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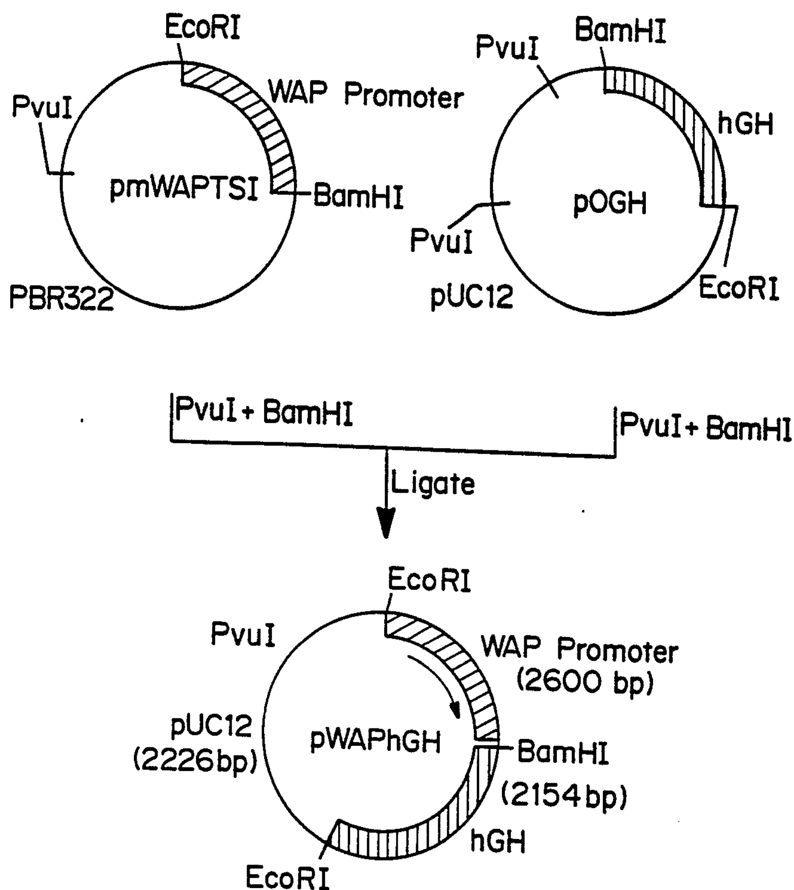
(54) Title: PRODUCTION OF GROWTH HORMONE IN TRANSGENIC ANIMAL MILK

(57) Abstract

DNA coding for human growth hormone was linked to mouse whey acid protein promoter fragment and microinjected into fertilized mouse ova. Females of the resulting transgenic mice were mated. After completion of gestation and birth of the litter, the milk from the mothers was assayed and found to contain human growth hormone.



* See back of page

DESIGNATIONS OF "DE"

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PRODUCTION OF GROWTH HORMONE IN TRANSGENIC ANIMAL MILK

Background of the Invention

5 This generally relates to the production of growth hormone in the milk of transgenic mammals.

Human growth hormone (hGH) is one member of the cascade of hormones responsible for normal growth in vertebrates. The cascade is initiated when, in response to neurological stimulation, 10 the hypothalmus is induced to release either a positive growth factor called growth hormone releasing factor (GHRF), or a negative factor, called somatostatin. GHRF stimulates the pituitary to release growth hormone (GH), which in turn acts on the liver to produce insulin-like growth factor I. This in turn binds to receptors on the cells of 15 peripheral tissue to modulate growth. Somatostatin acts on the pituitary to inhibit release of growth horomone.

In normal humans this cascade effectively modulates growth during childhood, usually resulting in adults of normal stature. However, there at least are two cases in which normal statures are not 20 attained. In one case, the short children are deficient in endogenous GH, probably as a result of some genetic defect. Administration of exogenous GH is effective in overcoming this deficiency in most of these individuals. In the other case, children of short stature have normal levels of endogenous GH, and thus are probably somewhat 25 resistant to the effects of exogenous GH. Although one might expect treatment involving administering exogenous hGH to be useless in these individuals, a study done at Emory University, reported by Shiner, G., Research Resources Reporter, U.S. Dept. Health and Human Services, vol. IV, pg 1-5 (1980), demonstrated that about 30% 30 of these children are responsive to exogenous hGH treatment. After this study was conducted, Rudman, et al, reported in Journal of Clinical Endocrinology and Metabolism, 49, 92-99 (1979), that the endogenous GH in the subset of short stature children who were responsive to the exogenous GH was defective in its ability to bind 35 GH receptors. This study effectively enlarged the population of short stature children who could be helped by hGH treatment.

Besides its use as a treatment for short stature in some children, new evidence has emerged which suggests a role for GH in immunoregulation. Kelsey, et al, reported in Nucleic Acids Research 15, 1459-1474 (1987), that GH can stimulate macrophages to produce
5 more than double the normal amounts of superoxide anion (O^{2-}) in rats. Superoxide anion is one of the intermediates responsible for intracellular killing of pathogenic microbes by macrophages, a function that is also carried out by interferons. Since macrophages are central to the induction and expression of many immune responses, the
10 discovery that GH acts on these macrophages in this way could lead to the discovery of other important macrophage activating properties of GH.

Other potential clinical applications of hGH include use in enhanced healing of wounds, cartilage damage and fractures, and
15 treatment of burn trauma, stress ulcers, hypercholesterolemia and osteoporosis.

Because growth hormone is species specific, hGH has been available only in limited quantities as a purification product from the pituitaries of human cadavers. Although the recent cloning and the
20 expression of this cloned gene in bacteria has increased availability of hGH, as first reported by Martial, et al., Science 205, 602-606 (1979), the relatively low yield and purification difficulties have caused the price of hGH treatment to be between $8,000 and $30,000 per patient per year. Clearly a cheaper, more efficient way of producing hGH
25 with higher yield would be beneficial both for patients and for use in the initiation of new studies to test for additional properties of hGH.

A proposed alternative method of production of GH is through expression in transgenic animals. Unfortunately, expression of the hormone in transgenic animals incorporating the gene for growth
30 hormone has had a number of unexpected side effects. For example, in pigs containing the gene for bGH in combination with an inducible metallothionein promoter, as described by Ramabhadran, et al., in

Gene 38, 111-118 (1985), the animals suffered from severe early onset rheumatoid arthritis. Transgenic mice having the gene for hGH fused with mouse metallothionein I promoter were infertile, as reported by A. Bartke, et al., J.Experimental Zoo. 248, 121-124 (1988). See also Kyung-Kwang, et al., Korean J. Anim. Sci. 31(3), 139-147 (1989).

One way to avoid the systemic effects and increase purification yield is to create transgenic animals incorporating the gene for GH in combination with a tissue specific promoter, for example, a cost effective alternative to production of recombinant hGH in bacteria would be its production in the milk of transgenic farm animals. By attaching the gene of interest to a tissue specific promoter for a highly expressed gene product, one can achieve specific expression of the gene of interest in tissues appropriate to the regulatory sequences. Some of the methodologies for making tissue specific sequences, and the problems associated with it, such as the lack of correlation between expression in cell culture in vitro and in vivo expression and the effect of regulatory proteins normally expressed by the targeted tissues, are discussed by S.A. Camper in Biotechniques 5(7), 638, 641-643 (1987).

Despite the problems, the production of foreign proteins in transgenic animals is an attractive alternative to bacterial or tissue culture fermentation as a means of producing large amounts of recombinant proteins. Successes have been reported, including the production of human alpha-1-anti-trypsin in mouse and sheep serum by Kelsey, et al. (1987), as well as the production of sheep beta-lactoglobulin and human t-PA in mouse milk by Simons, et al., Nature 328, 530-533 (1987) and Gordon, et al., Biotechnology 5, 1183-1187 (1987). Some proteins are present in milk at concentrations as high as 16 grams per liter, as reported by Clark, et al., Trends in Biotechnology, 5, 20-24 (1987).

It is impossible to predict whether it is possible to mimic these high levels by placing the hGH gene under control regions for

milk proteins which are selectively expressed in mammary tissues. However, even at 10% efficiency the expression levels could be as high as 1.6 grams per liter, which is significantly higher than current production levels in either bacteria or mammalian cell systems.

5 It is therefore an object of the present invention to provide transgenic animals capable of tissue specific expression of growth hormone, especially human growth hormone.

It is a further object of the invention to provide transgenic animals which stably transmit the gene for expression of growth
10 hormone in their milk.

It is a still further obect of the invention to provide vectors and regulatory sequences for expression of growth hormone, especially human growth hormone, for use in creating transgenic animals capable of tissue specific expression of the growth hormone.

15

Summary of the Invention

DNA coding for human growth hormone (hGH) was linked to mouse whey acid protein promoter fragment and microinjected into fertilized mouse ova. Females of the resulting transgenic mice were
20 mated. After completion of gestation and birth of the litter, the milk from the mothers was assayed and found to contain hGH protein.

Brief Description of the Drawings

Figure 1 is a schematic of the construction of the
25 pWAPhGH fusion vector, containing the WAP tissue specific promoter in combination with the gene for hGh.

Detailed Description of the Invention

The construction of transgenic mice expressing human
30 growth hormone in their mammary glands which can be isolated and purified for use as a pharmaceutical is described in detail below can, with minor variations, be used to incorporate the same genes and

tissue specific promoters into animals of others species, such as rats, rabbits, pigs, sheep, and cows, for expression and purification of human growth hormone. Similarly, genes for growth hormones of other origin, such as bovine or porcine growth hormone, can be incorporated into similar vectors and inserted into the genome of the desired species.

The production of the growth hormone in the transgenic animals has a number of advantages, including normal glycosylation and absence of bacterial contaminants, unlike recombinant growth hormone produced by bacterial fermentation processes.

Experimental Design and Methods:

Vector Construction:

pmWAPTSI, containing EcoRI-BamHI fragment of mouse WAP promoter obtained from Dr. Lothar Hennighausen and described by Pittices, et al., in Proc.Natl.Acad.Sci. 85, 5874-5878 (1988), was cut with PvuI and BamHI and ligated to pOGH cleaved with PvuI and BamHI. pOGH contains the DNA sequences coding for hGH and its polyadenylation signal. The resulting plasmid pWAPhGH was isolated after transformation into E. coli using the method of Maniatis, et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY 1982) and screening with appropriate enzymes such as EcoRI, BamHI, SmaI, SphI, and XhoI. The results are shown in Figure 1.

Preparation of DNA for microinjection:

pWAPhGH was digested with EcoRI and the 4754 bp fragment containing the WAPhGH fusion gene was isolated on 1% agarose gel followed by electroelution in a dialysis bag, as described by Maniatis, et al. (1982). The eluted DNA was precipitated, redissolved in water and purified by passing through an elutip-D column as per the instructions of the manufacturer (Schleicher and

Schuell, Inc., Keene, NH). The purified DNA was dissolved in 5mM Tris (pH 7.4) and 0.1 mM EDTA at 3 μg/ml concentration for microinjection.

5 **Animals and embryos:**

Mice were obtained from Charles River Laboratories, Boston, MA and Jackson Laboratories, Maine. Reagents such as bovine serum albumin, gelatin, and pronase were obtained from Sigma Chemical Co., St. Louis, MO. Hormones for superovulation, PMS and 10 hCG, were obtained from Organon, Inc., NJ. Hyaluronidase was purchased from Sigma. Restriction enzymes were obtained from Biolabs, Beverly, MA. The micromanipulator made by Nara Shige, USA, Inc., Rainin Instruments Co., Woburn, MA, was used to microinject DNA into the pronuclei. DMEM, fetal bovine serum, and 15 DPBS were supplied by GIBCO Laboratories, Gaithersville, MD.

Procedures for embryo manipulation and microinjection are described in "Manipulating the Mouse Embryo" by B. Hogan, F. Costantini and E. Lacy (Cold Spring Harbor Laboratory, 1986). Mouse zygotes were collected from six week old females that have 20 been superovulated with pregnant mares serum (PMS) follwed 48 hours later with human chorionic gonadotropin. Primed females were placed with males and checked for vaginal plugs on the following morning. Pseudopregnant females were selected for estrus, placed with proven sterile vasectomized males and used as recipients. 25 Zygotes were collected and cumulus cells removed by treatment with hyaluronidase (1 mg/ml).

Pronuclear embryos were recovered from B6D2 female mice mated to CDI males. Females were treated with pregnant mare serum, PMS, (5 IU) to induce follicular growth and human chorionic 30 gonadotropin, hCG (51 U) to induce ovulation. Embryos were recovered in a Dulbecco's modified phosphate buffered saline (DPBS)

and maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum.

Microinjection:

5 Microinjections were performed using Narishige micromanipulators attached to a Nikon diaphot microscope. Embryos were held in 100 microliter drops of DPBS under oil while being microinjected. DNA solution was microinjected into the largest visible male pronucleus. Successful injection was monitored by swelling of
10 the pronucleus.

Embryo transfer:

Immediately after injection embryos were transferred to recipient females, mature CDI mice mated to vasectomized male CD
15 mice. Recipient females were anesthetized using 2,2,2-tribromoethanol. Paralumbar females were made to expose the oviducts and the embryos were transformed into the ampullary region of the oviducts. The body wall was sutured and the skin closed with wound clips. Recipients were appropriately ear notched for
20 identification and maintained until parturition.

Sampling for DNA integration:

At three weeks of age about 2-3 cm long tail samples were excised for DNA analysis. The tail samples were digested by
25 incubating overnight at 55°C nutator in the presence of 0.7 ml 50 mM Tris, pH 8.0, 100 mM EDTA, 0.5% SDS and 350 μg of prokienase K. The digested material was extracted once with equal volume of phenol and once with equal volume of phenol:chloroform (1:1 mixture). The supernatants were mixed with 70 μl 3 M sodium acetate (pH 6.0) and
30 the DNAs were precipitated by adding equal volume of 100% ethanol. The DNAs were spun down in a microfuge, washed once with 70% ethanol, dried and dissolved in 100 μL TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA). 10 to 20 μl of DNAs were cut with <u>Bam</u>HI and

Bgl11 or EcoRI, electrophoresed on 1% agarose gels, blotted onto nitrocellulose paper and hybridized with ^{32}P-labeled hGH DNA sequences. Transgenic animals were identified by autoradiography.

5 **Propagation of transgenic mice:**

At five weeks of age transgenic female mice were mated to CDI males. At five days following parturition milk samples were taken and assayed for hGH. At six to seven weeks of age transgenic males were mated to two CDI females. The Fl litters were analyzed
10 for transgene. Four positive females were kept and mated at five weeks of age. At five days following parturition milk samples were assayed for hGH.

Collection of milk:

15 Milk samples (50-200 μl) were collected from anesthetized mice injected with 0.05 units of oxytocin, an inducer of lactation. The milk was collected in a glass capillary with the aid of mammary palpation.

20 **Radioimmunoassay:**

Human growth hormone produced in the mouse milk was assayed by an RIA kit available commercially from Nichols Institute Diagnostics, SanJuan Capistrano, CA.

25 After successful microinjection of DNA into 720 embryos, 69 live offspring were born. Fourteen of these, four males and ten females, were found to be transgenic and carrying different number of copies of WAPhGH. The females were mated and, after parturition, their milk samples were collected and assayed for hGH. The assay
30 results are tabulated as follows:

Table 1: Expression of hGH in milk of Transgenic Mice.

Transgenic female	hGH in milk (ng/ml)
Control nontransgenic mouse	0
10	<1
11	0
14	<1
25	525
26	<1
27	970
35	<5

5 Mouse #27 is producing hGH at the rate of 970 ng/ml (970 μg/liter) in its milk.

Stable lines of transgenic animals expression hGH in their milk are produced by mating the females expressing the gGH in their milk at the highest levels and by mating the offspring of the transgenic 10 males.

Despite the relatively high cost of generating these transgenic animals, scale-up costs are relatively low. In addition to conventional breeding as a means of proliferating these production animals, artificial insemination and embryo transfer techniques can be 15 employed to increase the number available for production purposes.

Modifications and variations of the present invention will be obvious to those skilled in the art from the foregoing detailed description of the invention. Such modifications and variations are intended to come within the scope of the appended claims.

20 We claim.

1. A transgenic mammal having incorporated into its genome the gene encoding growth hormone and a mammary tissue specific promoter, said gene expressed specifically by the mammary glands of a lactating female transgenic mammal.

2. The transgenic mammal of claim 1 wherein the mammal is selected from the group consisting of mice, rats, rabbits, sheep, pigs, and cattle.

3. The transgenic mammal of claim 1 wherein the growth hormone is human.

4. The transgenic mammal of claim 1 wherein the promoter is the Whey acid protein promoter.

5. The transgenic mammal of claim 1 wherein the mammal is a mouse having incorporated into its genome a gene for human growth hormone that is produced in the milk of a lactating mouse at levels of approximately 50 ng hGH/ml of milk or greater.

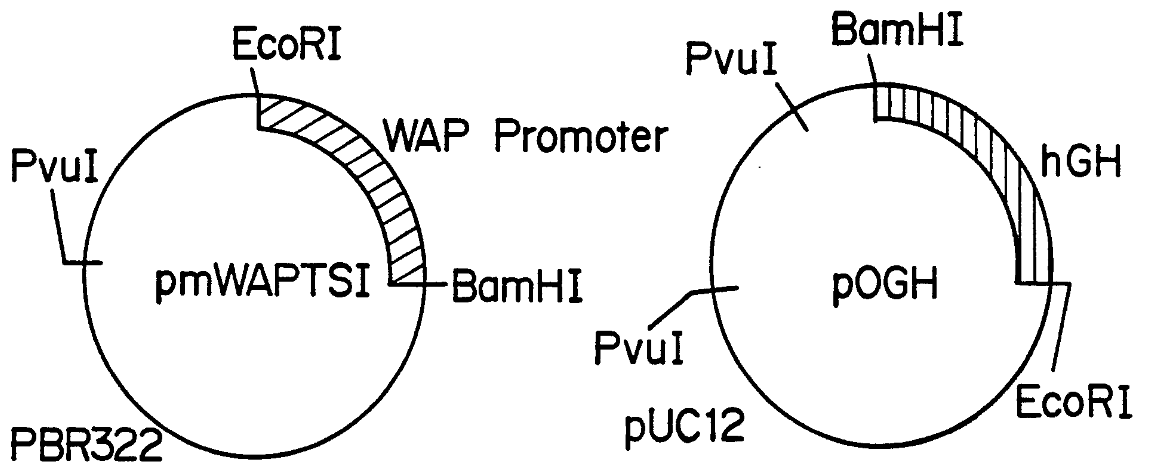
6. A method for making a transgenic mammal having incorporated into its genome the gene encoding growth hormone and a mammary tissue specific promoter, said gene expressed specifically by the mammary glands of a lactating female transgenic mammal comprising providing a vector containing the WAP promoter in phase with nucleotide sequence encoding growth hormone.

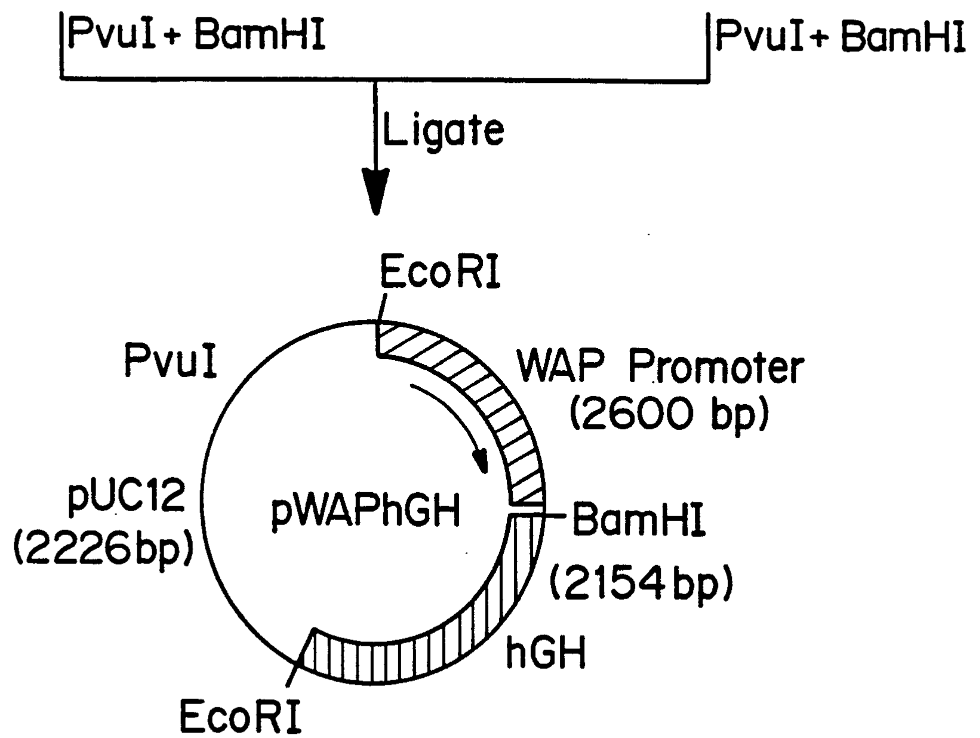
7. The method of claim 6 further comprising microinjecting the vector into the embryo of a mammal is selected from the group consisting of mice, rats, rabbits, sheep, pigs, and cattle.

8. The method of claim 6 wherein the growth hormone is human.

9. The method of claim 7 further comprising testing the animals for production of growth hormone in the milk of lactating females and mating the animals containing the highest levels of growth hormone in the milk.

FIGURE 1





INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/05130

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

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

IPC(5): C12N 15/00
U.S.Cl.: 800/2; 435/172.3

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	800/2, DIG 1 435/172.3,317.1,69.1 935/13,111

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Databases: DIALOG (Files 154,55,311,312), USPTO Automated Patent System (Files USPAT). See attachment for search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y,P	US, A, 4,873,316 (MEADE ET AL.) 10 October 1989, see the entire document and especially column 3, lines 30-40.	1-9
Y	Genes and Development, Volume 1, Issued 1987 (Cold Spring Harbor, USA), Pinkert et al., "An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice", pages 268-276, see the entire document.	1-9
Y	Proceedings of the National Academy of Sciences, Volume 85, Issued December 1988 (Washington, USA), Sweetser et al., "Transgenic mice containing intestinal fatty acid-binding protein-human growth hormone fusion genes exhibit correct regional and cell-specific expression of the reporter gene in their small intestine", pages 9611-9615, see the entire document.	1-9

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

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

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

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

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
29 October 1990	23 JAN 1991
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Jasemine C. Chambers Jasemine C. Chambers

Form PCT/ISA/210 (second sheet) (May 1986)

International Application No. PCT/US90/05130

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Proceedings of the National Academy of Sciences, Volume 85, Issued August 1988 (Washington, USA), Pittius et al., "A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice", pages 5874-5878, see the entire document.	1-9

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

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

1. ☐ Claim numbers , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers__________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International Application No. PCT/US90/05130

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	Proceedings of the National Academy of Sciences, Volume 84, Issued March 1987 (Washington, USA), Andres et al., "Ha-ras oncogene expression directed by a milk protein gene promoter: tissue specificity, hormonal regulation, and tumor induction in transgenic mice", pages 1299-1303, see the entire document.	1-9
Y	Bio/Technology, Volume 5, Issued November 1987 (Clinton, USA), Gordon et al., Production of human tissue plasminogen activator in transgenic mouse milk", pages 1183-1187, see the entire document.	1-9
Y	WO, A, 88/01648 (HOPP) 10 March 1988, see the entire document.	1-9

PCT/US90/05130

Attachment to PCT/ISA/210, Part II.

II. FIELDS SEARCHED SEARCH TERMS:

transgenic, milk, growth hormone, mice, human, tissue specific, secret, WAP, inventors' names