monoclonal antibodies do not complete with each other for Ep binding (unpublished results) and they most likely recognize different epitopes, a mixture of them may identify additional Ep cDNA clones as well as other Ep-related polypeptides. I have also not screened for the presence of additional Ep cDNA clones containing other inserts that would have remained undetected by the immunological screening." (Emphasis supplied.)

Based on the above-quoted portions of the Lee-Huang text, it is apparent that duplicatation of the erythropoietin cDNA isolation work described in the publication is not merely a formidable task, it is an impossible one.

Clearly, the key to Lee-Huang's initial mRNA isolation and subsequent cDNA isolation (resulting in 3 cDNA clones isolated from among 140,000 prepared) was the use of monoclonal antibody 7A7. Other monoclonals mentioned by Lee-Huang were described as having lower affinity and as reacting with different epitopes, not even alleged to be specific for erythropoietin. There is no notation in the P.N.A.S. article of public availability for antibody 7A7 or the hybridoma cells which produce it. If the skilled worker were to examine the "reference" (No. 11) cited by Lee-Huang as describing the monoclonal antibodies used in the published work, the worker would find only an abstract (previously submitted as Applicant's reference C-69) which describes three positive hybridoma cell lines, none of which are identified as producing antibody 7A7, and none of which are noted to be available from any public depository. Moreover, it is clear from the text that while the well-known Kohler and Milstein techniques may have been employed to generate 7A7 and the other monoclonal antibodies, the highly

pure immunogen used in the hybridoma development process was one which assertedly could only be "enabled" by practice of certain "improved" purification techniques. If the skilled worker were to examine the "reference" (No. 10) cited by Lee-Huang as relating to techniques for "improved Ep purification" which "enabled us to prepare sufficient quantities of purified material for the production of monoclonal antibodies to human Ep", the worker would find only a 1980 paper on hydrophobic interaction chromatography (HIC) (Applicant's Reference C-136). None of the erythropoietin preparations in the paper demonstrate the high specific activity of 66,000 units/mg which characterized the erythropoietin immunogen used to make the 7A7 antibody.

Without a public source for the 7A7 antibody, without a public description even of the purified immunogen used to raise the antibody, the skilled worker is simply without the wherewithal to take possession of the "discovery" related in the Lee-Huang publication.

The above conclusion as to unavailability to the ordinarily skilled worker of either the 7A7 antibody or the purified immunogen used to generate it is specifically confirmed by the statements of Dr. Lee-Huang in her PCT Application WO 85/03079 published July 18, 1985 (submitted as Applicant's Reference B-13). On page 1 of the published application, reference is first made to an alleged invention in cDNA clones of human erythropoietin, than to an "Anti-Ep Patent Application" (Serial No. 570,039, filed January 11, 1984) which relates to monoclonal antibodies to human erythropoietin, and finally to an "Ep Purification Patent Application" (Serial No. 570,075, filed January 11, 1984) which is said to relate to a novel method for purifying

[redacted]an erythropoietin. The interrelationship between these patent applications is set forth at page 2, lines 14-20 of the published PCT Application:

> "The progress made by the present inventor in native human Ep purification (described in the Ep Purification Patent Application) by direct and reverse immunochromatography, and in preparation of monoclonal Anti-Ep (described in the Anti-Ep Patent Application) has made it possible to attempt cloning of human Ep gene which, upon expression, can produce Ep protein."

Clearly, Lee-Huang's position was that an "invention" in means for purifying erythropoietin was needed to secure the immunogen employed in practice of an "invention" in monoclonal, anti-erythropoietin antibody production, which, in turn allowed for a third "invention" in the isolation of an erythropoietin gene. Neither of the "enabling" inventions in erythropoietin purification and monoclonal antibody production is disclosed in the P.N.A.S. article. This conclusion is further borne out by the text of the published application at pages 29 through 36 and 37 through 42 which respectively describes "Ep Purification" and "Monoclonal Anti-Ep".

In the "Ep Purification" section, an erythropoietin purification process is set out wherein the erythropoietin obtained by hydrophobic interaction chromatography (HIC) according to the 1980 Lee-Huang publication (C-136) serves as the starting material of the process. The material is subjected to further processing by direct immunoaffinity chromatography (DIAC) and then by reversed immunoaffinity chromatography (RIAC) to secure the final product. The HIC/DIAC/RIAC purification process is not found in the P.N.A.S. publication.

In the "Monoclonal Anti-Ep" section, the erythropoietin preparation, purified by HIC, DIAC and RIAC, is used as an immunogen to generate hybridomas secreting monoclonal antibodies including, specifically, the antibodies produced by clone "7A7". At page 38, lines 3-16, the immunization protocol is described. The required performance characteristics are set forth with the notation:

> "These performance characteristics are rather formidable, considering the weak immunogenic properties of Ep. Accordingly, the EP used for immunization should be the purest possible and the number of mice immunized should be relatively large. Generally, assuming careful selection and execution of the immunization protocol, about one mouse in six immunized will exhibit an acceptable immune response."

While the P.N.A.S. paper refers generally to the 1975 Kohler and Milstein procedures, page 38, lines 28-33 refer to a different reference for the fusion procedure and also refer to "modifications" of the procedure as actually applied by Lee-Huang. As noted at page 39, lines 14-17, the 7A7-producing clone was one of three stable clones isolated from a total of 6460 hybridomas generated from a total of 10 fusions.* At page 41, lines 11-13, the 7A7 clone was noted as a 10-fold higher producer than the other two clones. In the published patent application, the 7A7 antibody was distinguished from the others by type (IgG2a/k as versus IgG1/k) but the P.N.A.S. reference is wholly silent on this matter.

Support for the above conclusion that the skilled worker would not have been able to prepare the pure immuno-

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* This appears to be the type of "discovery" which has been characterized as patentable in Ex parte Old, 229 U.S.P.Q. 196 (Bd. App. & Int., 1985).

n employed to generate the 7A7 antibody without "inventive skill" is found in the recent issuance of U.S. Letters Patent 4,568,488 to Lee-Huang (Applicant's Reference A-22) based on the "Ep Purification Patent Application" Serial No. 570,075. Presumably, the U.S. Patent Office was convinced that extraordinary skill -- indeed a patentable invention -- was involved in the preparation of highly purified erythropoietin having a specific activity of 66,000 U/mg as described in column 15, lines 43-45. (This activity corresponds exactly to that ascribed to the starting material used in immunizations for monoclonal antibody 7A7 preparation set out in the Lee-Huang P.N.A.S. publication.) It is not known whether Lee-Huang's Application Serial No. 570,039 is still pending, and whether it claims an invention in the specific 7A7 antibody. The clear implication derived from the published PCT application, however, is that extraordinary skill in the art would be needed to produce the antibody.

Additional support for the conclusion that the 7A7 antibody was not publicly available to skilled workers at the time of the P.N.A.S. publication is provided by Applicant's attached Declaration (Exhibit No. 4 hereto) relating to attempts to secure a sample of the antibody. As set out in the Declaration, Applicant recently sought to obtain a sample of the 7A7 antibody from Dr. Lee-Huang and, after a long delay, his request was denied upon his refusal to assure that it would be used only for personal purposes, unrelated to work for his employer. As of this day, the high affinity, 7A7 antibody whose use was asserted to be critical to the practice of the isolation procedures of the Lee-Huang P.N.A.S. article remains unavailable to the public at large.

Because the record clearly demonstrates that the cDNA isolation work described in the Lee-Huang reference could not be duplicated without exercise of extraordinary skill, the publication is not properly urged as a bar to patentability of the present invention and the rejection of the claims under 35 U.S.C. §102(a)/103 may properly be withdrawn.

7. The Rejection of Claims 14, 15, 17-20, 24, 33, 34, 36, 58, 61-66, 69, 70 and 71 Under 35 U.S.C. §§102(a)/103 Based on Publications by Applicant and His Co-Workers May Properly Be Withdrawn

It was the Examiner's position that the prohibitions of Sections 102(a) and/or 103 dictate rejection of essentially all pending claims based on a 1984 Abstract jointly authored by Applicant and his co-workers [J.Cell. Bioch., Suppl. 8B, p. 45 (1984)]. As noted by Applicant's counsel in the Interview Outline, this Abstract and another of substantially the same caliber [appearing in Exp.Hematol., 12, 357 (1984)] do not provide any basis for ignoring Applicant's Declaration as to prior sole inventorship of the claimed subject matter filed in this and his "parent" applications dating back to December 13, 1983. Nonetheless, Applicant has attached hereto as Exhibit Nos. 5 and 6 two separate Declarations, consistent with the decision of In re Katz, 215 U.S.P.Q. 14 (CCPA, 1982), establishing sole inventorship of the claimed subject matter. Applicant submits that no basis exists for the continued rejection of claims 14, 15, 17-20, 24, 33, 36, 58, 61-66, 69, 70 and 71 based on Applicant's publications and that the outstanding rejection may properly be withdrawn.

8. The Rejections of All Claims Under 35 U.S.C. §103 Based Variously on Sugimoto, et al., Cohen, et al., Paddock, Farber, et al., Bennetzen, et al., Gouy, et al., and Lewin May Property Be Withdrawn

It was the Examiner's position that the subject matter of claims 14, 15, 17, 18, 20-24, 34-36, 58 and 61-72 was statutorily obvious upon consideration of Sugimoto, et al. (PTO Reference A, U.S. 4,377,513; Applicant's Reference A-8), in view of the disclosures of Paddock (PTO Reference B, U.S. 4,563,151; Applicant's Reference A-18), and Cohen, et al. (PTO Reference C, U.S. 4,468,464; Applicant's Reference A-17). The Examiner specific position was as follows:

> "Sugimoto, et al. teach cells from which erythropoietin RNA can be isolated, as they have a high erythropoietin production. Paddock teaches making cDNA from RNA, and Cohen, et al. teach cloning of a desired strand of DNA. Further, Sugimoto, et al. suggest that the erythropoietin gene could be so cloned. Thus it would be obvious to one or ordinary skill in the art to isolate and clone the erythropoietin gene, as the techniques for doing so are well known in the art and the expected result is obtained."

Claim 19, specifically directed to monkey species erythropoietin DNA, was rejected on the same grounds as above, in further view of the Farber, et al. Abstract appearing in Exp.Hematol. VII, Suppl. 4, Abstract 101 (PTO Reference T; Applicant's Reference C-32).

Finally, claims "25-30" (sic, 33?) were rejected based on Sugimoto, et al., Paddock and Cohen, et al., in further view of Bennetzen, et al. [J.Biol.Chem., 257(6), 3026-31 (1982); PTO Reference R[1]; Applicant's Reference C-133], Gouy, et al., [Nucleic Acids Res., 10, 7055-7074 (1982); PTO Reference U] and Lewin, [page 307 in Genes, John Wiley & Sons (1983); PTO Reference S[1]].

Applicant respectfully disagrees with the Examiner's conclusions of obviousness for the claimed subject matter and affirmatively submits that the Examiner's position is without factual support. The references relied upon neither disclose nor suggest the making of the invention claimed. As set forth with greater particularity below, it is Applicant's position that the "primary" Sugimoto, et al. reference provides no teaching of a source for erythropoietin RNA and the "secondary" references by Cohen, et al. and Paddock are wholly uninformative with respect to the correct methods and materials means for identifying erythropoietin encoding genetic material so as to allow the present invention to be attained. The remaining, "tertiary" references fail to bar patentability because the primary and secondary references do not suggest the invention. Finally, Applicant submits that the Lee-Huang P.N.A.S. article appears to describe a "failed experiment" which constitutes evidence of patentability of the present claims.

Turning first to the Sugimoto, et al. reference, at the outset of its text it is noted that the alleged invention resides in means for obtaining erythropoietin from human lymphoblastoid cells in culture. Beginning at column 1, line 55, the reference proposes culturing any erythropoietin-producing lymphoblastoid cells and goes on to note that such cells could include "human lymphoblastoid cells in which there has been introduced the human erythropoietin genetic sites" from a variety of normal and neoplastic cells. The reference then states that:

> "These erythropoietin production governing genetic sites may be introduced by means of cell fusion using polyethylene glycol or

Sendai virus, or by genetic recombination techniques using DNA ligase, nuclease and DNA polymerase." (Emphasis supplied.)

It is thus clear that the authors of the Sugimoto, et al. reference do not suggest that lymphoblastoid cells are a potential source of genetic material. Rather, the cells are proposed as a host for insertion of "genetic sites" borrowed (e.g., by cell fusion) from other cell sources. There is no mention at all of RNA or of the reverse transcriptase enzyme needed to secure cDNA from messenger RNA. Contrary to the Examiner's position, then there is no suggestion in Sugimoto, et al. that the erythropoietin gene could be cloned, nor any "teaching" of "cells from which erythropoietin RNA can be isolated, as they have a high erythropoietin production".* Even if there had been a direct disclosure in Sugimoto, et al. of a potential source of "high levels" of erythropoietin-encoding messenger RNA (such as might be attributed, for example, to the Farber, et al. disclosure of isolated kidney cells from phenylhydrazine-treated baboons), it still remains the case that neither Sugimoto, et al. nor any of the secondary references disclose any means whatever for ascertaining which message (from among the innumerable "messages" present in the cells) would encode erythropoietin or which reverse transcript (cDNA) of such a message would encode erythropoietin.

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* The Examiner will also recall that the "effect" of being able to isolate high levels of a secreted polypeptide from a culture may result from a variety of "causes" other than high levels of mRNA in the cells. As examples, one may note the possibility of more efficient translation of the same quantity of RNA, or of more efficient secretion of an essentially fixed quantity of RNA translation product, or even less efficient activity of destructive proteolytic enzymes in the cells or medium.

Even a most casual analysis of the secondary references to Cohen, et al. and Paddock reveals that the skilled worker can obtain no help from them in solving the problem of erythropoietin gene identification and isolation from erythropoietin-producing cells. One must note, for example, that the methods of the Cohen, et al. reference presume the independent existence of a means for identifying and isolating the "second DNA segment foreign to said cell and having at least one intact gene". Likewise, Paddock or any other reference which might address the successful "reverse transcription" of RNA to generate cDNA, also presumes either a means for isolating only the desired messenger RNA (prior to reverse transcription) or a means for isolating the desired cDNA sequences (following transcription and amplification of multiple messages).

Applicant submits that two separate pieces of evidence further support the conclusion that the Sugimoto, et al., Cohen, et al. and Paddock references do not "combine" to render the presently claimed invention obvious. The first source of evidentiary support is provided by consideration of the specific examples of the specification which describe how Applicant actually made his discovery. The second piece of evidence is provided upon analysis of the Lee-Huang reference previously distinguished.

It is highly pertinent to the issue of whether the cited references render Applicant's invention obvious that his isolation of DNA encoding human erythropoietin did not proceed by cDNA techniques attributed to the references and that his isolation of monkey cDNA encoding erythropoietin employed DNA/DNA hybridization methods and materials nowhere described or suggested by the references. As conspicuously

ted in the present specification, Applicant's isolation of human EPO-encoding DNA was by screening of a human genomic library of 1,5000,000 viral plaques in order to isolate what is now generally recognized as a single copy gene in the human genome. The improved methodology employed by Applicant is itself the subject of non-elected claim 60 which describes use of multiple mixed probes, specific substrates, protease enzyme treatment, specific probe concentrations and specific hybridization conditions. The success achieved by Applicant through practice of these procedures must be viewed in the context of the essentially concurrent pronouncement of the Anderson, et al. reference (Applicant's Reference C-2) that such screening methods are "...impractical for isolation of mammalian protein genes when corresponding RNA's are unavailable". It is also noteworthy that PCT Application 85/01961, published May 9, 1985 (Applicant's Reference B-15) relates the opinion of its authors that, as of its 1984 filing date (and perhaps as of the earlier priority dates listed), a patentable invention resided in mixed probe genomic library screening to isolate the human gene for Factor VIII:C. See, e.g., claims 21, through 31 of the application. The genomic library screening process employed by Applicant is nowhere "taught" in the cited reference.

As further conspicuously noted in the specification, Applicant's isolation of monkey cDNA involved hybridization screening as described above and the use of DNA probes whose sequences were determined by Applicant based on information unavailable from any published source, much less from the cited references to Sugimoto, et al., Cohen, et al. and Paddock.

outside the scope of the disclosure of the cited references is thus believed to support the conclusion of non-obviousness of the claimed invention.

Applicant next submits that non-obviousness is still further established by analysis of the Lee-Huang P.N.A.S. publication. A substantial basis exists for concluding that none of the cDNA sequences whose isolation is reported in the Lee-Huang publication actually encoded the polypeptide sequence of erythropoietin and, therefore, that the work of the publication constitutes a "failed attempt" to clone and express DNA encoding human erythropoietin. The principal indicators of failure are found in the publication's reports concerning the size and restriction enzyme digestion characteristics of the cDNA inserts alleged to encode erythropoietin, and in its reports concerning the molecular weights of the products of in vitro translation of messenger RNA used to generate the cDNA inserts.

It will be recalled at the outset that the publication's author concluded that erythropoietin cDNA had been cloned and expressed on the basis of following types of experimental evidence:

(1) polypeptide products of in vitro translation of a particular (sized) fraction of mRNA isolated from human renal carcinoma cells were immunologically reactive with a monoclonal antibody ("7A7") against human erythropoietin;

(2) the size (M_r) of these polypeptide translation products was about 29,000 and 15,000, and the larger of these appeared to correspond in size to the molecular weight

of "aglycosylated" erythropoietin as projected by the author*; and

(3) cDNA derived from the same fraction of mRNA provided for three "positive" clones which (a) upon "expression" provided β-lactamase fusion products immunoreactive with the 7A7 antibody, and (b) hybridized "back" to the mRNA fraction which had yielded the immunoreactive in vitro expression products.

While the publication purports to address the "cloning and expression" of human erythropoietin cDNA, no expression product of the cDNA was ever isolated and sequenced, nor were any of the cloned cDNAs analyzed for their nucleic acid sequence constitution. Restriction enzyme digestion of the three separate "positive" clones (designated by Lee-Huang as pEp1, pEp2 and pEp3) revealed that the three cDNA inserts had approximate sizes of 1,400 base pairs, 600 base pairs and 200 base pairs, respectively. It was Lee-Huang's conclusion (at page 2712) that:

> "Judging from the M_r of the native urinary Ep, the cDNA insert of pEp1 is probably close to the coding size, while those of clones pEp2 and pEp3 are too short to encode the complete sequence of Ep." (Emphasis supplied.)

Applicant respectfully submits that knowledge of the nucleic acid sequence of the human erythropoietin gene as provided by the present application and as substantiated by later analytical work (including the independent work of

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* Please refer to the publication text beginning with the second paragraph of the "Results" section on page 2709. Immunoreactive polypeptide product of in vitro mRNA expression in a system incapable of glycosylation were sized at M_r 29,000 and 15,000. A series of comparative tests using crude and purified natural EPO were said to confirm that the M_r 29,000 polypeptide "may represent the aglycosylated form of Ep."

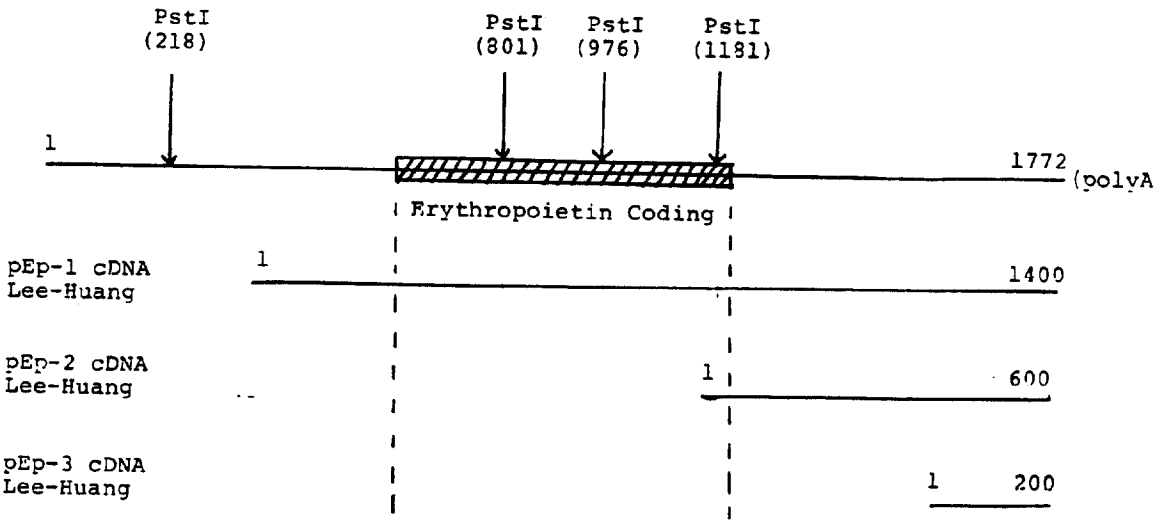
thers) indicates that it is highly doubtful that the Lee-Huang actually succeeded in cloning a DNA sequence that encoded human erythropoietin. This is the case despite the fact that mRNA translation products and cDNA fusion gene expression products described by Lee-Huang were noted to be immunoreactive with a particular monoclonal, anti-erythropoietin antibody.

Applicant has attached hereto as Exhibit No. 7 his Declaration relating to a computer-assisted restriction mapping analysis of cDNA sequence encoding human erythropoietin and to his experiments in restriction enzyme digestion of the cDNA. As described in the Declaration, computer analysis of the 1772 base pair cDNA reveals that there are a total of four 6-base pair recognition sites (5'-CTGCAG-3'), allowing for potential cleavage of the DNA by the restriction endonuclease enzyme PstI at four distinct sites. These occur at nucleotide numbers 218, 801, 976, and 1185. [Attached hereto as Exhibit No. 8 is a photocopy of Table VI of the present application whereupon red boxes enclose the four recognition sites. For comparative purposes, attached hereto as Exhibit No. 9 is a photocopy of Figures 2 and 3 of Jacobs et al., Nature, 313, 806-810 (1985) upon which the same recognition sites in the same locations, have been noted.] The Declaration further reveals that actual digestion of a human erythropoietin cDNA-containing circular plasmid with PstI in fact generated the expected total of four fragments. These comprise a large plasmid DNA fragment and three small fragments of sizes quite precisely corresponding in size to those predicted by the computer generated restriction map.

With this information in hand concerning the size and sequence of the mRNA reverse transcript (cDNA) isolated by both Applicant and independent investigators, analysis of Figure 5 of the Lee-Huang publication reveals that the 1400, 600, and 200 base pair cDNA sequences most likely do not encode human erythropoietin.

The 1772 base pair erythropoietin-encoding cDNA sequence analyzed by Applicant and the three cDNA clones isolated according to the publication are aligned for comparative purposes immediately below. In the illustration a scale of 1mm = 100 base pairs was employed and the position of the erythropoietin polypeptide coding region is represented by a block.

Erythropoietin cDNA (Lin, Jacobs, et al.)



It will be recalled that cDNA transcription occurs from the 3' end of the mRNA and therefore the linear alignment of the illustration is premised on the assumption that the Lee-Huang publication's cDNA clones were developed by reverse transcription from a polyadenyl (Poly A) site common to the 3' end all the mRNA's involved. The likelihood that the site of the polyA region of the erythropoietin mRNA from the kidney cell source used by Lee-Huang differed substantially from the polyA region of Applicant's messenger transcribed from a human genomic DNA sequence is quite small.

Clearly the cDNA inserts of pEp2 and pEp3 are, as Lee-Huang predicted, too small to encode human erythropoietin or. Upon examination of the restriction map characteristics of the largest cDNA fragment, present on Lee-Huang's "pEp1" plasmid as a "removable" 1400 base pair PstI insert into the plasmid pBR322*, one notes from Lane 3 of Figure 5 of the publication that three fragments were formed upon PstI digestion -- a large pBR322 DNA fragment and two small fragments said to be about 1100 and 300 base pairs, respectively. This number and size pattern of fragments would be accounted for by the presence of the two "designed" PstI recognition sites at the respective ends of the insert (allowing for the insert to be separated from the plasmid) and only one PstI recognition site within the 1400 base pair insert, located about 1100 base pairs from the 3' end. The restriction map of human erythropoietin cDNA as predicted by computer and verified by actual digestion

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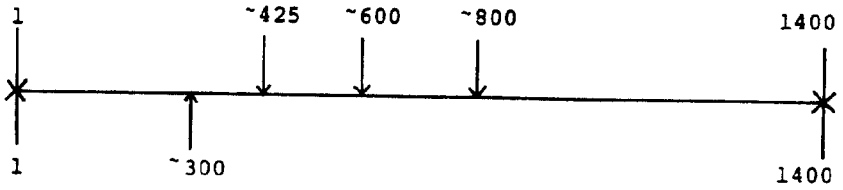
* See attached Exhibit No. 10 taken from Maniatis et al., "Molecular Cloning, A Laboratory Manual," Cold Springs Harbor Laboratory, 1982, which graphically illustrates the "standard" process carried by the Lee-Huang publication for cDNA preparation and insertion into pBR322 at its PstI site.

experiments, however, dictates that at least three PstI recognition sites should be present in a 1400 base pair, erythropoietin-encoding cDNA insert. Had the 1400 base pair cDNA insert in Lee-Huang's plasmid pEp1 been an erythropoietin-encoding DNA, then a total of five fragments would be expected upon digestion with PstI. Immediately below is an illustration of the projection.

Plasmid pEp-1

PROJECTED PstI Restriction Sites If EPO cDNA
Yield Upon Digestion = One Large (Plasmid) Fragment
Four cDNA Fragments



Plasmid pEp-1

ACTUAL PstI Restriction Sites
Yield Upon Digestion = One Large (Plasmid) Fragment
Two cDNA Fragments

In order for the cDNA insert of Lee-Huang's plasmid pEp1 to actually represent an erythropoietin-encoding DNA, the insert would have to somehow comprise a naturally-occurring allelic variant wherein a sufficient number of base pair changes are present to "kill" all three PstI sites within the polypeptide coding region and "create" a new PstI site in a different location within the coding region, about 300 base pairs from the 5' end. The likelihood that this could be the case is immensely small and one therefore must

conclude that the accuracy of Lee-Huang's identification of the cDNA as Epo-encoding is, at best, significantly in doubt.

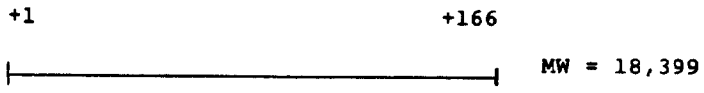
In addition to the above sizing and restriction mapping evidence casting doubt on the Lee-Huang's conclusion that erythropoietin-encoding cDNA had actually been cloned and expressed, there is still another piece of evidence indicating that the messenger RNA "template" used by Lee-Huang for cDNA preparation did not actually encode human erythropoietin. This evidence is manifested through comparison of the molecular weight (M_r) of the non-glycosylated in vitro translation products which Lee-Huang's mRNA gave rise to versus the calculated molecular weight of erythropoietin based on its amino acid sequence.

As indicated to the Examiner during the course of the recent interview, the carbohydrate-free, in vitro mRNA translation products described by Lee-Huang do not "correspond" in terms of molecular weight to products which would be expected to be obtained upon translation of an mRNA encoding human erythropoietin. The two immunoreactive expression products of the publication had 29,000 and 15,000 molecular weights, respectively. Lee-Huang projected that the M_r 29,000 product was an "aglycosylated" form of natural, glycosylated human erythropoietin, which had been reported to have an apparent molecular weight of 34,000. The M_r 15,000 translation product was projected to be an "aglycosylated" fragment of the larger polypeptide. However, calculation of the molecular weights of non-glycosylated products of erythropoietin mRNA translation reveals no potential for generation of an M_r 29,000 species, no potential for generation of an M_r 15,000 species, and no likely

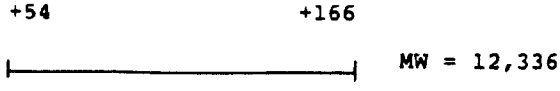
prospect that an M_r 29,000 species could be obtained by, e.g., dimerization of any potential species.

More specifically, if one were to assume that the product of human erythropoietin mRNA tranlsation in vitro was a "full-length" polypeptide having both the 27 amino acid residue leader sequence and a full complement of 166 residues of the mature polypeptide, the molecular weight of this 193 residue species would be 21,310.

If one were to assume that the in vitro translation system which Lee-Huang possessed the enzymatic wherewithal to process off the 27 residue leader sequence, the molecular weight of the resulting 166 residue species would be 18,399.



Finally, if one were to assume that the in vitro translation system employed by Lee-Huang somehow allowed for internal initiation of translation at the ATG codon for the methionine residue at position 54 of the erythropoietin polypeptide (rather than the methionine at -27) the calculated molecular weight of this species would be 12,336.



Clearly, none of the potential translation products of erythropoietin mRNA correspond to Lee-Huang's M_r29000 translation product nor could any homodimer of a potential translation product "weigh in" at 29,000.

The above analysis is believed to establish that available evidence of the nature and amino acid constitution of the human erythropoietin polypeptide, and the nature and base pair constitution of the DNA that encodes the human erythropoietin polypeptide (both revealed for the first time by this inventor and subsequently independently verified by others) fully supports the conclusion that the Lee-Huang publication describes a "failed attempt" to clone and express human erythropoietin cDNA. This, in turn, provides at least some evidence of the nonobviousness of the invention claimed herein.

Because none of the references relied upon disclose or suggest any suitable means for securing the claimed invention, Applicant respectfully submits that the rejections of the claims under 35 U.S.C. §103 may properly be withdrawn.

C. Remarks Concerning Preliminary Amendment and Information Disclosure Statement Dated April 23, 1986

As Exhibit 3 to Applicant's Preliminary Amendment dated April 23, 1986, there was provided a list of eight "closely related" references. This list included items A-13/B5 (Egrie, Published EPC Application 0,116,446), C-103 [Sue, et al., Proc.Nat'l.Acad.Sci.(USA), 80:3651-3655 (1981)] and C-106 [Sytowski, et al., J.Immunol.Methods, 69:181-186 (1983)]. In turn, these three were discussed at pages 4 and 5 of the concurrently filed Information

Disclosure Statement as references which relate "to synthetic peptides having structures based on prior attempts at identification of the sequence of amino acids at the amino terminal of urine-derived erythropoietin". At pages 5 and 6, under the heading "References Related to Isolation of Naturally-Occurring Erythropoietin by Immunological Means", a notation was made of Reference C-129 [Yanagawa, et al. J.Biol.Chem., 259(5), 2707-2710 (1984)].

In the recent past, Applicant's undersigned counsel determined that the Yanagawa, et al. reference (C-129), in addition to relating to immunological isolation of erythropoietin, also disclosed (at page 2710) a sequence of thirty amino terminal amino acid residues obtained by gas phase sequenator sequencing of immunopurified erythropoietin. Distinctions between this sequence and that of Sue, et al., reference (C-106) were drawn. Although no mention of synthetic peptides was made, it appears that the Yanagawa, et al. reference should probably have been listed as a ninth reference on Exhibit 3 to the Preliminary Amendment and included along with the Egrie, Sue, et al. and Sytowski, et al. as a references relating to amino terminal sequences of erythropoietin.

As was the case with the Sue, et al. and Sytowski, et al. references, the Yanagawa, et al. publication incorrectly identifies the amino terminal residues of erythropoietin -- fully five of the first thirty residues are incompletely or incorrectly noted (Pro^3 designated as "?"; Cys^7 designated as "Leu"; Asn^{24} designated as "?"; Thr^{27} designated as "Asp"; and, Cys^{29} designated as "Gly"). For the same reasons as advanced in the Information Disclosure Statement, the Yanagawa, et al. reference is not

believed to be relevant to patentability of the claimed invention.

CONCLUSION

The foregoing amendments and remarks are beleived to establish that pending claims 14 (amended), 15, 17-36, 58 and 61-72 are in condition for allowance and an early notice thereof is solicited.

Respectfully submitted,

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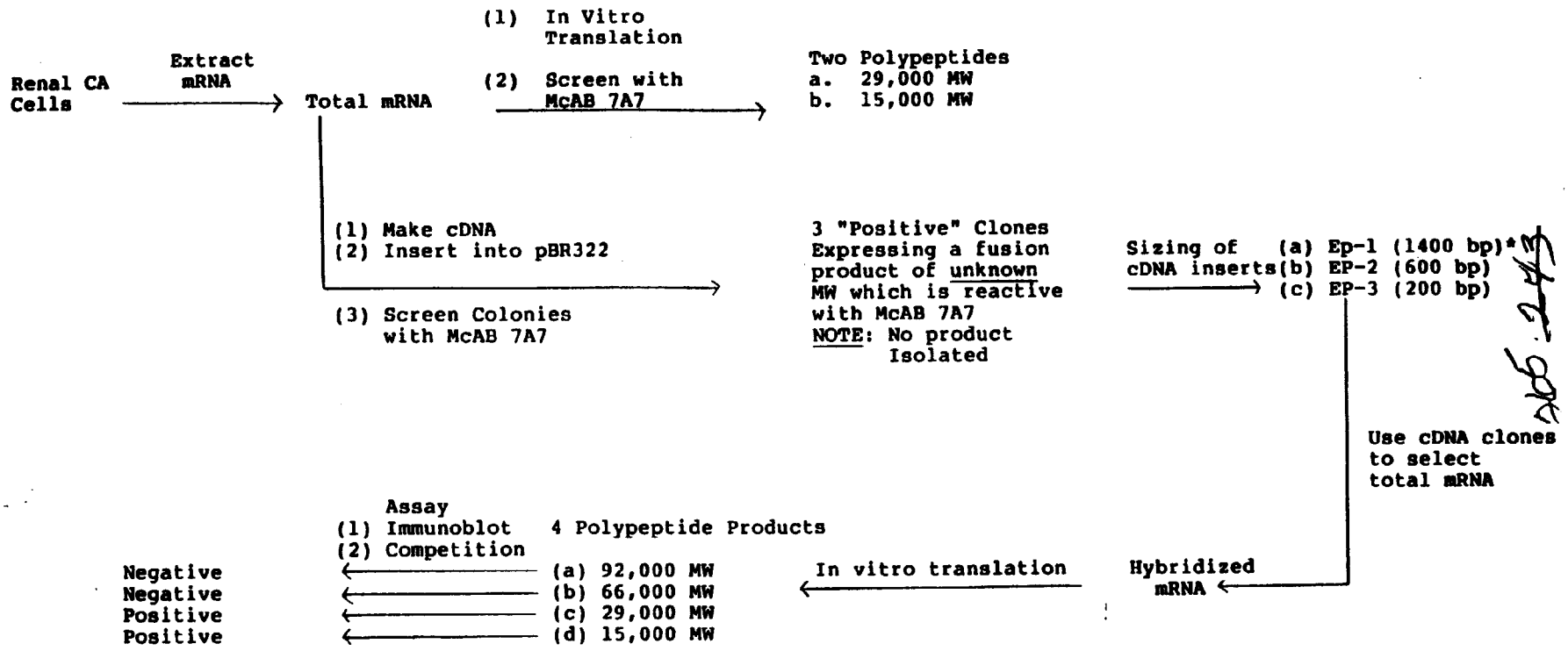
Chicago, Illinois
October 2, 1986

264 242

OUTLINE OF PROCEDURES DESCRIBED IN LEE-HUANG, PROC.NATL.ACAD.SCI. (USA), 81, 2708-2712 (1984)



"Probably close to coding size" (p. 2712)

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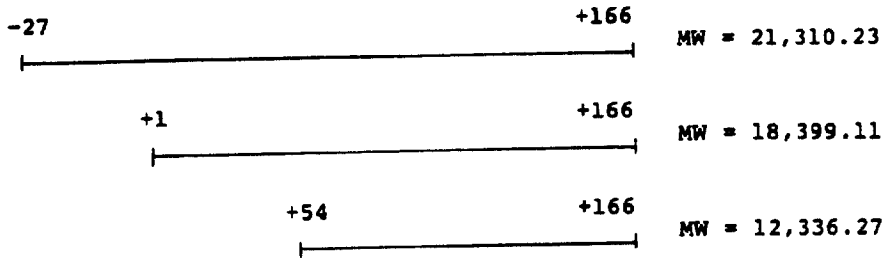
POTENTIAL TRANSLATION PRODUCTS OF mRNA ENCODING ERYTHROPOIETIN BASED ON SPECIFICATION TABLE VI



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2166-247

EXHIBIT B

Argument Map in DNA Strand hu13
from the '6cutter' file.

```
HIND111  STY1-1  HAE1-2  CFR1-3   BGL1  CFR1-3   HGIC1-1
      HGIJ11-2   STU1  NSPC1-2      AVA1-4    ACY1-4
         MST1     AVA1-4  SFI1       SMA1  HGIA-3  HAE1-1
            APA1  SMA1    BGL1        APA1     HAE11-4
            HGIJ11-4       CFR1-4    HGIJ11-4    KPN1
            AVA1-4         XMA3       BSTE2   HGIC1-4
            SMA1                      BSSH11  NAR1    PST1
              PST1                                    SFI1
                                                      BGL1
                                                      STY1-1

   HINC11-2    ACC1-2  TTHIII2-4  APA1  HGIC1-3  STY1-4
    HGIJ11-2       HAE1-2  HGIJ11-3  AHA111        HGIJ11-2
     PST1   PVU11           HAE11-2    ACY1-1          HAE1-3
     PVU11          STU1     NCO1  HGIJ11-4
     NSPC1-3        PST1     STY1-2     HGIA-4
      HAE1-3                     BGL11  HGIJ11-1
        HGIJ11-3                 XHO2-4
         HGIJ11-2                   BSM1-2
                                      SAC1
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  1  AAGCTTCTGCGCTTCCAGACCCAGCTACTTTGCGGAACTCAGCAACCCAGGCATCTCTGA
     TTCGAAGACCCGAAGGTCTGGGTCGATGAAACGCCTTGAGTCGTTGGGTCCGTAGAGACT
     ^
     1 HIND111,

 61  GTCTCCGCCCAAGACCGGGATGCCCCCCAGGGGAGGTGTCCGGGAGCCCAGCCTTTCCCA
     CAGAGGCGGGTTCTGGCCCTACGGGGGGTCCCCTCCACAGGCCCTCGGGTCGGAAAGGGT
                                                   ^
     104 HGIJ11,

121  GATAGCACGCTCCGCCAGTCCCAAGGGTGCGCAACCGGCTGCACTCCCCTCCCGCGACCC
     CTATCGTGCGAGGCGGTCAGGGTTCCCACGCGTTGGCCGACGTGAGGGGACGGCGCTGGG
                      ^     ^
     141 STY1, 148 MST1,

181  AGGGCCCGGGAGCAGCCCCCATGACCCACACGCACGTCTGCAGCAGCCCCGCTCACGCCC
     TCCCGGGCCCTCGTCGGGGGTACTGGGTGTGCGTGCAGACGTCGTCGGGGCGAGTGCGGG
      ^   ^                          ^
     182 APA1 HGIJ11, 185 AVA1 SMA1, 218 PST1,

241  CGGCGAGCCTCAACCCAGCGCTCCTGCCCCTGCTCTGACCCCGGGTGGCCCCTACCCCTG
     GCCGCTCGGAGTTGGGTCCGGAGGACGGGGACGAGACTGGGGCCCACCGGGGATGGGGAC
                      ^                    ^
     257 HAE1 STU1, 280 AVA1 SMA1,

301  GCGACCCCTCACGCACACAGCCTCTCCCCCACCCCCACCCGCGCACGCACACATGCAGAT
     CGCTGGGGAGTGCGTGTGTCGGACAGGGGGTGGGGGTGGGCGCGTGCGTGTGTACGTCTA
                                                            ^
     351 NSPC1,   267 345
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274

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361  AACAGCCCCGACCCCCCGCCAGAGCCGCAGAGTCCCTGGC        CCGGCCGCTCGCTCC
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     376 CFR1, 399 SFI1, 400 BGL1, 407 CFR1 ?. MAS,

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     520 BGL1,

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     552 AVA1 SMA1, 562 APA1 HGIJ11, 575 BSTE2, 584 BSSH11,

601  GAGGGACCCCCGCCCAGGCGCGGAGATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTT
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     610 CFR1, 631 HGIA,

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     703 ACY1 HAE11 HGIC1 NAR1,

721  ATCTGTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAGAATATC
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     746 HGIC1 KPN1, 758 HAE1, 759 SFI1, 760 BGL1, 761 STY1,

781  ACGACGGGCTGTGCTGAACACTGCAGCTTGAATGAGAATATCACTGTCCCAGACACCAAA
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     801 PST1,

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     949 HINC11,

961  CAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAGCCGTCAGTGGCCTTCGCAGCCTC
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     970 HGIJ11, 976 PST1, 979 PVU11, 984 NSPC1, 1005 HAE1,

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     1082 PVU11, 1135 ACC1,

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     1181 HAF1 STU1, 1185 PST1.  244 502
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      1324 NCO1 STY1,

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      1391 BGL11 XHO2, 1421 APA1 HGIJ11, 1437 BSM1,

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      1702 HAE1,

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277 269

Exhibit No. 2

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Isolation of Biologically Active Ribonucleic Acid from Sources Enriched in Ribonuclease†

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ABSTRACT: Intact ribonucleic acid (RNA) has been prepared from tissues rich in ribonuclease such as the rat pancreas by efficient homogenization in a 4 M solution of the potent protein denaturant guanidinium thiocyanate plus 0.1 M 2-mercaptoethanol to break protein disulfide bonds. The RNA was isolated free of protein by ethanol precipitation or by sedimentation through cesium chloride. Rat pancreas RNA obtained by these means has been used as a source for the purification of α-amylase messenger ribonucleic acid.

The preparation of undegraded ribonucleic acid from a number of cell types is hindered by the presence of active nucleases. An extreme example of this is the rat pancreas which contains over 200 μg of ribonuclease A per g of tissue wet weight (Beintema et al., 1973). Within the pancreatic exocrine cells, ribonuclease A as well as other digestive enzymes and zymogens appears to be synthesized on ribosomes bound to the cytoplasmic face of the endoplasmic reticulum, extruded directly into the cisternal side, and subsequently packaged in secretory granules. Thus, the functions of the cytosol are effectively sequestered from these strong hydrolytic activities. Disruption of the cells, however, inevitably results in rapid mixing of RNA and RNase.[1,2] One way to eliminate nucleolytic degradation of RNA is to denature all of the cellular proteins including RNase. This approach would be successful only if the rate of denaturation exceeds the rate of RNA hydrolysis by RNase. Deproteinization procedures using guanidine hydrochloride (Cox, 1968) or phenol even in the presence of RNase inhibitors such as heparin, iodoacetate, and detergent (Parish, 1972) are insufficiently effective to yield intact RNA from the pancreas.

We describe here a generally applicable method for the quantitative isolation of intact RNA. The rate of denaturation is maximized by the combined use of a strong denaturant, guanidinium thiocyanate, in which both cation and anion are potent chaotropic agents (Jencks, 1969), and a reductant to break protein disulfide bonds which are essential for RNase activity (Sela et al., 1956). This method has been employed in the isolation of intact biologically functional RNA from rat pancreas and the purification of mRNA for α-amylase, the most abundant pancreas-specific protein (Sanders & Rutter, 1972).

Experimental Procedure

Chemicals and Solutions. All glassware was rendered nuclease free by overnight treatment at 180 °C. Whenever possible [see Ehrenberg et al. (1974)], stock solutions were treated for 20 min with 0.2% diethyl pyrocarbonate and then thoroughly boiled to remove traces of the reagent. Buffers such as tris(hydroxymethyl)aminomethane, which contains a primary amine that reacts with diethyl pyrocarbonate, were avoided.

Guanidinium thiocyanate stock (4 M) was prepared by mixing 50 g of Fluka purum grade guanidinium thiocyanate (Tridom, Inc., Hauppage, NY) with 0.5 g of sodium *N*-lauroylsarcosine (final concentration 0.5%), 2.5 mL of 1 M sodium citrate, pH 7.0 (25 mM), 0.7 mL of 2-mercaptoethanol (0.1 M), and 0.33 mL of Sigma 30% Antifoam A (0.1%).

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[1] A brief note describing a version of this method has been published (Ullrich et al., 1977).
[2] Abbreviations used: RNase, ribonuclease; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid.

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Deionized water was added with warming and stirring until the volume equaled 100 mL at room temperature. The solution, which contained some insoluble material, was filtered, its pH was adjusted to 7 with a small amount of 1 N NaOH, and it was stored tightly closed for up to 1 month at room temperature. All handling of this solution and the initial homogenization were done in a fume hood, and all equipment which came into contact with 2-mercaptoethanol was subsequently treated with dilute aqueous hypochlorite solution (chlorine laundry bleach).

Guanidine hydrochloride (Sigma practical grade) was made up to 7.5 M, filtered, neutralized to pH 7.0, buffered with 0.025 volume of 1 M sodium citrate, pH 7.0, made 5 mM in either dithiothreitol or dithioerythritol, and stored for up to 1 month at room temperature.

Standard Guanidinium Thiocyanate Extraction Procedure. Freshly removed pancreases were trimmed free of lymph nodes, ganglia, and fat, weighed (the pancreas from a 300-g female rat weighs ~1 g), and then individually dropped into 16 mL of guanidinium thiocyanate stock solution in a 55-mL Potter–Elvehjem homogenizer tube and immediately homogenized for 30–60 s at full speed with an 18-mm diameter Tissumizer homogenizer (Tekmar Industries, Cincinnati, OH). The homogenates of two pancreases were combined in a 50-mL tube and centrifuged for 10 min at 8000 rpm in a Sorvall HB4 swinging bucket rotor at 10 °C to sediment particulate material. The supernatants were decanted into a flask and mixed with 0.025 volume (relative to the original volume of homogenizing buffer) of 1 M acetic acid to lower the pH from 7 to 5 and 0.75 volume of absolute ethanol. The flask was capped, shaken thoroughly, and placed at −20 °C overnight to precipitate nucleic acid. The material was sedimented by centrifugation for 10 min at −10 °C and 6000 rpm in an HB4 rotor. The tubes were drained of supernatant and any material which did not form a firm pellet. The pellet was then resuspended by vigorous shaking in 0.5 volume (relative to the original volume of homogenization buffer) of buffered guanidine hydrochloride stock solution. If necessary, the samples were briefly warmed in a 68 °C water bath to ensure complete dispersion of the pellets. RNA was reprecipitated by adding (relative to the amount of guanidine hydrochloride) 0.025 volume of 1 M acetic acid and 0.5 volume of ethanol. The solution was kept for at least 3 h at −20 °C and centrifuged as before. A final reprecipitation from guanidine hydrochloride was performed in the same way, with a further halving of the total volume. This reprecipitated material was centrifuged for only 5 min at 6000 rpm. From this point onward all procedures were carried out under sterile conditions to prevent nuclease contamination.

The final pellets were dispersed in ethanol at room temperature, triturated if necessary to extract excess guanidine hydrochloride, and again centrifuged for 5 min at 6000 rpm. Ethanol was removed from the pellet by a stream of nitrogen, and the RNA was dissolved with vigorous shaking in 1.0 mL of sterile water per g of original tissue. This solution was centrifuged for 10 min at 13 000 rpm and 10 °C to sediment insoluble material. The supernatants containing the RNA were decanted and saved, while the insoluble material was reextracted twice with 0.5 mL of sterile water per g of original tissue wet weight, followed by centrifugation for 10 min at 13 000 rpm and 10 °C. The combined aqueous solution was mixed with 0.1 volume of 2 M potassium acetate, pH 5, and 2 volumes of ethanol and left overnight at −20 °C.

RNA was sedimented from the ethanol suspension by centrifugation for 20 min at 10 000 rpm and −10 °C in Corex tubes in an H[illegible]. The pellets were thoroughly washed with 95% ethanol, dried with nitrogen, and dissolved in 1.0 mL of sterile water per g of starting tissue. Absorbance measurements were obtained by diluting the RNA solutions into 10 mM triethanolamine hydrochloride, pH 7.4. An $E_{1cm}^{1\%}$ of 200 at 260 nm was used to determine the concentration of RNA. The RNA samples were routinely stored as 70% ethanol suspension at pH 5 and −20 °C.

All of the extraction procedures were routinely carried out in polyethylene, polypropylene, or Corex centrifuge tubes. Exposure to guanidinium thiocyanate solutions resulted in a high rate of failure for polycarbonate tubes.

In view of the large amounts of ribonuclease in the guanidine homogenates and supernatants, it was imperative that no contamination of any glassware or chemicals by these solutions be permitted. Dilution of solutions of denatured RNase results in renaturation of the active enzyme (Sela et al., 1956).

A modification to the above procedure in which the RNA is separated from the guanidinium thiocyanate homogenate by ultracentrifugation through a dense cushion of cesium chloride (Glisin et al., 1974) was suggested by Dr. A. Ullrich. For this procedure, technical grade cesium chloride was made up to 5.7 M, buffered with 0.1 M sodium ethylenediaminetetraacetate, pH 7, or 25 mM sodium acetate or citrate, pH 5, sterilized with 0.2% diethyl pyrocarbonate, and passed through a 0.45-μm Millipore filter. Small amounts of tissue were homogenized in 4 M, filtered guanidinium thiocyanate with a small Tissumizer or Potter–Elvehjem and layered into ultracentrifuge tubes one-quarter filled with 5.7 M cesium chloride. In a typical experiment, a Beckman SW50.1 rotor was centrifuged for 12 h at 36 000 rpm and 20 °C. Dissolution of the RNA pellets in water was facilitated by brief heating in a 68 °C water bath or by first extracting excess cesium chloride with ethanol and drying with nitrogen. Since the supernatants in these experiments could contain large amounts of renaturable nuclease, great care was taken not to contaminate the pellets during their dissolution. This danger could be circumvented by suspending the pellets in a small volume of buffered guanidine hydrochloride stock solution and precipitating the RNA with ethanol as described above. Cesium chloride has been used for the preparation of samples of less than 100 μg of embryonic RNA (Harding et al., 1978) and for the isolation of rat parotid RNA free from polysaccharides (Swain and Rutter, unpublished experiments). When the maximum rotor speeds permissable for dense cesium chloride solutions are calculated, allowance must be made for the specific gravity of the guanidinium thiocyanate homogenates, which is between 1.1 and 1.2 g/mL.

Procedural Anecdotes and Variations. A large number of different experimental procedures were tested before reaching those described above. A summary of our experience is given here to facilitate adaptation of the procedure to other systems. First, the prevention of degradation by ribonuclease is dependent upon the efficiency of the initial seconds of the homogenization. For this reason, we have used the high-speed Tissumizer; the similar Polytron (Brinkmann Instruments, Westbury, NY) undoubtedly would be satisfactory. The use of a conventional blender or the homogenization of tissue which has been frozen and thawed or of tissue which has been pulverized in liquid nitrogen results in degradation of the RNA, as detected by the diminution in the 28S peak height and the concomitant appearance of lower molecular weight species on electrophoresis in denaturing gels. However, rat pancreases which have been lyophilized after pulverization in dry ice or liquid nitrogen can be satisfactorily extracted with the guani-

dinium thiocyanate procedure. [illegible]ies lacking significant nucleolytic activity, conditions [illegible]omogenization are less stringent. Embryonic pancreases can be prepared with a small Potter–Elvehjem homogenizer, and for some cultured cells no homogenization is needed since the cells lyse upon addition of the guanidine solution (Harding et al., 1977; Strohman et al., 1977).

The reprecipitations of the RNA aid to eliminate already denatured ribonuclease from the nucleic acid pellets. Thus, these steps can be varied according to the specific circumstances. The pH and temperature of the initial homogenization, 7.0 and ~20 °C, are optimal, but some variations can be tolerated. The use of the detergent sodium *N*-lauroylsarcosine is not essential but gives a cleaner initial precipitate of RNA and may accelerate the initial dissolution of the tissue. The 2-mercaptoethanol is essential for tissue containing RNase, but increasing its concentration beyond 0.1 M final concentration has no effect. Dithiothreitol can be used with the guanidine hydrochloride stock as a disulfide bond reductant, but it undergoes a chemical reaction with the thiocyanate anion to produce hydrogen sulfide and a green color.

The use of pH 7 and room temperature to dissolve the RNA and of pH 5, −20 °C, and the addition of 0.5 volume of ethanol to precipitate it follow the recommendation of Cox (1968) for guanidine hydrochloride. It is essential to determine empirically the time necessary for complete precipitation of a given RNA sample at −20 °C [viz., Strohman et al. (1977)]. Cooling to this temperature can be accelerated by the use of a 3:1 crushed ice–rock salt bath. It is also advisable to maintain RNA concentrations above 25 μg/mL in guanidine solution. Tissue can be homogenized in as little as 4 volumes of 4 M guanidinium thiocyanate, but the resultant solution may be too viscous to permit easy sedimentation of the RNA. The initial precipitation described above uses 0.75 volume of ethanol relative to guanidinium thiocyanate stock; this precipitates some DNA (eliminated by the reprecipitations) as well as RNA (Cox, 1968) but is necessary to prevent guanidinium thiocyanate from crystallizing out of solution at −20 °C. It is convenient to decrease the volumes of the successive precipitations to concentrate the RNA. Inclusion of a final organic solvent extraction step, for example, with phenol or chloroform, or of a 3 M sodium acetate precipitation at pH (Kirby, 1968) is unnecessary.

Some tissues may contain non-RNA molecules which coprecipitate with RNA by the methods described, necessitating further purification. We have, however, not encountered such contaminants in the tissues listed in Figure 2 or in rat brain, spleen, or muscle. Under the described conditions of centrifugation, yeast ^{3}H-labeled tRNA (provided by Dr. L. De Gennaro) was not sedimented. Similarly, in the standard procedure tRNAs (and DNA) are not precipitated from guanidine hydrochloride plus 0.5 volume of ethanol, as noted by Cox (1968).

Since the early steps of the procedure are always carried out in the presence of denaturants, sterile procedures and glassware are unnecessary, but as soon as the RNA is no longer in the presence of guanidine, stringent precautions against adventitious nucleases must be taken.

Preparation of Polyadenylated RNA. Polyadenylated species were separated from rRNA by two cycles of binding to oligo(dT)–cellulose (Type T-2, Collaborative Research, Waltham, MA). The procedure of Aviv & Leder (1972) was modified by the use of 0.5 M lithium chloride, 0.2% dodecyl sulfate, and 10 mM triethanolamine hydrochloride, pH 7.4, as the binding buffer. For minimization of nonspecific ribosomal contamin[illegible] of the polyadenylated RNA, the samples were heated for [illegible] min at 68 °C at a concentration of no more than 2.5 mg/mL in sterile water and then rapidly quenched on ice immediately prior to the other additions listed above and application to the column. Bound polyadenylated RNA was eluted with 10 mM triethanolamine hy[illegible]hloride, pH 7.4, without an intermediate 0.1 M salt wa[illegible]

Resolution Analysis of Isolated RNA Species. The mRNA preparations were subjected to gel electrophoresis in 3% agarose, 6 M urea, and 25 mM sodium citrate, pH 3.5, according to a modification of the procedure of Woo et al. (1975). Agarose was dissolved in buffered 6 M urea plus 0.02% Antifoam A by holding in a boiling water bath until uniformly in solution and free of bubbles. The solution was poured at 30 °C and allowed to gel overnight at 5 °C. Cylindrical gels were removed partially from their tubes, cut into 98-mm lengths, returned to the tubes, and held in place with gauze. After application of a 20-μg sample to a 5-mm diameter gel in buffered urea plus 10% sucrose, electrophoresis was conducted for 4 h at 100 V and room temperature with rapid recirculation of reservoir buffer (25 mM citrate, pH 3.5). The gels were washed for at least 1 h in sterile 25% glycerol and scanned at 260 nm.

For analysis of electrophoretically resolved mRNAs, agarose gels were cut with a razor blade immediately after being scanned and the slices were extracted 3 times each with a volume of oligo(dT)–cellulose binding buffer equal to the volume of the gel slices at room temperature for 24 h. RNA was recovered from the combined extracts by ethanol precipitation. The samples were dissolved in water, centrifuged to remove particles of agarose, and analyzed by translation either directly or after rebinding to and elution from oligo(dT)–cellulose.

The RNA preparations were tested for biological activity by translations in a cell-free system as described by MacDonald et al. (1977) after nuclease pretreatment according to Pelham & Jackson (1976). In addition, cDNA was prepared from these samples and hybridization analyses were performed as described by Harding et al. (1977).

Results

Our interest in specific pancreatic genes and their expression has led to rather extensive investigations of methods for preparation of biologically functional RNA from tissues rich in RNase. A number of methods were tested; some were useful, but none were found to be completely satisfactory.

An extensive analysis of the hybridization characteristics of rat pancreas RNA and cDNA made from it with reverse transcriptase has been published by Harding et al. (1977). These experiments were conducted with RNA prepared by a precursor to the present procedure, one in which the tissues were homogenized in 7.5 M guanidine hydrochloride plus 1% diethyl pyrocarbonate (Zsindely et al., 1970). This procedure gave undegraded RNA which, however, appeared to be partially modified by the diethyl pyrocarbonate (Ehrenberg et al., 1974). When rat pancreas RNA prepared with guanidine hydrochloride plus diethyl pyrocarbonate was translated in vitro, there was a marked lower efficiency of synthesis of higher molecular weight polypeptides, especially amylase. This effect was much more pronounced in the reticulocyte lysate than in the wheat germ cell-free system of Roberts & Paterson (1973). Attempts to circumvent this effect by scavenging unreacted diethyl pyrocarbonate by adding excess 2-mercaptoethanol 30 s after the start of homogenization were unsuccessful. Decreasing the initial concentration of diethyl pyrocarbonate only resulted in partially degraded RNA. These deleterious effects

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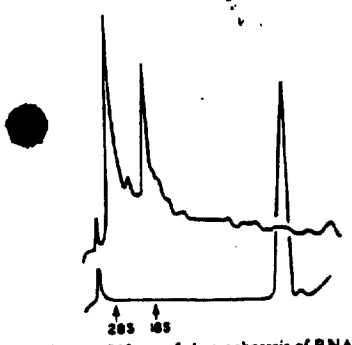

FIGURE 1: Scan at 260 nm of electrophoresis of RNA on 4% acrylamide gel in 98% formamide (Pinder et al., 1974). Direction of migration was from left to right. Upper trace: RNA prepared with guanidinium thiocyanate plus 2-mercaptoethanol by ethanol precipitation. Lower trace: RNA prepared by dropping a pancreas into a blender running at full speed at 4 °C containing 0.1 M sodium acetate, pH 5, 5 mM iodoacetate, 2 mg/mL heparin sulfate, and 0.5% sodium dodecyl sulfate plus an equal volume of buffer-saturated phenol.

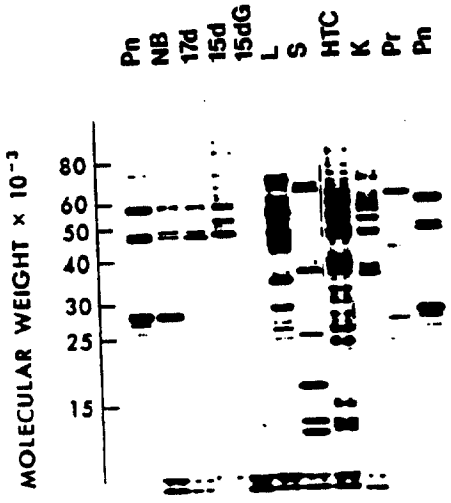

FIGURE 2: Autoradiogram of [^{35}S]methionine-labeled peptides synthesized from purified RNAs in a rabbit reticulocyte lysate (Pelham & Jackson, 1976). Analysis was as described by MacDonald et al. (1977). Total RNA samples (all from rat) were the following: Pn, adult pancreas (24 μg); NB, newborn pancreas (23 μg); 17d, 17-day embryonic pancreas (21 μg); 15d, 15-day embryonic pancreas (21 μg); 15dG, 15-day embryonic gut (22 μg); L, adult liver (40 μg); S, adult submaxillary gland (41 μg); HTC, hepatoma cell line (37 μg); K, adult kidney (27 μg); Pr, adult parotid gland (21 μg).

of diethyl pyrocarbonate led us to develop the improved guanidinium thiocyanate method described here.

The recovery of RNA from adult rat pancreas by the guanidinium thiocyanate procedure (see Experimental Procedure) was ~20 mg/g of wet tissue, varying by 10%. RNA preparations had a A_{260nm}/A_{280nm} ratio of 2.1 or better, indicating low protein contamination. The DNA content was less than 0.5% by the diphenylamine assay (Burton, 1956). Total RNA prepared by this procedure was analyzed by electrophoresis under denaturing conditions in 4% acrylamide gels in 98% formamide (Pinder et al., 1974). Figure 1 shows the absorbance profile of such a gel at 260 nm, demonstrating the

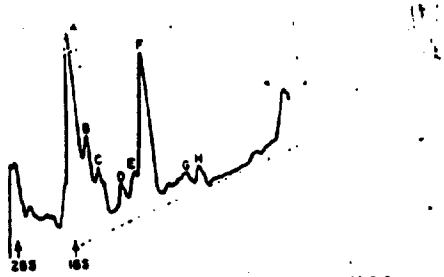

FIGURE 3: Scan at 260 nm of a 3% agarose–6 M urea gel, pH 3.5 (Woo et al., 1975). A 40-μg sample of polyadenylated rat pancreas RNA was run on a 6 × 98 mm gel, which was sliced as indicated.

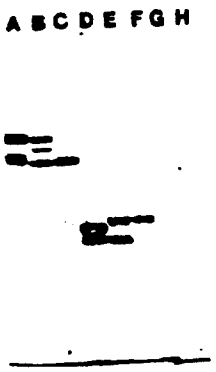

FIGURE 4: Translational specificity of pancreatic mRNAs resolved by agarose gel electrophoresis. Autoradiogram was prepared as in Figure 2. The in vitro translations were performed on the entire extracted aliquots of RNA from the slices indicated in Figure 3, except for bands A and F of which only half of the aliquot was translated. Lane T contains the translation products of unfractionated pancreatic polyadenylated RNA.

presence of intact 28S and 18S ribosomal RNAs in the sample prepared with guanidinium thiocyanate. The lower trace in Figure 1 demonstrates the degradation of these species when isolated by conventional phenol extraction in the presence of a panoply of ribonuclease inhibitors: heparin, iodoacetate, and sodium dodecyl sulfate. Electrophoresis in 3% agarose gels in 6 M urea, pH 3.5 (Woo et al., 1975), gave results (not shown) very similar to those found with formamide gels. Between 1.0 and 1.5% of the total RNA was contained in the polyadenylated fraction after two passages over oligo(dT)–cellulose (Aviv & Leder, 1972).

The products of translation in vitro of RNAs isolated from a variety of rat tissues with guanidinium thiocyanate are displayed in Figure 2. The discrete, tissue-specific products seen for embryonic and adult rat pancreas, liver, kidney, submaxillary, parotid, and HTC cell (a rat hepatoma cell line) RNAs indicate that the guanidinium thiocyanate procedure yields RNA suitable for protein synthesis.

The relatively simple set of proteins synthesized in vitro in Figure 2, lane Pn, suggested that polyadenylated pancreatic RNA should contain a limited number of discrete messages. This was borne out by the profile of oligo(dT)–cellulose-bound RNA on a denaturing agarose–urea gel (Figure 3). When the indicated RNA peaks were eluted from the gel, they were found to be enriched in their template activities for specific polypeptide bands (Figure 4). The major protein band in lane

A has been demonstrated to be a major (58000 molecular weight) of α-amylase by specific immunoprecipitation (MacDonald et al., 1977; Przybyla et al., 1979). The RNA coding for amylase (Figure 3, peak A) comigrated with 18S rRNA. On the basis of the in vitro translation data and cDNA–mRNA hybridization complexity (Harding & Rutter, 1978), the isolated amylase mRNA was judged to be greater than 80% pure.

Since it is obviously not possible in this system to compare the quality of the RNAs prepared by the guanidinium thiocyanate and conventional phenol methods, we have performed comparative experiments using dog pancreas, which lacks detectable levels of RNase A (Zendzian & Barnard, 1967). Total polysomal RNA was prepared by conventional phenol–chloroform extraction by MacDonald et al. (1977). The data obtained for dog pancreas were very similar to those shown in Figures 1–3 for rat pancreas. In both species, α-amylase was the dominant tissue-specific protein and its RNA comigrated with 18S rRNA. The patterns of the translation products and of the polyadenylated RNAs were similar. When copied with reverse transcriptase, rat pancreas RNA gave the highest incorporation of nucleotides per gram when the RNA had been prepared with guanidinium thiocyanate (0.13 nmol of [^{3}H]dCMP per μg of polyadenylated RNA) and the lowest when prepared with guanidine hydrochloride plus diethyl pyrocarbonate (0.06 nmol of [^{3}H]dCMP per μg). In comparison, phenol-prepared dog RNA gave an incorporation of 0.11 nmol of [^{3}H]dCMP per μg. All pancreatic cDNAs displayed the same size distribution on alkaline sucrose gradients.

When phenol-extracted dog pancreas RNA was hybridized in excess to cDNAs made from RNA templates prepared either with phenol or guanidine hydrochloride plus 1% diethyl pyrocarbonate, indistinguishable results were obtained. The curves were very similar to those seen for rat pancreas RNA–cDNA hybridizations (Harding et al., 1977).

Discussion

Because of the high concentrations of RNase and RNA in the rat pancreas, polyanionic competitive inhibitors of RNase such as heparin, polyvinyl sulfate, and macaloid (Parish, 1972) cannot be brought practically to high enough concentrations to be useful. Similar limits to attainable concentration preclude the use of antibodies against or protein inhibitors of RNase (Brown et al., 1959; Gribnau et al., 1969). The well characterized covalent inactivators of bovine pancreatic RNase A such as 3-bromopyruvate and iodoacetate react much too slowly to be of use (Heinrikson et al., 1965). Diethyl pyrocarbonate is an effective active-site histidine reagent against pancreatic RNase, but unfortunately this reagent also modifies nucleic acids (Ehrenberg et al., 1974). Such modification may account for the loss of amylase mRNA translation activity and template activity for RNA-directed DNA polymerase described above. Diethyl pyrocarbonate has been reported to destroy ovalbumin message activity (Palmiter, 1974). As demonstrated by Figure 1, phenol plus sodium dodecyl sulfate does not denature RNase sufficiently rapidly to prevent massive degradation of pancreatic RNA.

Although ribonuclease is the bane of molecular biologists, it has been a boon to physical biochemists. It is a thoroughly investigated model of protein denaturation. The transition state for denaturation of pancreatic RNase A is close to the denatured state, so that reagents of increasing effectiveness for equilibrium denaturation will denature with increasing rapidity (Tanford, 1968). Thus, the half-life of RNase is 3 min in 8 M urea (Barnard, 1964) and 10 s in 4 M guanidine hydrochloride (Miller & Bolen, 1978). Both Von Hippel & Wang (1964) and Castellino & Barker (1968) found that guanidinium thiocyanate was about 2.5-fold more effective on a molar basis than guanidine hydrochloride as an equilibrium denaturant. In the former salt both cation and anion are strong chaotropes, while in the latter only the guanidinium cation is chaotropic and hence active in denaturation (Hencks, 1969). Thus, it was expected on the basis of the rate dependency upon denaturant strength that guanidinium thiocyanate would be a much more rapid denaturant of RNase than guanidine hydrochloride, thus permitting the isolation of intact rat pancreatic RNA without recourse to diethyl pyrocarbonate.

RNA prepared from pancreas with guanidinium thiocyanate can be translated in vitro to give products (Figure 2) very similar to those seen for phenol-isolated dog pancreas RNA (MacDonald et al., 1977) and very similar to the contents of pancreatic secretory granules (Przybyla et al., 1979). Since α-amylase is the major secretory product of the pancreas, its message should be an abundant polyadenylated species. Figures 3 and 4 suggest that this is the case. The 18S polyadenylated RNA (Figure 3, peak A) is the predominant component resolved, although its abundance may be exaggerated by contaminating rRNA. Hybridization between this purified amylase mRNA and the cDNA made from it indicates that the message is more than 80% composed of a sequence of 1500-nucleotide complexity, just large enough to code for the pre-amylase polypeptide (Harding & Rutter, 1978).

In addition to hybridization experiments with purified rat pancreatic amylase mRNA, total rat pancreas RNA from adults and developing embryos has been analyzed by cDNA hybridization kinetics (Harding et al., 1978). In no case is there any indication that isolation with guanidinium thiocyanate plus 2-mercaptoethanol introduces any artifactual modifications into RNA or causes the selective loss of any species other than 4S RNA. Recovery of RNA from the organ was quantitative within the experimental uncertainty of such determinations (Schneider, 1946).

RNA prepared with guanidinium thiocyanate from rat islets of Langerhans has been used to synthesize double-stranded cDNA. When this DNA was inserted into a bacterial plasmid, cloned, and analyzed, it was found to contain nucleotide sequences correctly coding for the complete amino acid sequence of rat proinsulin I (Ullrich et al., 1977), thereby confirming that the information content of the starting RNA was retained during the procedure.

The predicted utility of guanidinium thiocyanate was fully confirmed by the results described above; RNA isolated with the reagent was physically intact and fully active in translation, specific message purification, hybridization, and recombinant DNA experiments. The variety of tissues from which active RNA has been obtained with this method (Figure 2) suggests that the guanidinium thiocyanate procedure offers a useful alternative to phenol-based methods, particularly for nuclease-containing cells.

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Measurement of the Transcription of Nuclear Single-Copy Deoxyribonucleic Acid during Chloroplast Development in *Euglena gracilis*†

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ABSTRACT: The fraction of nuclear single-copy deoxyribonucleic acid (DNA) transcribed at different stages of chloroplast development in *Euglena gracilis* (Z strain) was measured by RNA–DNA hybridization. *Euglena* cells were grown in a heterotrophic medium in the dark to stationary phase and transferred to the light. Total cell RNA was isolated at various stages of chloroplast development and hybridized in a vast excess to ^{125}I-labeled single-copy DNA. The fraction of ^{125}I-labeled single-copy DNA in the form of a duplex was measured by using S1 nuclease. The amount of RNA–DNA hybrid in the duplex mixture was determined by correcting for the contribution of DNA–DNA renaturation. The fraction of single-copy DNA transcribed was calculated by multiplying by 2 the amount of DNA in the form of an RNA–DNA hybrid and correcting for the reactivity of the single-copy DNA probe with total DNA. In dark-grown cells (i.e., prior to the initiation of chloroplast development), the complexity of total cell RNA derived from single-copy DNA was 8.0×10^7 nucleotides. After initiation of chloroplast development, the complexity of the total cell RNA derived from single-copy DNA first increased slightly to 8.9×10^7 nucleotides and then progressively decreased to 7.4×10^7 and 6.4×10^7 nucleotides after 12, 48, and 72 h of exposure to light, respectively. Total cell RNA isolated from cells which had never been cultured in the dark had a complexity of 6.5×10^7 nucleotides.

Chloroplasts are complex organelles which require a multitude of membrane structures, enzymes, and electron-transport constituents to carry out photosynthesis. The development of a functional chloroplast from a proplastid, the progenitor of chloroplasts, presents an interesting example of the need for the coordinate expression and interaction of two distinct genomes within the plant cell. Both the chloroplast and the nuclear DNAs contribute genetic information required for the production of a photosynthetically competent organelle (Schiff,

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Exhibit No. 3

human Epo vs. Phe 91 in monkey Epo). This result points out the extreme specificity of mixed-sequence short oligo probes and the requirement for exact aa sequence data.

Epo obtained from mice, rats, sheep, rabbits, dogs and baboons has been previously shown to compete with human Epo for binding to anti-human Epo antibodies (Garcia et al., 1979). It is also well known that human Epo is biologically active in mice and rats (Anagnoston et al., 1978; Miyake et al., 1977). These findings imply that some functional domain(s) and antigenic determinant(s) of Epo have been highly conserved during the course of mammalian evolution. With the exception of aa 28–35 and 83–123, the aa sequences of monkey and human Epo are highly conserved. The conserved regions may be involved in determining the function and antigenic determinants of the Epo molecule.

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