others) 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.

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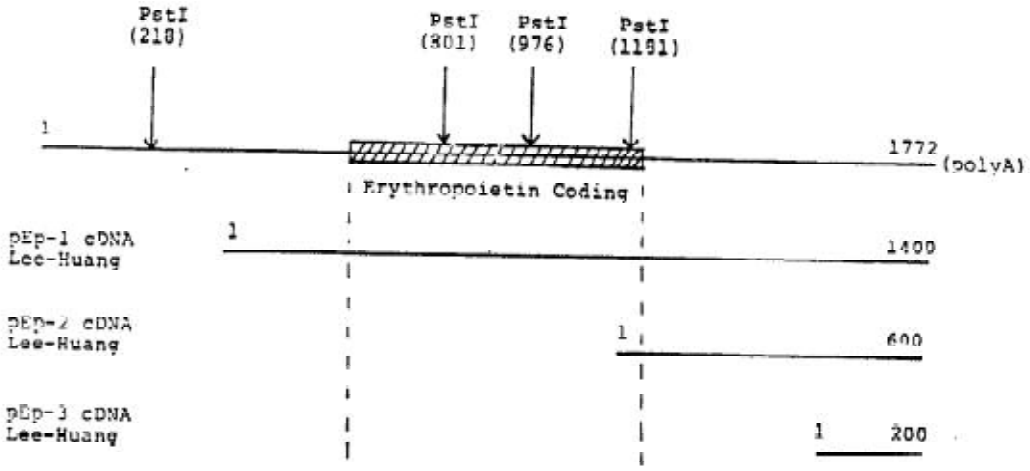
With this information in hand concerning the size and sequence of the mRNA reverse transcript (cDNA) isolated by both Applicant and independent investigators, analysis of Figure 5 of the Lee-Huang publication reveals that the 1400, 600, and 200 base pair cDNA sequences most likely do not encode human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



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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 of. 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

* 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.

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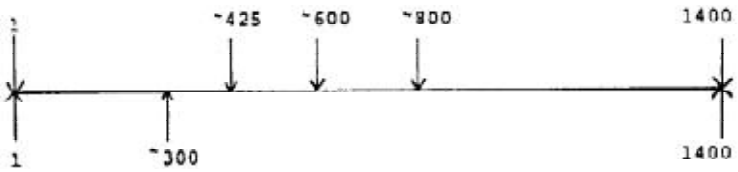
experiments, however, dictates that at least three PstI recognition sites should be present in a 1400 base pair, erythropoietin-encoding cDNA insert. Had the 1400 base pair cDNA insert in Lee-Huang's plasmid pEp1 been an erythropoietin-encoding DNA, then a total of five fragments would be expected upon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

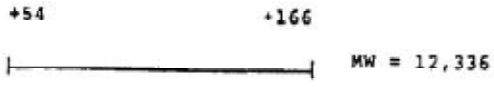
As indicated to the Examiner during the course of the recent interview, the carbohydrate-free, in vitro mRNA translation products described by Lee-Huang do not "correspond" in terms of molecular weight to products which would be expected to be obtained upon translation of an mRNA encoding human erythropoietin. 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 translation 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_r 29000 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

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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

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believed to be relevant to patentability of the claimed invention.

CONCLUSION

The foregoing amendments and remarks are believed 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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