COMPARISON OF ECTOPIC HUMAN PROLACTIN GENE EXPRESSION BY THE IM-9-P B-LYMPHOBLASTOID CELL LINE TO THAT OF THE NORMAL PITUITARY AND UTERINE DECIDUA

BY

GABRIEL E. DiMATTIA

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

Department of Physiology
Faculty of Medicine
University of Manitoba
Winnipeg, Canada
May 1990

Copyright © Gabriel E. DiMattia, May 1990

National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format; making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-63379-4

Canada

COMPARISON OF ECTOPIC HUMAN PROLACTIN GENE EXPRESSION BY THE IM-9-P B-LYMPHOBLASTOID CELL LINE TO THAT OF THE NORMAL PITUITARY AND UTERINE DECIDUA

BY

GABRIEL E. DIMATTIA

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

© 1990

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this thesis, to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film, and UNIVERSITY MICROFILMS to publish an abstract of this thesis.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

To my wife, Jacqueline
and to my daughter, Alessandra

"Love expands: it not only sees more
and enfolds more, it causes its
objects to bloom."

H. Prather, 1977

ACKNOWLEDGEMENT

The past 4 1/2 years in Winnipeg have been a tremendous growth experience for me. The person who most influenced that experience is Dr. Henry G. Friesen, my supervisor. Through his extraordinary insight and sharp intellect I have been taught not only the principles of good scientific research but also much about the old values: unselfishness, loyalty, honesty and courage -- those qualities that traditionally have been thought to be the elements of character in a good person. Above all he has taught me that there is another way to go through life besides being pulled through it kicking and screaming. I am deeply grateful for his kindness and support over the years.

Dr. Mary Lynn Duckworth has been a constant source of knowledge and encouragement without which I would have never succeeded. Day after day she had the patience to deal with the fact that no matter how good things became, my capacity to make myself unhappy was always equal to it! Surprisingly she endured my often irascible nature and I feel very fortunate and enriched to have had the opportunity to work with an individual of such high calibre.

I am certainly indebted to many present and former members of Dr. Friesen's laboratory for their advice and generous friendship, especially Drs. Paul Murphy and Birgit Gellersen without whom this thesis would have been somewhat thinner. I am also very appreciative of the many encouraging words from, and stimulating conversations with, Drs. R. Matusik and R. Shiu. Special thanks to Dr. Jim Davie for having done the most incredibly thorough critique of a thesis that we naively thought was "perfect"!

To a very close and dear friend, Dr. Liisa Selin, I owe an enduring debt of thanks. A friend through the good and bad times, always generous, supportive, and too damn honest about my mildly abrasive character . You helped keep me on track.

Finally, my mind is blank. I have no eloquent prose nor are there words, only the actions of my heart, with which to express my eternal gratitude to my wife, Jacqueline. She typed this thesis with a vigor and sense of purpose that can only be rewarded with co-authorship. Thank you Jacqueline for all that is wonderful and good in my life.

This page would not be complete without some mention of my joy, Alessandra. You made completing this thesis an even greater challenge than I had imagined but you have brought a joy and completeness to my life that I never dreamed.

ABSTRACT

Prolactin (PRL) gene activity exhibits a high degree of cell specificity being transcribed in the anterior pituitary lactotrope and the decidualized stromal cell of the primate endometrium. The production of protein hormones by cells other than those of the gland commonly associated with that hormone can provide unique insights into previously unknown functions of various hormones. In an effort to identify expression of PRL or PRL-like genes in other tissues and cell lines, PRL expression was discovered in the B-lymphoblastoid cell line, IM-9. Interestingly, this phenotype was found to be unique to the cells in this laboratory and thus the PRL-producing line was designated IM-9-P. These cells were a variant subline of the classical IM-9 line as determined by HLA-typing, and analysis of immunoglobulin gene rearrangement. Immunophenotyping had indicated that cell lines of the IM-9-P class had undergone significant genotypic changes which may have contributed to the ectopic activation of the PRL gene. The clonal IM-9-P3 line was found to secrete 40-50ng PRL/10^6cells/24hr which was found to be indistinguishable from its counterpart normally produced in the pituitary based on immunological reactivity, bioactivity and electrophoretic analysis. The PRL secreted by IM-9-P cells did not act in an autocrine fashion and therefore was not important for the growth and viability of these cells.

IM-9-P3 PRL mRNA was approximately 150 nucleotides longer than its pituitary counterpart but identical in size to the decidual PRL message. Sequencing of IM-9-P and decidua PRL cDNAs demonstrated an identical elongated 5' untranslated region (UTR). The IM-9-P/decidua PRL 5' UTR extended 41 nucleotides upstream of the normal transcription start-site for pituitary hPRL. This was preceded by an additional unique sequence of up to 93 bases which was not homologous to the published DNA sequence found 5' to the pituitary hPRL cap site. This elongation of the 5' end was confirmed by primer extension experiments and identical multiple start sites on the IM-9-P and decidua

PRL gene were found which produced predominant 5' UTRs of 140-178 nucleotides. Extensive genomic Southern blot hybridization analysis indicated that the IM-9-P PRL gene was intact and that the unique IM-9-P/decidua 5' UTR was localized as a new 5' non-coding exon about 5.9kb upstream of the pituitary-specific cap site. It is likely that new *cis*-acting elements and corresponding nuclear DNA-binding proteins function in the vicinity of the new 5' non-coding exon 1a and thus constitute a novel mechanism conferring decidual and lymphoblast PRL expression. Cell lines of the IM-9-P class therefore represent a unique and easily manageable model system with which to compare and contrast the molecular mechanism of ectopic PRL expression with that of the lactrotrope and uterine decidual cell.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

REAGENTS:

BSA	bovine serum albumin
CM	conditioned culture medium
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
RNase	ribonuclease
DTT	dithiothreitol
FCS	fetal calf serum
HS	horse serum
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
ETOH	ethanol
IPTG	isopropylthiogalactoside
SDS	sodium dodecyl sulfate
PBS	phosphate-buffered saline
Xgal	5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside
Con-A	Concanavalin-A

BIOLOGICAL FACTORS AND PREFIXES:

GH	growth hormone
PRL	prolactin
PL	placental lactogen
PLP-A	prolactin-like protein A
PLP-B	prolactin-like protein-B
PLII	placental lactogen II
IL	interleukin
Ig	immunoglobulin
h	human
r	rat
TF	transcription factor
EBV	Epstein-Barr virus
POMC	proopiomelanocortin
ACTH	adrenocorticotropic hormone
CRF	corticotropin releasing factor
TSH	thyroid stimulating hormone
GRF	growth hormone releasing factor
CG	chorionic gonadotropin
LH	luteinizing hormone
VIP	vasoactive intestinal peptide

o	ovine	**cAMP**	cyclic adenosine 3',5'-mono-
d	decidual		phosphate

UNITS OF MEASURE:

A	Absorbance	**vol/vol**	volume per volume
"n"C	degrees centigrade	**ml**	millilitre
g	gravity	**µl**	microlitre
EC_{50}	50% effective concentration	**gm**	gram
M.W.	molecular weight	**mg**	milligram
min	minute(s)	**µg**	microgram
hr	hour(s)	**ng**	nanogram
bp	basepair(s)	**M**	molar
kb	kilobase(s)	**µM**	micromolar
cpm	counts per minute	**Ci**	curie
%	percent	**µCi**	microcurie
mm	millimeter	**rpm**	revolutions per minute
cm	centimeter		

MISCELLANEOUS:

G	deoxyguanosine
A	deoxyadenosine
T	deoxythymidine
C	deoxycytidine
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
cDNA	complementary DNA
RNA	ribonucleic acid
cRNA	complementary ribonucleic acid
hnRNA	heterogeneous nuclear RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
dNTP	deoxyribonucleotide triphosphate
poly(A)	containing polyadenylic acid tail

UTR	untranslated region
PAGE	polyacrylamide gel electrophoresis
RIA	radioimmunoassay
NIAMDD	National Institute of Arthritis, Metabolism and Digestive Diseases
NIADDK	National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases
ATCC	American Type Culture Collection
HPLC	high pressure liquid chromatography

LIST OF FIGURES

INTRODUCTION

Preamble

The work presented in this thesis impinges on a variety of diverse subject matters in cell biology and for the sake of coherence and lucidity a preliminary explanation of the various introductory sections is necessary. The objective of the research presented here was to characterize a human B-lymphoblastoid cell line, IM-9-P3, that spontaneously produced PRL. As a logical extension, the molecular mechanism by which this event occurred was investigated and compared to the molecular features of normal PRL expression in the pituitary lactotrope and uterine decidual cell. It became evident that the ectopically produced lymphoblast PRL transcript was identical to the message transcribed in uterine decidual cells, thus the IM-9-P3 line constituted a convenient *in vitro* cell system to study decidual-specific PRL transcription.

To properly prepare the reader for the implications of these results, the "Introduction" is composed of sections dealing with PRL physiology and secondly with molecular mechanisms of eukaryotic cell-specific gene transcription. The "Introduction" begins with a brief overview of general PRL biology. This is followed by a more extensive review of the current literature on human decidual PRL which is appropriate in view of the results presented here which show that IM-9-P3 PRL gene transcription is similar to the decidual mode of transcription rather than mechanism of pituitary PRL expression. A short section on ectopic hPRL secretion is included to place in perspective the discovery that a human lympohoid cell line produces PRL inappropriately. Next a description of the rapidly expanding field of hormone and immune system interaction seemed pertinent given the fact that IM-9-P3 cells are of B-cell origin and could reflect an authentic *in vivo* function of leukocytes. In that section a review of the literature on

production of classical hormones by cells of the immune system is featured as well as work on the effect of lymphokines on pituitary function. This section would not be complete without a discussion of the recently described putative immunomodulatory properties of PRL.

The second half of the "Introduction" deals with the molecular biology of cell-specific expression since production of hPRL by IM-9-P3 cells appears to be an example of how the molecular constraints on cell-specificity have gone awry. Central to the discussion of tissue-specificity is the identification of DNA elements which bind cell-specific transcription factors. A brief introduction, therefore, into the basal *cis*-acting DNA elements of gene transcription is presented followed by examples of how alternative *cis*-acting promoters and RNA splicing can facilitate cell-specific gene expression. The latter subject is directly related to this thesis since IM-9-P3 and decidual PRL gene transcription occur at identical locations far removed from the site of pituitary-specific PRL transcription initiation. A brief description of how the structural conformation of chromatin affects cell-specific gene expression is included to complete this section. The last part of the "Introduction" deals with the current knowledge of the molecular mechanisms of lactotrope PRL gene transcription in rat and human so as to provide a foundation for comparison to the unique features of IM-9-P3/decidua-specific PRL gene expression presented in this thesis.

I trust that these prefatory statements adequately explain the rationale behind the various topics presented in the following "Introduction".

Prolactin: A Brief Overview

Fundamental to the definition of a mammal is the ability to provide nourishment to its newborn young in the form of milk produced and released from a mammary gland.

Clearly, from an evolutionary perspective such a biological function is critical to the perpetuation of a mammalian species and any biological factor essential to maintain lactation must necessarily be considered indispensable in the wild. It is now well-established that one of those mammotropic factors is prolactin (PRL), the existence of which was first implied by the work of Striker and Greuter (1) in 1928 when they showed that crude pituitary extracts initiated lactation in pseudopregnant female rabbits. The term prolactin, however, was given to this pituitary lactogenic principle in 1932 by Riddle, Bates and Dykshorn (2,3) who were the first to demonstrate prolactin as an entity separate from that of the growth and gonad stimulatory activities of the pituitary (4). At that time the preferred assay system involved the differentiation and proliferation of the crop sac mucosal epithelium in pigeons and doves. The crop sac is an organ of food storage located between the oral cavity and stomach of birds. The greatly thickened epithelium actually sloughs off and is the primary constituent of "crop milk" used to feed the hatchlings. The assay simply involved weighing of crop sacs after birds had been injected intramuscularly once daily for four days with the test material presumably containing PRL . The pigeon crop assay is still used as one means of calibrating international standards of human PRL (hPRL) (5). A second assay employed by these investigators involved injection of pseudopregnant rabbits or hysterectomized pregnant guinea pigs for a period of seven days. The condition of the mammary gland was then subjectively assessed with regard to the amount of milk in the glands. At that time the pigeon crop assay was considered more reliable and quantitative than the mammary gland assay and became the method of choice.

The purification of PRL from the human pituitary was not accomplished until the early 1970's, essentially simultaneously by two groups (6,7). One of the major difficulties in obtaining a purified preparation of hPRL was contamination by the structurally related and abundant pituitary growth hormone (GH) at a time when the prevailing view was that hGH functioned as the hPRL . This concept was reasonable as primate GHs uniquely among vertebrates also possess lactogenic properties. Moreover,

because of the structural similarity of hGH and hPRL and the greater abundance of hGH in the pituitary, separation of the two hormones proved elusive until a more discriminating immunological technique was applied. The development of a radioimmunoassay for hPRL in 1971 (8) proved to be a pivotal breakthrough and catalyst for the subsequent heightened interest in PRL biology and the hitherto unsuspected clinical importance of PRL was soon established.

In 1977 Shome and Parlow (9) reported the entire amino acid sequence of human PRL as a single polypeptide consisting of 198 amino acids. Then, in 1981, Cooke and colleagues published the predicted amino acid sequence of hPRL as deduced from its cloned complementary DNA (cDNA) sequence (10). They showed that the human pre-prolactin molecule consists of 227 amino acids (25,880 M.W.) of which 28 amino acids constitute the hydrophobic signal sequence required for secretion into the lumen of the endoplasmic reticulum. The corrected sequence of the mature peptide is 199 amino acids long and presence of six half cysteines allows putative folding of the molecule into three intra-molecular loops in series stabilized by the disulfide bridges.

In serum and from pituitary gland extracts hPRL occurs in three distinct forms, namely the monomeric form, "big" prolactin and "big-big" prolactin which appeared in protein fractions after gel filtration corresponding to a Mr of 45,000 and greater than 100,000 daltons, respectively (11,12). These larger forms of hPRL likely represent polymers of the monomeric form which may or may not be associated with other unknown proteins. The treatment of these aggregates of hPRL with reducing agents (ß-mercaptoethanol) results in the resolution of only monomeric PRL (13,14). Other variants of prolactin in human and rodent species have been described (15) and these not only include the larger forms but also smaller immunoreactive peptides of Mr 21,000 (16) as well as 16,000 and 8,000 M.W. (17). The physiological relevance of these smaller variants was put into question, however, by the recent discovery that they may arise via a preparative artifact involving a non-specific acid protease cleavage of monomeric PRL

(18). There is also evidence that rat PRL can be phosphorylated *in vivo* to a small degree (19), the physiological significance of which has yet to be determined.

The best documented variant of prolactin is a glycosylated form which is found in ovine, porcine, rodent and human pituitary glands (20). Glycosylated ovine PRL was the first to be characterized and was found to contain a carbohydrate unit at asparagine 31 linked through an N-acetylglucosamine (21). The amino acid sequence of hPRL contains the same potential glycosylation signal at asparagine 31 and is likely to contain the sugar moiety at that position. Markoff and Lee (22) showed that in humans the predominant form of PRL in serum is glycosylated and, interestingly, this post-translational modification reduces the bioactivity of the hormone in various assays (23). To date no particular physiological property has been ascribed to the glycosylated variant, although it appears to diminish in concentration as pregnancy progresses (24).

It is well established that PRL is a member of a gene family which includes growth hormone and placental lactogen (PL) or chorionic somatomammotropin (CS) (25). These three hormones share considerable amino acid homology, on the order of 80% for hGH and hPL and more than 50% for hGH and hPRL when conservative amino acid changes are included (10). It is believed that PRL and GH genes arose via a common ancestral gene which diverged approximately 400 million years ago to give the present day GH and PRL lineages. In humans the placental lactogen genes, of which there are three, exhibit very high nucleotide sequence homology (92%) with the GH genes and thus it is postulated that the hPL genes arose from one of the GH genes via an intrachromosomal duplication event about 10 million years ago (10). Sequencing of placental lactogens from other species such as rat (26) and cow (27) indicated that they were derived from the PRL gene rather than from the GH gene. It would appear that primate PLs are unique in that they are slightly divergent duplicates of GH whereas sequence comparisons of all other mammalian PLs studied thus far clearly suggest evolution from the PRL gene. Recently there has been a large expansion of the PRL -GH gene family with the discovery of new

members expressed in rat (28,29), mouse (30,31) and bovine (32,33) placenta. All of these newly discovered genes are more highly related to PRL than GH. The mouse placental members of this gene family are located on chromosome 13 with mouse PRL , whereas mouse GH is located on chromosome 11 (34). The most intriguing feature of these new placental PRL-like proteins is that none of the members are conserved across species. Each PRL-like protein appears unique and highly related to the other members expressed in that species but are not conserved across species. It is clear that this is a rapidly evolving gene family which has undergone divergent evolution. The function of these proteins has yet to be elucidated and is no doubt the subject of intense investigation.

In the human genome the PRL gene is located on chromosome 6 (35). The cDNA sequence as cloned from a human pituitary adenoma library included the entire protein coding region of 681 base pairs (bp), a 3' untranslated region of 148bp with an accompanying poly(A) tail and just 3bp of 5' non-coding sequence (10). Truong and colleagues (36) reported the structure and sequence of the hPRL gene; it spans 10 kilobase pairs (kb) of DNA and consists of 5 exons split by 4 introns. It is a single copy gene and there is no evidence, to date, of a human PRL-like gene family as observed in other mammalian species.

Although PRL was discovered because of its mammotropic properties, it is also found in many non-mammalian species where its functions are diverse. Well over 100 actions have been attributed to prolactin in fish, amphibians, reptiles, birds and mammals (37,38,20) which relate to osmoregulatory and ectodermal effects, growth and developmental actions as well as modulation of metabolic and reproductive functions. In mammals the most established function of PRL is the promotion of mammary growth and lactation in synergy with estrogen, progesterone, cortisol, and growth hormone. Prolactin is particularly important in the development of the lobulo-alveolar ductal system of the mammary gland and in lactation or ongoing milk production through stimulation of the milk protein genes, casein and whey acid protein (39,40) as well as fat synthesis by the

mammary gland. In rodents the action of PRL on the ovary is well-documented, being both luteotropic and luteolytic (37). In human ovarian cells, however, the data on PRL action is conflicting (41) even though specific receptors for PRL have been documented in the ovary (42). The function of PRL in male mammals is much less clear than in females. In male rodents PRL receptors have been found in Leydig cells and accessory sex glands (43,44). PRL can promote the synthesis of testosterone (45) as well as the growth and function of the rodent prostate (46). In the human male the level of circulating PRL is only slightly lower than that of a non-pregnant, non-lactating female. Evidence for a direct effect of normal levels of PRL on male reproduction is lacking however. Perhaps the most intriguing evidence implicating PRL in male reproduction is in the pathological state where excessive PRL release from an anterior pituitary tumor is associated with a dramatic loss in libido and potency which is accompanied by infertility (47). Although this would imply a direct effect of PRL on the testes, investigators have shown that increased PRL levels stimulate dopaminergic neurons in the hypothalamus which in turn suppress gonadotropin releasing hormone (GnRH) secretion contributing to infertility (48).

The multiplicity of PRL actions is mediated through its cell surface receptor which has been identified in more than 13 different tissues from rodents and rabbits by classical binding studies (49). These results in some cases have been verified by the identification of PRL receptor mRNA in prostate, testis, kidney, ovary, adrenal gland, and mammary gland, and estrogen treated female liver (50). The intracellular second messenger signalling system required to transduce the membrane receptor-ligand binding event into a physiological response is not known. The classical intracellular messengers such as calcium, cyclic nucleotides, prostaglandins, and metabolites of phosphatidyl inositol have all been implicated but individually cannot completely mimic the effects of PRL on the lactogenic process (51,52). Now that the prolactin receptor cDNA has been cloned, it is conceivable that the post-receptor signalling system can be more closely

controlled *in vitro* cell system with transfected receptor and appropriate reporter gene such as that for beta-casein.

Human Uterine Prolactin

The production of PRL in subprimate mammals is generally restricted to lactotropes of the anterior pituitary gland. Uniquely in humans and other primates, PRL is also expressed in cellular components of the human uterus. Physicochemical and biosynthetic data have established that PRL is produced in the myometrium (53), by uterine fibroids (54) and by leiomyoma of the uterus (55). The most comprehensive data existing for an extra-pituitary source of hPRL involve the endometrial layer of the uterus, specifically its decidualized form. Before proceeding, a description of this reproductive tissue is necessary.

Transformation of the endometrium leading to decidualization

The term decidua was introduced by William Hunter in 1774 (cited in 56) to describe the lining or mucous membrane of the pregnant uterus. In general, the term decidua has been used to describe material which can be shed, such as baby teeth or deer antlers. With regard to the uterus, decidualization is the process whereby this organ undergoes distinct morphological changes under the influence of ovarian steroids in preparation for implantation of a fertilized ovum or blastocyst. The differentiation of decidual cells is preceded by a series of cellular changes in the endometrium which are repetitive and cyclic in nature (proliferation, differentiation and sloughing) and follow the normal menstrual cycle. In the normal reproductive lifetime of a human female, the menstrual cycle averages 28 days. The first 14 days, post-menstruation, are generally referred to as the follicular or proliferative phase during which there is repair to the lining or

endometrium of the uterus. The endometrium at the beginning of the follicular phase is thin, there are few uterine glands which are straight, with little lumen. As this phase proceeds and ovarian estradiol secretion increases, the thickness of the endometrium increases three to five times. Both the stroma and the glands of the endometrium undergo mitosis; the stroma which was initially compact begins to accumulate interstitial fluid (57-59). The surface epithelium becomes columnar and the rapidly growing glands begin to curve and eventually become tortuous around day 14. The stromal cells which appear fibroblastic in nature change such that they have a high nucleus to cytoplasm ratio and are generally described in many texts as essentially naked nuclei with scanty cytoplasm (57-60). The length of this stage of the menstrual cycle can be highly variable and is dependent on the development of the ovarian follicle.

In an ideal cycle, ovulation occurs on day 14 and the next 12-14 days of the cycle are termed the luteal or secretory phase. After ovulation the rise in progesterone levels from the corpus luteum causes further changes in the endometrium. The uterine glands become increasingly tortuous and glycogen is accumulated in large vacuoles at the base of each cell. As this phase progresses, these vacuoles move from the base of the glandular epithelial cells to the apex adjacent to the lumen of the glands resulting in a large increase in glandular secretion which peaks on day 20. At the same time there is an increase in stromal edema and the spiral arteries continue to elongate and assume a coiled appearance (57-61). In a normal fertile cycle, blastocyst implantation usually occurs on day 21, in the absence of this event the corpus luteum begins to slowly regress.

On day 23, a predecidualization stage is evident. This is described as a change in the stromal cells from being small with dense nuclei and filamentous cytoplasm, to epitheloid or polygonal in appearance with greatly enlarged and clear cytoplasm. The cells are proliferating and found surrounding or cuffing the spiral arterioles. This change in the stroma spreads rapidly through the endometrium and by day 27 there is a continuous sheet of decidual cells in the upper part of the stroma beneath the endometrial epithelium. At the

ultrastructural level, the decidualization of stromal cells is easily distinguishable and includes the accumulation of glycogen, enlarged nucleoli, and an increase in polyribosomes and size of the rough endoplasmic reticulum as well as large Golgi apparatus (57,60). When conception and implantation do not occur the loss of estrogen and progesterone from the regressing corpus luteum causes spasmic contractions of the spiral arteries. The endometrial stromal cells disintegrate and interstitial hemorrhage occurs followed by sloughing of the superficial endometrial cells as well as glands along with blood at menses.

Should implantation of a blastocyst occur, the decidualization reaction continues with the decidual cells becoming binucleate and polyploid (60). At the time of implantation the endometrium has a thickness of 5-7mm with a surface area of about 1,000mm^2 and containing about 15,000 glands (56). The decidualization reaction spreads through the upper two-thirds of the endometrium and this layer forms the zona compacta of the functionalis which is that area of the decidua directly beneath the site of implantation. It is demarcated by a lack of uterine glands and a high density of decidual cells. Beneath the compacta is a highly glandular mucosal layer with minimal stroma termed the zona spongiosa and beneath it is the zona basalis which exhibits no hypertrophy or edema. The placenta, when shed at birth, contains maternal decidua and is thus termed deciduate. The layer of the decidua overlying and separating the conceptus from the uterine lumen is the decidua capsularis; it is most prominent around the second month of pregnancy consisting of stromal cells covered by a single layer of flattened epithelium and no uterine glands. During the first four months of pregnancy the conceptus is smaller than the uterine cavity and the rest of the pregnant uterus is lined by the decidua parietalis. The decidua parietalis and capsularis eventually fuse as the fetus grows to fill the uterine cavity, but by the 22nd week of gestation the capsularis degenerates due to a reduced blood supply, leaving the parietalis which thins to 1-2mm at full term (56,62,63). As fetal placentation occurs, it is the decidua functionalis which is extensively infiltrated by trophoblastic cells in the form of

villi leaving decidual tissue wedges called septa which divide the fetal placenta into 10-38 cotyledons (63).

The physiological significance or function of the decidua and its secretions has yet to be determined. Many investigators believe that the secretory products are somehow important in supporting the blastocyst prior to implantation. The decidua may act as a buffer zone curtailing the invasion of fetal trophoblast. Some believe the degeneration of a decidual layer may form the cleavage zone to facilitate separation from the uterus at parturition. Others speculate that the decidua in some way protects the fetoplacental unit from rejection by the maternal immune system (57).

Decidual prolactin synthesis and regulation

Prolactin is expressed in the decidualized endometrium late in the luteal phase and on through pregnancy should blastocyst implantation occur (64-66). Endometrial PRL synthesis is detectable as early as day 22 of the menstrual cycle, which coincides with the first histological signs of decidualization (67) but can be induced in preovulatory proliferative endometrial explants and cell culture by progesterone (68-71). Immunohistochemical studies have localized PRL to the secretory endometrium (72) and parietal decidual cells (73). *In situ* hybridization analysis of PRL gene expression in these tissues has not been reported. This latter technique would provide conclusive evidence as to the specific cell types in the uterus responsible for expression of PRL . The presence of PRL mRNA in samples of decidua-chorion tissue was demonstrated by Northern blot analysis using the hPRL cDNA as a probe (74). The only other demonstration of PRL mRNA in human uterus was in RNA isolated from cultured secretory and proliferative endometrial cells (70). Decidual PRL cDNAs have been cloned and nucleotide sequencing has confirmed that the protein is identical to that produced in the pituitary (75). Thus, it was assumed that the decidual PRL mRNA was identical to its pituitary counterpart and that the gene structure and transcription of PRL was not different in these two tissues.

The regulation of PRL synthesis and secretion by the decidualized endometrium appears to be different from mechanisms which operate at the level of the pituitary lactotrope. The classical modulators of pituitary PRL synthesis and release; thyrotropin releasing hormone (TRH), dopamine and estradiol have essentially no effect on decidual PRL secretion (76). As stated above, progesterone appears essential for nonfertile endometrial PRL secretion. It is not known whether progesterone acts directly on the PRL gene or indirectly by stimulating decidual differentiation of which PRL gene activation is an end result. The importance of progesterone for the synthesis of uterine PRL has also been demonstrated in non-human primates (77). In combination with estradiol and a synthetic progestin, medroxyprogesterone acetate, the synthesis of PRL by nongestational endometrial cells in culture was enhanced by relaxin (70). Insulin-like growth factor I (IGF-1) has also been shown to modestly stimulate the synthesis and release of PRL from gestational decidual cells in culture after a 48 hour treatment (78). Handwerger and colleagues (79,80) have shown that decidual PRL synthesis may be controlled by local factors. A purified 24,000 M.W. placental protein stimulates the release of prolactin from decidual tissue *in vitro* in a dose-dependent manner (79). The identity of this releasing factor has not been reported. A polypeptide having a molecular weight of approximately 40,000 which is released by decidual explants *in vitro* can inhibit the release of PRL by such explants (80). Overall, few factors regulating decidual PRL synthesis have been characterized relative to the extensive knowledge of and multiplicity of factors controlling pituitary PRL secretion primarily because there are no easily manageable human endometrial cell lines available which express the PRL gene. To date the only known critical regulatory factor of decidual PRL synthesis remains progesterone.

Suggested functions of decidual PRL

It is generally accepted that PRL synthesized by the decidua is transported into the amniotic fluid (AF) of the fetal compartment. Amniotic fluid PRL at ten weeks peaks at levels (1-2μg/ml) approximately 100-200 fold higher than that of maternal serum and decreases during the latter half of pregnancy (0.5μg/ml). There is no significant correlation between levels of AF PRL and those of the maternal or fetal circulation throughout gestation which suggests an alternative tissue source. This was supported by the work of Josimovich *et al* (81) who found that radiolabelled PRL injected into the maternal or fetal circulation of rhesus monkeys did not accumulate appreciably in the AF (<3%). That endometrial decidua is the major source of AF PRL became apparent when it was found that AF PRL concentration continued to rise normally in pregnant women treated with bromocriptine (82) or hypophysectomy (83) to alleviate pituitary hyperprolactinemia. Moreover the hypophysectomy of pregnant rhesus monkeys or death of the fetus *in utero* did not alter AF PRL levels (84). When a large volume of AF in pregnant Rhesus monkeys was replaced with saline, the pre-replacement levels of PRL were re-established within one to two hours. This could not be accounted for by maternal or fetal plasma PRL levels (81). In addition, two groups (85,86) showed that concentrations of PRL released from decidual tissue paralleled and were highly correlative with AF PRL levels throughout gestation strongly implying that decidua is the source of AF PRL.

The transport of PRL across human fetal membranes was demonstrated directly in a device called an Ussing apparatus (87) in which two lucite chambers are separated by a piece of biological membrane. Riddick and Maslar (88) found that 40% of the detectable PRL in decidual tissue adherent to fetal membranes placed in an Ussing apparatus was transported across the amniochorion to the other chamber. This result was supported by McCoshen *et al* (89) when they demonstrated that transport of PRL to the fetal side required the presence of decidual tissue and that the decidua must be in contact with intact fetal membrane consisting of amnion and chorion. Conclusive evidence for the transport of

decidually synthesized PRL to the AF was elegantly shown by McCoshen and Barc (90). Tritiated leucine was placed on the isolated maternal side of the amniochorion with adherent decidua, the labelled amino acid was biosynthetically incorporated into PRL which was then detectable on the fetal side of the membrane within four hours. Thus the amniochorion facilitates the transport of newly synthesized decidual PRL to the amniotic compartment. These observations collectively and unequivocally indicated that the primary source of PRL in the AF is produced locally in the decidualized endometrium surrounding the fetal membranes *in utero* .

The function of decidual PRL has yet to be elucidated although some have speculated about possible roles (91). The human fetus *in utero* develops in an aquatic environment floating freely in AF. It has been well established that PRL functions as an osmoregulatory hormone in fish and amphibians; therefore, it was suggested by some investigators that AF PRL may be serving a role in maintaining electrolyte and fluid balance of the AF. In order to perform such a function, PRL receptors must be present on the amnion-chorion membrane. Prolactin receptors have been identified on the chorion laeve of the human placenta (88-90,92-94) and this has been confirmed recently by the detection of PRL receptor mRNA in this tissue (95).

The first study to test the effect of PRL on AF homeostasis involved the intraamniotic delivery of 1 or 10mg of ovine PRL to pregnant Rhesus monkeys (81). Within two hours a 50% decrease in AF volume was detected; the volume being transferred to the maternal extracellular fluid. Moreover when the AF was replaced with a hypertonic or hypotonic solution, ovine PRL was capable of preventing or reversing environmental tonicity effects on the fetal extracellular fluid. Leontic and Tyson (96), using isolated human amnion placed between two lucite hemichambers, tested the permeability of this membrane after addition of ovine PRL, hGH or hPL to one chamber. At a concentration of 10μg/ml, ovine PRL caused a significant decrease (65%) in the permeability to tritiated water of the human amnion after a latent period of about 1.5 hours.

This effect was specific for the fetal side of the membrane and hGH or hPL had no effect on permeability. The decreased permeability of the amniotic membrane in the presence of PRL occurred through the PRL receptor since antiserum to the receptor blocked the effect (97). In later studies Tyson and co-workers (98,99) found that the reduction in fetomaternal water flow due to decreased membrane permeability was dependent on the membrane used in the experiment. When intact amniochorion was used instead of just amnion, the action of ovine PRL was restricted to the maternal side of the membrane and resulted in a reduced membrane permeability toward the fetal direction. The results of these latter studies conflict with earlier conclusions, but utilization of an intact fetal membrane more closely reflects the *in vivo* situation and these results therefore are more likely to be physiologically relevant.

In this context, Healy and colleagues (94) have reported a significant decrease in the specific binding of hGH to chorion laeve lactogenic receptor from cases of chronic idiopathic polyhydramnios. This is a condition where one of the symptoms is excessive AF volume. The reduced ligand binding was due to a decrease in receptor number and they postulated that this reduction in receptor number could be a causal factor in the development of chronic polyhydramnios.

Clearly, the role of decidual PRL in gestation requires further investigation. Since dPRL is expressed only in humans and primates, progress in this field has been slow owing to the obvious and significant ethical problems associated with the experimental system.

Other extra-pituitary sources of prolactin

Reports regarding the extra-pituitary synthesis of PRL by organs other than the uterus sporadically appear in the literature. Recently it has been suggested that PRL is ubiquitously produced by normal connective tissue of the human body (100). These investigators did not, however, measure directly the synthesis of PRL by fascial cells.

The amount of PRL released into the culture medium by fascial cells was inconsistent over time and extremely low, on the order of 4ng/ml after seven days in culture medium containing 20% fetal bovine serum. Another report claimed the identification of PRL mRNA in porcine luteal cells using a 30 base oligonucleotide of human PRL cDNA sequence as a probe in RNA dot blot hybridizations (101). Immunocytochemical evidence for the presence of PRL specifically in the luteal cells of human corpora lutea has also been published (102). This form of identification does not, however, prove synthesis. A rather unusual report by Roux and colleagues (103) suggested the presence of a PRL-like substance in the cytoplasm of testicular interstitial cells, Sertoli cells and spermatocytes using immunohistochemical techniques, however, they conclude that the primary result was artifactual.

The brain has on several occasions been identified as a source of extra-pituitary PRL, particularly the neurons of the hypothalamus. The majority of these reports rely on immunohistochemical identification, the first of which (104) stated that PRL-like immunoreactive material was still present one month after hypophysectomy. In the majority of subsequent studies, immunohistochemistry remained the primary tool and it was repeatedly suggested that the PRL immunoreactivity in the rat brain was of extra-hypophyseal origin (105-107), since the content of brain PRL was not dependent on pituitary or plasma concentrations perturbed in a variety of ways (i.e., hypophysectomy, pregnancy, lactation). However, in a more recent report, Harlan and co-workers (108) placed serious doubt as to the authenticity of these data. They discovered that the PRL antiserum used in the majority of these immunohistochemical studies recognizes a number of different proteins and that pre-absorption of the antiserum with the 16,000 M.W. N-terminal fragment of proopiomelanocortin (POMC) eliminated the immunoreactivity. These investigators had also previously reported the presence of PRL mRNA in the brain (109). In their more recent work (108), however, it was stated that they have been unable to detect prolactin mRNA in hypothalamic neurons using *in situ* hybridization. Despite this

contradictory evidence, others (110) continue to report on the putative authenticity of rat brain PRL . It is curious that since the first immunohistochemical description in 1977 no direct evidence for the synthesis of PRL by the rat brain (i.e., metabolic labelling) has been reported. Hence the concept of rat brain PRL production remains unproven.

Ectopic Prolactin Production

The term ectopic hormone secretion is a phrase used clinically to describe a situation where a hormone is synthesized by a neoplastic tissue whose normal progenitor cells do not normally synthesize that hormone. The concept was first introduced by Liddle and co-workers (111) in 1965 and has been found to apply to many polypeptide hormones. Today the term "ectopic" must be used cautiously when describing the production of a hormone by a cell type which had previously been thought not to be a normal physiological source of that hormone. With the advent of the powerful and sensitive tools of recombinant DNA technology, the boundaries delineating eutopic tissue-specific expression of a particular hormone are continuously being redefined. This has been documented for a number of classical endocrine hormones, most notably for the POMC precursor of ACTH (112,113) and its releasing factor CRF (114). Given the ever-expanding list of normal cell types capable of synthesizing classical endocrine hormones, the definition of ectopic or inappropriate hormone synthesis must be re-evaluated. Howlett and Rees (115) in their review of the subject make the appropriate suggestion that the term be applied to any peptide in situations where a "recognizable clinical syndrome is apparent or even when there is elaboration of hormones in the absence of clinical symptoms". The process of re-defining ectopic hormone synthesis has been evolving since Baylin and Mendelsohn's comprehensive review of the topic in 1980 (115-117). The abnormal expression or ectopic production of hPRL associated with different tumours in humans is a rare event. Molitch

and co-workers (118) assessed 215 patients with various neoplasms for hyperprolactinemia and found fifteen patients with above normal levels of serum PRL . However, in the majority of these cases it was deduced that drugs or physical stress was the causal factor for hyperprolactinemia. They concluded at that time that there was no clear evidence for the secretion of PRL by the tumour. Evidence for direct synthesis by these neoplasms was not obtained. Only three other reports exist claiming the inappropriate production of PRL: by bronchogenic carcinoma (119), renal cell carcinoma (120) and gonadoblastoma (121). In the latter case, although PRL synthesis was not demonstrated directly, immunohistochemical analysis of the tumour revealed cell-type specific staining and a significant PRL gradient between the venous drainage from the tumour and the peripheral vein PRL concentrations.

With regard to established cell lines, Rosen and co-workers (122) reported the detection of PRL by RIA in cell extracts when different human malignant cell lines (none of pituitary origin) were studied. Actual synthesis of PRL by these cell lines was not measured and secretion into the culture medium was not detectable. Moreover, they were unable to confirm the identity of the immunoreactive material as authentic PRL because the quantity detected (0.1-0.5pmol/mg cell protein) was very small.

Ectopic expression of the structurally related GH gene is also a rare event and in most reported cases the evidence was based on a fall in elevated circulating GH levels after surgical removal of the neoplasm. GH immunoreactivity has been detected in extracts of bronchogenic carcinoma and gastric adenocarcinomas, as well as cancers of the breast and ovary (123,124). In none of these cases was there any evidence of biosynthesis shown. In one patient Greenberg *et al* (125) demonstrated secretion of GH from a lung carcinoma *in vitro*. The ectopic production of the other member of this gene family, hPL, is somewhat more convincingly demonstrated since normally it is exclusively a fetoplacental product. Immunoreactive PL has been detected in the serum of patients of both genders having a variety of trophoblastic and non-trophoblastic malignancies (126-128).

It is clear from the literature that inappropriate gene activation of the PRL-GH gene family does not occur with great frequency or is a rare event. *A priori,* there is no obvious reason why ectopic PRL , GH or PL should occur less frequently than for instance that of the beta-subunit of human chorionic gonadotropin; the explanation potentially lies in the mechanistic constraints of cell-specific gene activation.

Hormones and the Immune System

Since this thesis deals in part with the production of PRL by a lymphoblastoid cell line, a discussion of the current literature regarding hormones and the immune system is appropriate. Recently this concept has gained credence with the emergence of data suggesting that cells of the immune system may potentially synthesize what were previously believed to be classical pituitary derived hormones. One of the most prominent laboratories in this field is that of Edwin Blalock and colleagues. These investigators have reported that human and rodent peripheral blood lymphocytes are capable of synthesizing ACTH (129), thyrotropin (TSH) (130), chorionic gonadotropin (CG) (131) and GH (132) when stimulated by various antigens or mitogens. Of these reports only one case, that of lymphocyte ACTH production has been substantiated by other laboratories. Oates *et al* (113) provided definitive evidence for the presence of POMC transcripts in EBV-transformed B-lymphocyte cell lines and tonsillar B-cells by RNA blot hybridization analysis. Buzzetti and co-workers (133) confirmed these results and went on to prove that the POMC transcripts were translated into a post-translationally processed polypeptide elaborating ACTH. In an earlier dissenting report, however, Lacaze-Masmonteil and colleagues (134) had shown that the POMC transcript in non-pituitary tissues, including the thymus, was truncated and could not code for a mature ACTH peptide. These transcripts were sized at 800 bases, similar in size to those seen in the reports (113,133) described

above. The normal POMC transcript is 1200 nucleotides in length. Thus even though POMC gene activity has been documented in cells of lymphoid origin, it remains questionable whether these transcripts give rise to a biologically active ACTH-like molecule. A similar situation has been described for vasopressin mRNA in bovine corpus luteum (135).

There are relatively few other examples of lymphoid cells producing peptide hormones or opioids. The expression of preproenkephalin mRNA in activated mouse T-helper cell lines has been documented (136). Vasoactive intestinal peptide (VIP) can be synthesized by rat basophilic leukemia cells; it has been purified and subjected to amino acid sequencing which revealed structural variants of the VIP normally found in neuroendocrine tissues (137). The existence of oxytocin and its carrier, neurophysin, have been documented in human thymus by HPLC analysis and biological assay at concentrations well above those found in the circulation, thus implying local synthesis. In a recent review (138), Blalock claimed that lymphoid cells were also capable of producing growth hormone-releasing factor (GRF) as well as gonadotropin-releasing hormone (GnRH), although no data was presented to confirm those claims. According to Blalock, cells of the immune system are mini-circulating hypothalamo-pituitary glands. It should be emphasized, however, that Blalock's results have not been corroborated in the literature and the passive acquisition by lymphocytes from the circulating pool of peptides remains a possibility.

On the other hand, it has been recognized that interleukin cytokines can be produced by endocrine glands. A variety of biological effects have been attributed to interleukin-6 (IL-6) including stimulation of B-cell growth and differentiation, stimulation of T-cells as well as acute phase protein production. Two groups (139,140) have indicated that cells of the anterior pituitary *in vitro* can synthesize IL-6; the latter demonstrated by biological assay. It would appear that the cells responsible for the IL-6-like bioactivity are the folliculo-stellate cells which are macrophage-like and form part of the matrix of the

pituitary in which the classical hormone-secreting cells are embedded (140). Breder and colleagues (141), by immunohistochemical means, demonstrated an IL-1-like substance in the neural elements of the hypothalamus. It should be noted that the synthesis of these interleukins by cells other than those of the immune system is not surprising as it has been shown that interleukins can be produced by fibroblasts (142) and endothelial cells (143).

To date the most strongly documented interaction between the neuroendocrine and immune axes has involved the effect of IL-1 on the release of ACTH from pituitary corticotropes. This effect was first shown using the pituitary tumour cell line AtT-20 (144). This finding would appear to have been supported by the work of Besedovsky *et al* (145) when they showed that circulating levels of ACTH could be increased 4- to 5-fold two hours after mice were injected with recombinant human IL-1. However, more recent studies from three different laboratories indicated that IL-1 could increase ACTH release from pituitary corticotropes but in an indirect manner via stimulation of CRF release from the hypothalamus (146-148). The direct site of action of IL-1 in the hypothalamo-pituitary-adrenal axis remains controversial with the report of Bernton *et al* (149) and Fukata *et al* (150) who have provided direct independent evidence for the stimulation by IL-1 of ACTH secretion from primary pituitary cell monolayers in the first case and AtT-20 tumour cells in the latter study. Thus the evidence for a bidirectional communication between immunomodulatory peptides and classical endocrine factors is conclusive. The precise nature of this interplay has not been universally confirmed although the preponderance of evidence appears to support a central mechanism utilizing the hypothalamus as the transducer of the peptide signal from the immune system.

A number of reports have also implicated IL-1 as a regulator of steroidogenic function in mammalian cells in culture. Several reports have indicated that IL-1 can suppress luteal function. This occurs by down-regulating luteinizing hormone (LH) receptors in granulosa cells as well as reducing the synthesis of progesterone by these cells under basal conditions and also after stimulated by LH (151-154). IL-1 has a biphasic

effect on sex-steroid production by cultured Leydig cells being initially stimulatory and then inhibitory after a six to eight hour latent period (155,156). While it appears that IL-1 is a major mediator of the putative functional link between immune and endocrine systems, more work is required to determine whether interleukins exert a physiological role in regulating sexual activity or stress responses typically mediated by classical endocrine factors.

Prolactin and the immune system

It is only in recent years that PRL 's role as a putative immunomodulatory factor has become a subject of popular interest. In general the earlier studies on PRL regulation of immune system function utilized supraphysiological doses of PRL in order to obtain a response. For instance, Harris and colleagues (157) found that a significant decrease in human lymphocyte chemotaxis could be observed only at PRL concentrations greater than 1000ng/ml. Only pharmacological doses of PRL (i.e., 75ng/ml) were found by Karmali *et al* (158) to reduce the mitogenic response of lymphocytes to lectin stimulation. The specificty of PRL action in such studies seems questionable because at such high concentrations contaminants in the PRL preparation may have played an integral role in the measured response. This is especially relevant now that IL-6 and possibly others of this group of immune regulators have been found to be actually synthesized by the pituitary.

The work of Berczi and colleagues (159) made clear the importance of the pituitary in normal immune function in rodents. In hypophysectomized rats, these researchers found that the immune response to various challenges (i.e., sheep red blood cells or lipopolysaccharide) was significantly suppressed and this could be reversed in large part by pituitary grafts. Giving back PRL had the same effect which clearly implicated PRL as a trophic factor of the immune system. In a later publication Nagy, Berczi and Friesen (160) presented dramatic evidence that either GH, PRL or hPL when given individually were capable of restoring the hypophysectomized rodent antibody response to

sheep red cell challenge. Although provocative the authors were cautious about suggesting that the results meant that these lactogenic hormones were acting directly on lymphoid cells through specific receptors. They concluded that it was more likely that PRL and GH effects on immunocompetence occurred through some intermediate factors. At that time there was no good evidence for the presence of GH or PRL receptors on normal cells of the immune system. Additional evidence for a stimulatory role for PRL on the immune system was presented by Berczi and co-workers (161) when they showed that low doses of PRL (20μg/day) could reinstate the inflammatory reaction to painting dinitrochlorobenzene onto the backs of rats. In hypophysectomized rats, the contact dermatitis reaction was absent. In a recent report Kelley and colleagues (162) were able to reverse the normal atrophy of the thymus in aging rats as well as T-cell function by implantation of GH_3 pituitary tumour cells for 2 months. These researchers suggested that the principal secretory products of these cells, PRL and GH, may have a direct stimulatory effect on the thymocyte population. However GH_3 cells secrete a number of different products some of which may be stimulatory to the thymic gland. Moreover, no data regarding PRL and GH levels in the tumour cell-implanted rats was given. It is conceivable that during the two month treatment period the GH_3 tumours acquired new secretory characteristics compatible with restoring thymic activity to old rats. This study was not sufficiently specific in its design to allow critical conclusions regarding the effects of GH and PRL on thymic aging to be formulated.

In 1984 Russell proposed that the immunosuppressive agent, cyclosporin, used extensively to minimize human organ transplant rejection, competed for PRL binding sites on human lymphocytes (163,164). It was reported that human peripheral blood mononuclear cells contain approximately 360 high affinity PRL receptors/cell. This concept was promoted by Hiestand *et al* (165) who went on to present unconvincing evidence for the expression of a PRL-like gene in splenic lymphocytes after antigenic stimulation. The size of the putative PRL-like mRNA was more than 10kb and more likely

represented genomic DNA contamination of lymphocyte RNA preparations. Nevertheless, Russell's group (166) attempted to characterize this putative PRL-like gene product in murine peripheral lymphocytes. Hiestand *et al* (165) had proposed that stimulated lymphocytes might produce an autocrine PRL-like factor which could then begin a stimulatory cascade on neighbouring lymphocytes in an attempt to explain why, when PRL is reduced to an almost undetectable level, the T-cell mediated immune response is still completely functional. Russell's group (166) utilized the rat Nb2 lymphoma mitogenic assay as a method for the detection of a PRL-like molecule in the supernatant of Concanavalin-A (Con-A) stimulated murine splenocytes. After 48 and 72 hours of stimulation, supernatants contained Nb2 lymphoma mitogenic activity which was as much as two-fold greater than that produced by a maximum mitogenic dose of rat PRL. In immunoneutralization experiments of this novel PRL-like activity, rather peculiar results were obtained. The PRL antiserum (obtained from the NIADDK) decreased the mitogenic effect of the Con-A stimulated splenocytes supernatant 50% better than the antigen (PRL) to which it was originally raised. Moreover this antiserum, when applied to Con-A, PRL or alloantigen stimulated splenocytes, caused a severe reduction in splenocyte proliferation. Russell *et al* interpreted these results as indicating the production of a PRL-like molecule essential for all forms of lymphocyte proliferation. The equally plausible explanation of a non-specific toxic effect of the antibody on splenocytes or Nb2 cells was not considered nor were appropriate controls to rule out such a possibility included. It is also noteworthy that the classical method of obtaining large amounts of interleukin-2 is by Con-A stimulation of splenocytes. Interleukin-2 is a potent mitogen of Nb2 cells (167). Thus the results of Russell and colleagues could be due entirely to the production of interleukin-2.

The interaction of cyclosporin with putative PRL receptors on lymphocytes was challenged by Varma and Ebner using the Nb2 cell line (168,169), the only lymphoid cell line known to contain an abundance of PRL receptors and which is exquisitely sensitive to lactogens for mitogenesis. These investigators found that cyclosporin could

indeed inhibit PRL stimulated Nb2 cell proliferation; however, cyclosporin and PRL clearly did not compete for a common binding site on Nb2 cells (170). Cyclosporin acts to suppress lymphocyte proliferation at a site downstream of the ligand binding event at the cell surface. The lack of interaction between PRL and cyclosporin on the PRL receptor was also demonstrated using rabbit mammary gland preparations which contain well characterized PRL receptors (171).

Recently the cellular receptor to which cyclosporin binds, cyclophilin, has been purified and its sequence determined (172). It is a highly abundant, ubiquitously distributed peptidyl-prolyl *cis*-trans isomerase whose activity is inhibited by the binding of cyclosporin (173). In yeast and Neurospora crassa, the cytotoxic effects of cyclosporin are mediated by cyclophilin (174). It has been postulated that cyclophilin, an enzyme which facilitates folding during protein synthesis, may be a fundamental component of various intracellular signal transduction processes. Hence the inhibitory activity of cyclosporin on Nb2 cell proliferation induced by PRL may be somewhere in the pathway from the ligand binding event at the membrane to the replication signal in the nucleus.

A role for PRL in directly regulating the immune system as suggested by Russell *et al* necessarily implies the presence of high-affinity, saturable receptors on the surface of immune cells. Russell's data on lymphocyte PRL receptors has been corroborated by one group who even detected PRL receptors on human erythrocytes at 0.24% of total radiolabelled PRL added to $2x10^6$ cells (175). Others have been unable to find PRL receptors on quiescent or stimulated rodent splenocytes (176). In addition, PRL receptor mRNA was not detected in poly(A^+) mRNA isolated from rat spleen or thymus (95).

Thus, the existence of lymphocyte PRL receptors has not been universally confirmed; nevertheless, data continues to sporadically accumulate implicating PRL as an immunoregulatory factor (177-179). Of these reports the most provocative was that of Bernton *et al* (177). They tested the effect of significantly lowering PRL levels with

bromocryptine in mice and analyzed macrophage tumouricidal activity, lymphocyte proliferative responses to T- and B-cell mitogens as well as the production of macrophage activating factors by T-cells. In general bromocryptine had a significant suppressive effect on the immune system which could be reversed by administration of PRL . Bernton's work concurs with the earlier studies of Berczi and colleagues (159-161) and provides credible *in vivo* data supporting a trophic effect for PRL on the immune system.

All of the studies reviewed thus far have dealt with establishing PRL as an immunomodulatory factor. A reciprocal effect of the immune system on PRL release from the pituitary has received little attention. A lone report has documented that IL-6, which can be released by the anterior pituitary gland, is capable of stimulating the release of PRL , GH and LH from pituitary cells in culture at picomolar concentrations (180). The presence of IL-6 receptors on cells of the anterior pituitary has not yet been determined. Certainly these results create an intriguing scenario where IL-6 secreted by folliculo-stellate cells of the pituitary could act in a paracrine fashion to modulate the release of pituitary hormones.

Common Mechanisms of Eukaryotic Cell-Specific Gene Transcriptions

Cis- and trans-acting elements

It is generally accepted that the temporal activation of specific genes is an obligatory step which directs the primary level of cellular organization. This process is manifested as the differentiation of cells into specific types to constitute a tissue of particular function and which in turn develop into an organ component of a physiological system. An identical complement of genes is found in every cell but each differentiated cell synthesizes a different set of specialized proteins along with the essential "housekeeping" gene products (181). This line of developmental progression obviously requires precise

molecular regulatory mechanisms to ensure the switching on or off of the transcription of a specific complement of genes which results in the phenotypic identity of that cell.

In recent years there has been an explosion of information regarding the role of sequence-specific DNA binding proteins in the selective activation of genes in a temporal or cell-specific manner. Much of our present knowledge about the transcriptional regulation of genes in higher eukaryotes has been elucidated from studying the biochemical response of target cells to steroid hormones. Steroid hormones and other inter- and intracellular messengers exert their controlling influence over the physiology of a cell primarily by altering the various steps of RNA metabolism. It is now well established that the first step in RNA biogenesis can be controlled by these messengers via short DNA sequences called promoters and enhancers located primarily 5' to the transcription start-site of a particular gene (182-186). These sequences act as recognition sites for binding of proteins which can then interact with RNA polymerase II to initiate and modulate the activity of the enzyme on the gene.

The primary or basal promoter region in the vast majority of mammalian genes studied thus far is located within 100bp of the transcription initiation site and is composed of at least two easily recognizable motifs. The first conserved sequence being TATAAG called the "Hogness" or "TATA" box is usually located about 30bp upstream of the RNA start site. When this sequence is deleted or mutated a decrease or obliteration of transcription initiation occurs (182,186,187), as well as a loss of the fixed physiological transcription start site (188-189). It is to this core sequence that general transcription factors bind in an ordered manner to interact with RNA polymerase II and form the "preinitiation complex". The factor which binds to the TATAA core sequence, transcription factor (TF) IID has just recently been purified to homogeneity and its cDNA cloned from yeast (190). TFIID is but one of a number of protein factors designated TFIIA, TFIIB, TFIIE/F which interact in a coordinated fashion on the core promoters centred on the TATA box sequence. These components of the class II transcriptional

machinery have been semipurified and used in cell-free systems to reconstitute transcription initiation. This was done on a defined template in order to determine the sequence of binding or interactive events which result in a commited transcriptional complex with RNA polymerase II (191-193). Buratowski and colleagues resolved seven distinct complexes in the pathway to the formation of a competent transcriptional apparatus. The binding of TFIID to the TATA element is the first step in the pathway. This results in the formation of a stable so-called "committed" complex (194,195) since TFIID will not dissociate readily from the promoter when challenged with competitor DNA or even nucleosomes (196).

The other highly conserved sequence in the basal promoter is GGPyCCAATCT commonly referred to as the "CCAAT box". Deletion analysis of this sequence indicated that it functions primarily to set the basal level of transcription (182). The CCAAT recognition motif is not recognized by a single ubiquitous factor, rather there is a family of CCAAT transcription factors (CTF) which have been isolated and some members cloned (197-199). These structurally related CTFs arise by alternative splicing of a single gene. Numerous other reports have suggested that there exists a very large group of distinct DNA-binding proteins which recognize the CCAAT motif (197 and refs. therein) such as C/EBP (200). By cDNA cloning, however, C/EBP bears no homology to the CTFs isolated by R. Tjian's group. Chodosh *et al* (199) postulated from the mixing of chromatographic fractions containing DNA-binding activity, that the CTFs are multimeric complexes composed of heterologous polypeptides thus generating a high degree of diversity and potential gene-specificity of binding. The specificity of the various CCAAT binding proteins on different genes is controversial, but there is no doubt a large family of these proteins which recognize the CCAAT core sequence in the basal promoter of most mammalian genes.

A growing number of genes have been described as lacking the hallmarks of a basal promoter region (i.e., TATA and CCAAT boxes) immediately upstream of the RNA start site. Examples of such "TATA-less" transcription units includes the gene for

transforming growth factor-beta (201), human interstitial retinoid-binding protein (202), human c-fms (203), murine lck protein tyrosine kinase (204), human sex hormone-binding globulin (205), human nerve growth factor receptor (206), murine thy-1.2 glycoprotein (207), hCG-ß subunit (208), rat acyl-CoA oxidase (209), human CD3-gamma and sigma chains (210) and references cited therein. Some of these genes contain another recognizable binding motif close to the transcription start site called the GC box (GGGGCGGGGG) which was originally described in the 72bp repeat of simian virus 40 promoter region (211). The GC box binds the glutamine-rich transcriptional activator, Sp1, which has been purified and cloned (212). Genes lacking a TATA and CCAAT boxes but having a number of GC boxes can be generally divided into two groups. One category consists of genes with constitutive GC-rich promoters expressed in a wide variety of tissues which encode proteins that perform housekeeping functions (e.g., 209); typically these genes have multiple Sp1 binding sites and transcription initiation sites. The other group of genes lack any recognizable DNA binding motifs immediately upstream of the transcription start site. These genes are typically not constitutively expressed, usually code for proteins involved in cell growth or differentiation (e.g., 204,213), and often have one or a few tightly clustered transcription start sites. These categorizations are not without exceptions (201,206). Attempts to characterize the basal promoter elements of genes lacking the conventional binding sites have met with little success. Recently Smale and Baltimore (214) have localized the basal promoter of the mouse terminal deoxyribonucleotide transferase (TdT) gene to a 17bp region which includes the transcription start site. The activity of this basal promoter could be enhanced by the upstream insertion of a TATA box or Spl binding sites in a strictly position dependent manner (i.e., TATA box about 30bp and Sp1, 40-50bp from the start site). This initiator basal promoter sequence has not been found universally distributed in mammalian genes; however, its discovery may help to re-shape our notion of a basal promoter.

The set of DNA sequences which increase the rate of transcription are termed enhancers. Unlike the promoter elements described above, enhancers have the unique property of amplifying transcription from promoters in a position and orientation independent manner. There are basically two types of enhancers; those which confer temporal and/or cell-specific expression (215,216) and those that are inducible such as the cAMP response element (217) and the steroid hormone receptor binding elements (183). In recent years this has been an extremely active area of research with *cis*-acting enhancers and the corresponding *trans*-activating proteins having been characterized for a large number of genes (218). As stated above enhancers can be located almost anywhere relative to the RNA start site. Enhancers, which act over very large distances such as the cell-specific element of the T-cell receptor alpha-locus which exerts its influence over 69kb (219), can be present in the 3' flanking DNA of a gene (220-222). Moreover enhancers and promoters can be located within intronic regions of a gene (223,224) which is not surprising since for some genes the terms intron and exon are ambiguous and the same segment of DNA can function as one or the other. This applies to genes with multiple transcripts initiating from heterogeneous points where an intron could potentially contain promoter and enhancer elements. In embryonal carcinoma cells the platelet-derived growth factor receptor mRNA initiates immediately upstream of exon 6 (225); a similar strategy exists for the transcription of the band 3 anion exchange protein mRNA in rat kidney (226). Traditionally the 5' flanking region of a gene is a good hunting ground for transcriptional enhancers. It is increasingly evident, however, that one can no longer ignore internal areas of a gene as potential regulatory regions. Eukaryotic transcriptional regulatory proteins are generally thought of as activators; however, equally plausible is the selective repression of gene transcription via binding of proteins to so-called "silencer" elements. Glucocorticoid down-regulation of the rat PRL gene is mediated through binding to negative glucocorticoid response elements which exhibit some homology to glucocorticoid response elements that activate transcription (227). Other examples of transcriptional repression include

glucocorticoid receptor inhibition of human glycoprotein hormone alpha-subunit gene, thyroid hormone receptor negative regulation of Xenopus vitellogenin gene, and various combinations of Drosophila homeobox containing transcriptional regulators which result in suppression of a reporter promoter (228). Recently Kageyama and Pastan (229) have cloned a DNA-binding protein that binds to GC-boxes (which are Spl activating sites) causing transcriptional repression in co-transfection experiments.

The complexity of transcriptional regulation is quickly coming to light and it is apparent that the global regulation of any gene will involve a pyramid of proteins interacting with specific DNA elements as well as each other on and off the DNA.

Alternative promoter use and RNA processing

Another mechanism by which cell-specific gene expression is achieved is by the utilization of alternative transcription start sites which usually imply the use of alternative promoter and enhancer elements. There are numerous examples of alternative use of exons and promoters as a means to generate tissue-specific RNA diversity. This now classical mechanism is operational on the gene for mouse alpha-amylase 1 (230), Drosophila alcohol dehydrogenase (231), rat acetyl-coenzyme A carboxylase (232), rat gastrin-releasing peptide (233), and the rat gamma-glutamyl transpeptidase (234). In these cases two different mRNA species are synthesized having different 5' untranslated regions transcribed from separate promoters which are active in different cell types, but direct the synthesis of identical proteins. In some cases the distance between the cell-specific promoters can be considerable. In the human c-fms gene, multiple transcription start sites facilitate placental trophoblast-specific expression with the inclusion of a unique 5' non-coding exon located 25kb upstream of the monocyte-macrophage specific cap site (203). The lck protein tyrosine kinase gene in non-transformed lymphoid cell lines initiates at a site more than 30kb upstream of the cap site activated in non-lymphoid tumour cell lines (235).

A variation on this mode of tissue-specific regulatory flexibility is when the alternative promoter occurs in an intron as discussed in the previous section. In those situations multiple forms of a protein can be generated in a cell-specific fashion as is the case for the cartilage versus calavaria alpha-2(I) collagen mRNAs where the resultant cartilage protein is not of the collagenous type (236). Splicing of a common primary transcript to yield multiple proteins in specific tissues can also occur (237-239). Differential splicing coupled with the use of alternative polyadenylation sites allows for the neuron-specific synthesis of calcitonin gene-related peptide as well as different immunoglobulin heavy chain genes in B-lymphocytes (240).

In the PRL-GH gene family alternative splicing of the hGH-N and hGH-V gene has been documented. The N-gene primary trancript can be differentially processed such that the 5' portion of exon 3 is deleted to produce a smaller hGH of 20kd (241,242). The expression and alternative splicing of the hGH-V gene has been detected in placenta and involves the differential splicing of the fourth intron to produce an hGH-V protein containing a highly divergent and longer COOH-terminal region (243). The selective retention of intron 4 in a GH hnRNA was initially described for the bovine pituitary transcript (244). The differential processing of the hGH genes is not constrained by cell-type since both transcripts are present in transfected cells. However, *in vivo* hGH-V gene expression has been detected only in placenta and hGH-N expression is restricted to the pituitary (245). There appears to have been no documentation of alternative splicing of PRL mRNA.

Potential intrinsic gene regulatory factors

The conversion of a gene from a transcriptionally quiescent state to a locus of active tissue-specific expression involves not only *cis*- and *trans*-active elements, but also a structural organization and protein composition unique to active chromatin. It may be that the ability of extrinsic regulators such as steroid hormone receptors to bind to DNA

depends on the chromatin structure. These structural properties have been discovered because their presence has been correlated with actively transcribed chromatin. These parameters include changes in the packaging of the chromatin, DNA methylation, histone and non-histone protein alterations and chromatin interaction with the nuclear matrix.

Whether these biochemical changes are a cause or consequence of gene transcription has yet to be clarified. From the context of tissue-specificity, the role of methylated cytosine residues has received considerable attention. There are numerous examples in the literature which have correlated the expression of a gene in a particular tissue with a state of hypomethylation in and around the gene (246 and references therein). However, it is becoming clear that this is not a general predictive scheme (247,248). The effect of methylating *cis*-active DNA elements on subsequent binding of corresponding *trans*-acting factors has resulted in conflicting data (249,250). Understandably then, only cautious predictions regarding the functional importance of 5' methyl cytosine in cell-specific gene expression can be ventured from such a spectrum of non-uniform results.

It is well established that the transcriptionally active fraction of the eukaryotic genome is somehow more susceptible to digestion by DNase I than bulk chromatin. That chromatin decondensation is associated with the expression of genes in a tissue-specific manner is indisputable (251). The 5' promoter regions of genes are usually the focus of attention in studies of DNase I hypersensitivity because nucleosome-free regions of the chromatin fibre presumably facilitate the binding of cell-specific DNA-binding proteins to sequence recognition motifs.Regions of DNase I sensitivity can be altered by transcriptional regulators such as steroid hormone receptors (252). Sites of DNase I hypersensitivity in the controlling regions of a gene are often set during development in a cell-specific manner. As is the case of methylation, it is not yet clear whether nucleosome displacement is a cause of or consequence of other factors which contribute to cell-specific gene activation.

Mechanisms of pituitary-specific expression of the rat PRL gene

The prolactin gene has become a popular candidate for defining the molecular basis of cell-specific gene transcription since its expression is generally restricted to the anterior pituitary gland. Using the sophisticated techniques of DNase I footprinting, cell-transfection assay, *in vitro* transcription, band shift analysis, and DNA-affinity chromatography, a number of *cis*-active DNA sequences in the 5' flanking DNA of the rat PRL gene as well as one of the corresponding DNA binding proteins have been isolated (253-257). The now seminal paper by Nelson *et al* (253) delineated two clusters of tissue-specific *cis*-elements lying between -38 to -200 (proximal enhancer) and -1386 to -1728 (distal enhancer) relative to the rat PRL gene start site. The proximal enhancer contained three binding sites, and the distal, four binding sites consisted of the consensus sequence A $^{A}/_{T}$ $^{A}/_{T}$ TATNCAT. These sites competed with each other for the binding of a single factor termed Pit-1. Interestingly the binding of this factor could be competed for by the tissue-specific elements of the rat GH gene. These data were verified in a number of other laboratories (254-257). Shortly thereafter, Ingraham *et al* (258) and Bodner *et al* (259) cloned the cDNA which corresponded to the *trans*-acting protein which bound to the tissue-specific enhancers of the rat PRL and GH genes, respectively. The deduced amino acid sequence of the 33kd Pit-1/GHF-1 contained two domains which exhibited a high degree of conservation with other DNA-binding proteins. Near the COOH-terminus of the 291 amino acid Pit-1 molecule exists a 60 amino acid region strongly resembling the homeobox domain found in several Drosophila and vertebrate developmental regulatory proteins. Just upstream is another region of 67 amino acids which was found to be conserved in three other DNA-binding proteins (Oct-1, Oct-2 and unc 86) which was named the POU-domain (260). The combined POU-homeodomain is required for DNA binding (261), although others (262) dispute the importance of the POU-subdomain in binding to the Pit-1 enhancer.

The specificity of Pit-1 for PRL and GH gene expression in the rat anterior pituitary has been demonstrated by transient co-transfection assays in heterologous cell systems in which the amount of Pit-1 expressed was not supraphysiological (263). Using transgenic mice, the Pit-1 enhancer regions were shown to specifically target the cells of the anterior pituitary (264). Saturation mutational analysis of the Pit-1 binding site showed both *in vivo* and *in vitro* that this *trans*-activator interacts with the GH and PRL gene cell-specific enhancers (265). These results firmly establish Pit-1 as an essential pituitary-specific transcriptional activator of the PRL gene in rodent, but do not discount the distinct possibility that other yet undiscovered factors are important to set the physiological levels of PRL and GH gene expression.

Human prolactin gene transcription

The structure and sequence of the hPRL gene has been elucidated; however, relatively little work has been done on regulation at the transcriptional level largely because of a lack of a human cell system. Organ culture of human pituitary adenomas or fetal pituitaries can remain viable for up to almost one year (266-268) but during this time there is a loss of specific differentiated function, i.e., hormone secretion. As a result the establishment of human pituitary cell lines has met with little success, and this has been attributed to the slow growth of hormone secreting cells coupled with eventual overgrowth by fibroblastic cells (269). Recently, two groups have reported the establishment of human pituitary tumour cell lines that secrete PRL and are responsive to regulation by a variety of factors (270-271). In the case of decidual PRL , no immortalized cell lines of the endometrium have been reported to secrete PRL . This is not surprising because the majority of endometrial cell lines available, seven from the American Type Culture Collection (Rockville, MA) (272), possess epithelial cell characteristics and not those of endometrial stroma from which PRL is normally synthesized.

In the previous section it is clear that the availability of rat pituitary tumour cell lines producing PRL and GH has greatly facilitated the study of the cell-specific expression of these genes. Truong and co-workers (36) recognized the high degree of sequence homology that exists in the immediate 5' flanking region between the human and rat PRL genes. Those sequences in and around the binding site of Pit-1 in the proximal enhancer were almost totally conserved. A single report has appeared describing the interaction of extracts prepared from subclones of a rat pituitary tumour cell line with the 5' flanking DNA of the hPRL gene. By DNase I footprinting, *in vitro* transcription and gel retardation assays, these investigators showed that the human PRL gene can be controlled in a tissue-specific manner from enhancer sequences almost identical to those described by Nelson *et al* (253) in the rPRL gene proximal enhancer. Only binding site 2P recognized by Nelson (253) at -115 to -130 of the rPRL gene 5' flanking DNA was not detected in the corresponding sequence of the hPRL gene. This site is proposed to be non-tissue-specific (254). Moreover, Lemaigre and co-workers (273) found that the tissue-specific factor which binds to the hPRL gene enhancers is competed for by the hGH gene enhancers and probably represents Pit-1/GHF-1 described previously. Thus it is reasonable to assume, despite the use of heterologous cell system, that the cell-specificity of the hPRL gene is conferred by a mechanism analogous to that so far delineated for the rPRL gene.

Rationale and Objectives of this Investigation

Initially, the objective of my Ph.D. research was to focus on identifying the expression of PRL and PRL -related mRNAs in rat and human tissues other than the pituitary. This was founded on the reports of Duckworth *et al* (28,29) who discovered two new members of the PRL-GH gene family expressed in rat placenta each of which possesses 35-45% amino acid sequence homology to PRL. Prior to this point it had been well established that the rat placenta synthesized two other distinct polypeptides (rat

placental lactogen I and II) which were considered PRL-like by virtue of the fact that they possessed lactogenic activity. In addition to these four new members of the rat PRL-like gene family, two other distinct polypeptides sharing high homology with PRL were discovered in the mouse placenta (46,47) bringing the total to six PRL-like proteins expressed in rodent placenta. The existence of a large PRL-like gene family has also been documented for bovine fetal cotyledons (48,49). These PRL-like proteins are not cross-species homologues of one another and it appears that this is a rapidly evolving family of genes.

Given these precedents it was of great interest to determine which, if any, of these PRL-like genes might have been conserved and expressed by human placenta. The initial purpose of my investigation was to establish the presence and determine the primary molecular characteristics of putative new members of the human PRL -GH gene family. It was considered that purification of lactogen-like polypeptides, other than human chorionic somatomammotropin, from the human placenta would have significant clinical ramifications. As a first step, RNA from a variety of human and rat tissues and cell lines was analyzed for the expression of PRL or related mRNAs. Extensive low and high stringency hybridization screening of a number of human term placenta lambda GT10 cDNA libraries with the various rat PRL-like cDNAs as probes did not reveal human counterparts. Moreover, low stringency genomic Southern hybridization experiments with these rat cDNAs did not show reproducible or convincing cross-hybridizing human genomic DNA fragments. Therefore, after considerable efforts it was concluded that the novel PRL -related gene family expressed in the rat placenta is either not present in the human genome or not expressed in term placenta.

During the screening of rat tissues and cell lines for PRL-like mRNAs, however, what appeared to be high stringency hybridization of the rPRL cDNA (274) was detected to an RNA whose electrophoretic mobility was slightly faster than 18S ribosomal RNA at approximately 1.6kb. This putative PRL-like RNA was found to be ubiquitously

expressed in rat tissues. In the course of these studies, a number of human cell lines were also screened by Northern hybridization with hPRL cDNA and an mRNA was detected in a human B-lymphoblastoid cell line, IM-9, under high stringency conditions. It was a novel observation that led to the work described in this thesis for the following reasons:

1. At the time this work began there were no other human cell lines available which synthesized and secreted hPRL . Therefore, this cell line represented a potentially valuable tool for the study of hPRL synthesis *in vitro*. Early studies using human pituitary tumours or fetal pituitaries were very limited because of scarcity of tissue and because of the gradual loss in culture, over time, of the PRL producing phenotype.

2. The ectopic expression of PRL is rare and has never been demonstrated directly. Thus the production of PRL by the human B-lymphoblastoid line would be the first conclusive report of inappropriate PRL secretion.

3. The most exciting potential use for this PRL-producing B-cell line pertains to the question of cell-specific gene transcription. As stated in an earlier section the expression of the PRL gene is highly tissue-specific and significant progress has been made regarding the mechanism by which this occurs, largely due to the availability of rodent pituitary cell lines. The PRL-producing IM-9 line represents a unique and easily manageable resource with which to analyze the mechanism by which human PRL gene transcription is constrained by cell-type. By studying the presumably aberrant, or at least inappropriate expression of PRL in these cells, some insight could be gained into how normal cell-specific PRL gene expression occurs in the lactotrope or uterine decidual cell.

With these facts in mind, the objective of my work was to characterize the expression of PRL by the B-lymphoblastoid cell line. By analysis of the PRL gene, mRNA and protein, I hoped to provide some understanding of how the PRL gene became inappropriately expressed and what effect this phenotype had on the physiology of the B-lymphoblast. During the course of these studies, it was discovered that the PRL mRNA in IM-9-P and decidual cells was identical. These studies, therefore, would form the foundation for further use of this cell line in experiments designed to elucidate elements which confer transcriptional capability to the PRL locus in uterine decidual cells.

MATERIALS AND METHODS

Materials

Restriction enzymes, DNA and RNA modifying enzymes were purchased from Pharmacia (Canada) Inc. (Baie d'Urfe, Quebec) and Boehringer-Mannheim (Indianapolis, IN). Oligo(dT)-cellulose was supplied by Collaborative Research Inc. (Waltham, MA) and Pharmacia. The lambda phage packaging kits came from Amersham Corp. (Arlington Heights, IL) as did the nick-translation and random-primer DNA labelling kits which utilized [alpha-^{32}P]dCTP. All radionucleotides were purchased from DuPont-New England Nuclear (Boston, MA). Reagents for generating [^{32}P]UTP labelled cRNA probes were obtained from the Pharmacia Transprobe-T kit and Promega Biotech Riboprobe Kit (Madison, WI). Hybridization transfer membrane, Hybond-N matrix was from Amersham Corp., nitrocellulose was from Schleicher and Schuell (Keen, NH) and Nitroplus-2000 from Micron Separations Inc. (Westboro, MA). Plasmids (pGEM-3) and DNA sequencing reagents adapted for double-stranded templates were purchased from Promega Biotech. The B-cell and T-cell-specific human DNA probes were obtained from Cedarlane Laboratories (Hornby, Ont.). Synthesis of oligodeoxyribonucleotides was done on an Applied Biosystems DNA synthesizer Model 380A. Unless otherwise stated, all cell lines were obtained from the American Type Culture Collection (Rockville, MA).

The human and rat PRL cDNAs (10,274) as well as rat GH cDNA (275) were provided by Dr. J. Baxter (University of California, San Francisco). The hPL cDNA clone (276) came from Dr. G. Saunders (M.D. Anderson Hospital and Tumour Institute, Houston, TX). A recombinant plasmid containing a fragment of the human DNA repeat element AluI (277) and the human Y-chromosome-specific DNA probe pY3.4 (278) was provided by Dr. Yun-Fai Lau. The rat probasin or M-40 cDNA was kindly provided by

Dr. R. Matusik (Dept. of Physiology, University of Manitoba) (279). Recombinant plasmids containing different fragments of the human PRL gene were the generous gift of Dr. Joseph A. Martial (University of Liege, Liege, Belgium) (36). A 5' end-specific EcoRI fragment of the hPRL gene (phPRLg2750) was provided and consisted of 978bp of 5' flanking DNA, exon 1 and approximately 1740bp of intron A. The hPRL gene 3' specific genomic clone (phPRLg3900), also an EcoRI fragment, spanned 645bp of intron C, exon 4, intron D, exon 5 and 392bp of 3' flanking sequence. These genomic fragments were cloned into the EcoRI site of pBR322. An additional 5' flanking genomic DNA clone (phPRLg4800) was sent later and had been isolated from an EMBL3 genomic library as a 6.0kb fragment whose sequence overlapped with the previously published sequence of the hPRL gene (280). The most 5' 4800 bp SalI-EcoRI fragment was subcloned into pUC19.

Human pituitaries were obtained at autopsy and stored frozen at -70C. Human maternal decidua was obtained from 9-12 week aborted conceptus. Permission for the procurement of maternal decidua was obtained from the University of Manitoba Faculty Committee on the Use of Human Subjects in Research (Reference:E88:199). Human term placental tissue was obtained from the laboratory of Dr. Peter Cattini (Dept. of Physiology, University of Manitoba). A lambda GTII cDNA library constructed from human term placenta was procured from Clontech (Palo Alto, CA). A human uterine decidua lambda GTII cDNA library (281) was kindly provided by Dr. Olli A. Janne (Population Council and Rockefeller University, NY).

Cell Culture

IM-9 cells, originally supplied by Dr. Ron Rosenfeld and kept in our laboratory for several years (referred to as IM-9-P) as well as IM-9 cells recently obtained from three different sources, Dr. R. Rosenfeld (Stanford University, Stanford, CA), Dr. Maxine

Lesniak (NIH, Bethesda, MD), American Type Culture Collection (ATCC, Rockville, MD), were maintained in RPMI-1640 medium (Gibco, Burlington, Ontario) supplemented with 10% fetal calf serum (Gibco), 50U/ml penicillin, and 50μg/ml streptomycin (Gibco, RPMI/FCS). Serum-free medium consisted of RPMI-1640 supplemented with 0.1% BSA. Cell numbers were determined using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) and had a coefficient of variation of less than 5%. For production of conditioned medium, cells were placed at 1 x 10^5cell/ml, incubated for four days unless indicated otherwise, and sedimented by centrifugation (500 x gravity) at 4C for 5-10 min, and supernatants were passed through filters of 0.45μm pore size to remove any remaining cells. The following six human lymphoblastoid and leukemic cell lines were obtained from the ATCC and cultured as recommended by the supplier (RPMI 1788, RPMI 7666, RPMI 6666, RPMI 8226, HS-Sultan, CCFR-SB). The Nb2 11c clone of the rat T-cell lymphoma line established by Gout *et al* (282) was cultured in Fischer's medium (Gibco), as previously described (168).

Small aliquots of the above cell lines were stored frozen in liquid nitrogen. These aliquots were derived from nearly confluent cell cultures which were centrifuged as above to facilitate resuspension to a concentration of up to 4 x 10^6cells/ml in freezing medium. The freezing medium consisted of 10% DMSO/90% FCS which was filter sterilized through a 0.22 micron filter. The spent media was carefully removed from the cell pellet, the pellet was dislodged and 6ml of freezing medium was added. The cells were resuspended by gentle swirling and 2ml dispensed to Corning 2ml cryogenic vials (Corning Glass Works, Corning, NY) and placed at -70C overnight. The next day the vials were submerged in liquid nitrogen.

Culturing of cells from these frozen stocks was done by quickly thawing the cells in sterile 37C distilled water. The vial was then wiped with 70% ethanol, opened and the cell suspension pipetted into 10ml of complete medium in a centrifuge tube. The cells were dispersed by a slow swirling motion, centrifuged as above and the supernatant

discarded. Another 10ml of complete medium was added to the washed pellet and cells resuspended by gentle pipetting. Cells were then transferred to tissue culture flasks for incubation at 37C.

Cells of the IM-9-P class generally obtained confluency at a concentration of 1 x 10^6cells/ml with a doubling time of approximately 18hr. All manipulations of cells in culture were performed under sterile conditions in a laminar flowhood.

All IM-9 lines and the Nb2 cells were tested for the presence of mycoplasma contamination using the Mycoplasma T.C. Detection Kit purchased from Gen Probe Inc. (San Diego, CA). This test is based on liquid hybridization of mycoplasma ribosomal RNA in cell culture medium to a specific [^{3}H]-labelled DNA probe. Only the original PRL-producing IM-9-P cell line tested positive for mycoplasma contamination and was subjected to a curing regime dictated by the BM-Cycline kit from Boehringer-Mannheim. This involved a three-cycle treatment of antibiotics over a four week period from November 7-28, 1986. The cells subsequently tested negative for mycoplasma using the Gen Probe mycoplasma detection kit.

In February 1987 the original PRL-producing IM-9-P cell line was cloned by limiting dilution by plating 100µl/well in 96-well plates of a suspension containing 3 cells/ml. The cloning medium consisted of 60% RPMI-1640/FCS and 40% conditioned medium removed from a three-day culture of the parent population. After eleven days of growth 19% of the 384 wells seeded showed growth of which 56 clones were tested for PRL production by RIA. One clone was picked for further expansion, designated IM-9-P3, because of its significant PRL production, and another, IM-9-P6, which did not secrete PRL into the culture medium was also chosen for further experimentation. The IM-9 line from the ATCC was cloned in the same manner and no PRL producers were found.

Immunophenotyping

Immunophenotyping of the classical IM-9 cell line obtained from ATCC and the IM-9-P3 clonal line was done by assaying for the binding of specific monoclonal antibodies to cell surface antigen. Monoclonal antibodies to the T8 (suppressor/cytotoxic T-cell), I2 (HLA-D/DR), T4 (thymocytes and peripheral T-cells), B1 (B-cells), J5 (common ALL antigen), Kappa (B-cells), Lambda (B-cells), immunoglobulin M (IgM) and IgG cell surface antigens were obtained from Coulter Immunology (Burlington, Ontario). Monoclonal antibody T3 (T-cell receptor) was purchased from Ortho Diagnostic Systems, Inc. (Raritan, NJ). Tests using the above monoclonal antibodies were performed by Dr. E. Rector (University of Manitoba, Winnipeg, Man.). The following monoclonal antibodies were also procured from Coulter Immunology: Mol (monocytes and granulocytes), Mo2 (monocytes and macrophages), MY9 (monocytes), NKH-1 (natural killer cell), and IL-2R1 (interleukin-2 receptor). Cell surface leukocyte antigens detected by the preceding monoclonal antibodes were tested by the Immunoprotein Laboratory (Health Sciences Centre, Winnipeg, Manitoba). Binding to the cell surface was determined by indirect immunofluorescence, as analyzed by a fluorescence-activated cell sorter. Results were expressed as the percentage of fluorescing cells corrected for background fluorescence when second antibody was used alone.

HLA typing was performed by the Transplant Immunology Laboratory (Health Sciences Centre, Winnipeg, Manitoba), as described previously (283). The presence of IgG in the culture medium was detected by enzyme-linked immunosorbent assay (ELISA) as described (284). The labelled antibody was alkaline phosphatase conjugated goat anti-human IgG antibody and the resultant color reaction was read at 410nm. The sensitivity of the assay ranged from approximately 50-1000ng/ml.

Nb2 Bioassay

To demonstrate the biological activity of IM-9-P PRL, the rat Nb2 lymphoma bioassay was used. This line proliferates in a dose-dependent fashion in the presence of lactogenic hormones (PRL, hGH, and hPL) (169) or interleukin-2 (167). The bioassay for lactogenic hormones was carried out essentially as described (168). Briefly, Nb2 cells were grown in Fischer's medium for leukemic cells plus 20mM beta-mercaptoethanol, 10% FCS and 10% HS and then 18hr prior to assay, cells were transferred to a medium containing only 10% HS and Fischer's medium at a density of 1.5 x 10^5cells/ml. This was the arrest procedure and done when cells were confluent in 75cm^2 flasks (approximately 1.2 x 10^6cells/ml). One millilitre of the arrested population of cells was counted and then aliquoted into 24-well plates at 1ml/well. Standard hormone preparations (pituitary hPRL extracted and immunoaffinity purified in our laboratory and standardized against WHO 75/504 hPRL or ovine PRL NIAMDD PS-14) and experimental samples were added in a volume of 25µl/well. All standard hPRL stocks were dissolved in phosphate-buffered saline (PBS), 0.1% BSA and when added to 1ml of Nb2 cells, ranged from 12.5pg/ml to 2500pg/ml. Each concentration of standard hPRL and experimental samples was tested in triplicate. The Nb2 cells plus added lactogenic samples were allowed to grow for three days after which the cells were resuspended, diluted in 9ml Isoton (Fisher Scientific, Pittsburgh, PA) and cell number determined with a Coulter counter. The cell count in control cultures with no added mitogen after three days was used to determine fold stimulation.

For immunoneutralization experiments, 25µl of monoclonal anti-hPRL antibody 9C3 (generated in our laboratory) was added from a 1:100 dilution to give a final dilution of 1:4000 in each well of cells. To insure that a maximally stimulating dose of IM-9-P conditioned medium was added, two volumes of conditioned media were chosen (12.5µl

and 25µl) for addition to Nb2 cells. The hPRL standard was added at a maximally stimulating quantity of 2.5ng/ml. After a 3-day incubation cell numbers were determined.

hPRL RIA

Concentrations of hPRL immunoreactivity in conditioned medium were assayed by the double antibody RIA technique previously described (285). A polyclonal antiserum to hPRL raised in our laboratory was used at a dilution of 1:20,000. The same hPRL standard preparation as that employed in the Nb2 bioassay was used for the RIA. IM-9-P conditioned medium was concentrated 5-fold by lyophylization and dialyzed against PBS. The final IM-9-P sample was in PBS, 0.5% BSA. The within-assay coefficient of variation was less than 2%.

For determining IM-9-P3 hPRL intracellular content, log phase IM-9-P3 cells were centrifuged at 500 x g for 1 min and washed several times in serum-free medium. Cells were resuspended in PBS-0.2% TritonX-100 at 2 x 10^7cells/ml and kept on ice for 15 min, followed by polytron homogenization (Brinkmann Instruments, Westbury, NY) (286) and the resultant supernatants were assayed for hPRL by RIA.

Immunoaffinity Purification of IM-9-P PRL

IM-9-P cells were grown to confluency in 0.1% BSA/RPMI 1640 until 400mls of conditioned medium (CM) was collected. Hepes was added to the CM to a final concentration of 10mM to maintain pH 7.4. An immunoaffinity column of monoclonal antibody 9C3 coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) was provided by Helle Cosby (Department of Physiology, University of Manitoba). A 1ml

immunoaffinity column was equilibrated with PBS, then 400ml of IM-9-P CM was passed through the column at 4C over a 2-day period. The flowthrough was checked for hPRL content by RIA and hPRL was not detectable. The column was then washed with several changes of 0.1M $NaHCO_3$-0.5M NaCl (pH 6.8 and pH 8.2, alternatively) at 10 x column volume until the $O.D._{280}$ of the eluant was no longer measurable. This was done to remove non-specifically bound protein. Bound antigen was eluted with 0.2M CH_3COOH-0.5M NaCl, pH 2.5 as 400µl fractions into 100µl 1M Tris-HCl, pH 9.0 to neutralize each fraction. Eighteen fractions were collected and 1µl of each fraction was assayed by RIA for hPRL content. Peak fractions were dialyzed for 24 hr against several changes of 0.01M ammonium bicarbonate at 4C, vacuum dessicated and used for determination of bioactivity and electrophoretic mobility. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) the immunoaffinity purified IM-9-P PRL was reconstituted after vacuum dessication, in 30µl of SDS-sample buffer. A $^1/_{20}$ volume of ß-mercaptoethanol was added to each sample, boiled for 5 min and applied to 15% polyacrylamide gel slabs (287) for electrophoretic fractionation. Gels were subsequently silver-stained (288).

Plasmid DNA Amplification and Recovery

Competent bacterial cells for transformation by recombinant plasmid DNA were generated using a calcium dependent protocol (289). Briefly, a single bacterial colony was used to innoculate 5ml of L-broth medium (290) which was grown overnight in a shaking incubator at 37C. One ml of the turbid culture was transferred to 100ml L-broth and vigorously agitated at 37C on a rotary shaker for 1.5-2hr or until the $O.D._{550}$ was approximately 0.5. The culture volume was split between two 50ml centrifuge tubes and cells were pelleted by centrifugation at 1500 x g (4C) for 15 min. The cell pellets were

resuspended in half-volume of cold 0.05M $CaCl_2$ and left on ice for 20-30 min. The cells were centrifuged again as above and the cell pellets resuspended in 6.5ml of cold 0.05M $CaCl_2$ and stored on ice until use. The bacterial strains used in these studies were MV1193 or TGI-lambda .

Transformations were carried out by adding a maximum of 4µl of the plasmid DNA solution to 100µl of competent cells on ice for 45 min. This mixture was subjected to heat-shock by immersion in a 42C waterbath for 90 sec. Then 1ml of L-broth was added to the mixture and the transformed cells allowed to grow for 1hr without agitation at 37C. Then 100-200µl of the transformation was spread on L-broth-agar plates (290) containing the appropriate antibiotics for selection of transformed colonies.

Plasmid amplification and recovery was performed using two different but related protocols. The first procedure involved amplification of the transformed bacterial colony in 500ml of rich medium according to Maniatis *et al* (290). Recovery of plasmid DNA began by centrifugation of the culture medium at 1,000 x g (4C) for 10 min. The cell pellets were resuspended in a total of 9.5mls of ice-cold lysis buffer (0.025M Tris-HCl pH 8.0, 0.01M EDTA, 0.05M glucose) in the 250ml centrifuge bottles on ice. Then 500µl of cold, freshly prepared 20-50mg/ml lysozyme solution (in lysis buffer) was added to the resuspended transformed bacterial cells. Prior to this step the resuspended bacteria had been transferred to 30ml screw-cap Oakridge tubes (Nalgene Co., Rochester, NY) and placed on ice. With the lysozyme solution added, the cells were incubated on ice for 15 min with occasional gentle rolling of the tubes. Subsequently 10ml of 0.2M NaOH, 0.2% SDS solution was added to the partially lysed bacterial slurry and incubated for 15 min on ice as above, followed by the addition of 10ml 3M $NaCH_3COO$ pH 5.6. This latter addition facilitated precipitation of bacterial chromosomal DNA and was left on ice for 30 min. This viscous slurry was centrifuged at 40,000 x g (4C) for 1 hr to pellet the bacterial debris. The approximately 30ml supernatant was transferred to a 150ml Corex centrifuge bottle and allowed to equilibrate to room temperature before adding 0.6 volume of

isopropanol in order to avoid precipitation of SDS in the supernatant. The isopropanol was added to precipitate the remaining DNA and left at room temperature for about 1 hr or until a floculent white precipitate was evident. This was then pelleted by centrifugation at 9,000 x g for 20 min at room temperature. The white pellets were then dessicated and dissolved in 6ml of sterile TE pH7.5 (0.01M Tris-HCl pH 7.5, 0.001M EDTA) and transferred to 15ml Corning tubes containing 6.6g of CsCl which was then dissolved by gentle manual agitation. Five hundred microlitres of 10mg/ml ethidium bromide (EtBr) was added and gently mixed and centrifuged at maximum speed in an IEC model HN-S benchtop centrifuge (Needham Heights, Mass) for 10-20 min. Aspiration of the solid pink pellet was avoided by using a long glass Pasteur pipet to transfer the supernatant to Beckman quick-seal Ti75 polyallomer centrifuge tubes via a 10ml syringe-16 gauge needle. The Ti75 centrifuge tubes were filled with mineral oil, balanced and heat sealed. Centrifugation was at 55,000rpm in a Beckman Ti75 rotor at 23C for a minimum of 16hr.

Visualization of the lower plasmid band in the CsCl gradient was performed by fluorescence under long wavelength ultraviolet light. The air space in the centrifuge tube was penetrated with an 18-gauge needle and recovery of the plasmid band was facilitated by insertion of a 3ml syringe fitted with an 18-gauge needle just below the plasmid band. Slowly, the plasmid band (1-1.5ml) was drawn into the syringe and then expelled through its open end into a sterile 15ml glass Corex tube which contained 2ml of water-saturated butanol. This mixture was gently mixed and the upper pink organic phase discarded. This butanol extraction was repeated several times until no pink color was evident in the upper phase. The majority of the EtBr having been removed; 2ml of sterile water was added followed by 2.5 volumes of -20C ethanol (EtOH), agitated and placed at -20C for at least 2 hr to precipitate DNA. The plasmid DNA was then centrifuged at 10,000 x g (4C) for about 10 min, the pellet washed with 70% EtOH, dessicated and dissolved in an appropriate volume (100-200µl) of TE pH 7.5 and stored at 4C. The quantity of plasmid DNA isolated was determined spectrophotometrically by absorbance at 260nm.

The other procedure used for large scale preparation was devised by Drs. Paul Kreig and Doug Melton (Harvard University) and presented in the Promega Biotech Sequencing Protocol booklet. This procedure did not involve amplification of the plasmid DNA. A single bacterial colony was used to innoculate 250ml of L-broth containing the appropriate selective antibiotic (ie 200μg/ml ampicillin) and the cells were allowed to grow overnight in a shaker at 37C. The cells were then harvested and lysed as described above. After the 40,000 x g (4C), 1 hr centrifugation the supernatant was transferred to a 50ml polypropylene Corning centrifuge tube to which 50μl of 1mg/ml RNaseA was added and incubated at 37C for at least 20 min. This was then extracted with an equal volume of phenol:chloroform which had been equilibrated with 0.01M Tris-HCl pH 8.0. The DNA was then precipitated by the addition of two volumes of EtOH on ice for 30 min, followed by centrifugation at 9,500 x g (4C) for 20 min. The DNA pellet was dissolved in 1.6ml sterile distilled water, then 0.4ml of 4M NaCl was added, mixed and followed by 2ml of 13% polyethylene glycol (M.W. 8,000). This solution was mixed and placed on ice for at least 1 hour before centrifugation as described previously for pelleting purified plasmid DNA. At this stage the yield was usually 1-2mg of DNA; however, it was often contaminated with bacterial chromosomal DNA. In order to remove the contaminating DNA, the plasmid DNA solution was made up to 6ml with TE pH 7.5 and subjected to CsCl gradient centrifugation as described above. The final yield of plasmid DNA was somewhat less than without the CsCl purification step but overall this procedure was faster than the first protocol described.

Restriction Enzyme Digestion and Agarose Gel Electrophoresis

Purified plasmid DNA was digested with various restriction endonucleases as dictated by the manufacturer. The total reaction volume consisted of plasmid DNA,

restriction buffer, restriction endonuclease, sterile distilled water and amounted to between 20-40µl depending on the quantity of DNA digested. Approximately 1µg of plasmid DNA was completely digested after 1 hour incubation at 37C.

The fragmentation pattern of the restricted plasmid DNA was analyzed by electrophoresis through horizontal agarose slab gels as described (290). The choice of gel medium was determined by the molecular weight range of the DNA fragments of interest. Visualization of the restriction fragmentation pattern was facilitated by the addition of EtBr to the melted agarose gel at 0.5µg/ml of gel solution. The banding pattern was photographed while the gel was transilluminated with long wavelength ultraviolet light. Fragment sizes were determined by plotting the distance travelled (mm) from the gel wells against the log derivation of the size (basepairs) of the DNA markers which were lambda phage DNA digested with HindIII and ØX174 digested with HaeIII. A best-fit straight line was drawn through the points and the sizes of the test restriction fragments of interest were determined given their respective migratory distance through the gel.

Subcloning of DNA Fragments

To facilitate subcloning of DNA fragments from other recombinant plasmids, the DNA subfragment of interest was purified from low melting point agarose gels as previously described (291). Usually approximately 50µg of plasmid DNA was digested with the appropriate restriction enzymes at a ratio of 2 units/µg DNA overnight in a volume of 60µl. Long thin wells were made in the low-melting point agarose gel to facilitate loading of the large digest volume and reduce the possibility of smearing of the DNA due to overloading. The purified DNA fragment was quantitated by A_{260}. All subcloning was done into the Promega Biotech vector pGEM-3. Routinely the ligation reactions consisted of 200ng of pGEM-3 vector digested to produce compatible ends with the fragment to be

subcloned, from 500ng to 1000ng of the purified DNA fragment, 5 x ligase buffer (0.3M Tris-HCl pH 7.5, 0.04M $MgCl_2$, 0.005M ATP, 50µM beta-mercaptoethanol, 250µg/ml BSA), 0.01M ATP, and 1-6 units of T4 DNA ligase in a total volume of 20µl. The reaction was allowed to proceed overnight at 15C or at room temperature for at least 4 hr. The reaction was terminated by incubation at 65C for 10 min. Then 4µl of the ligation mixture (up to 240ng) was used to transform 100µl of bacterial cells as described above. The transformed cells (100-200µl) were spread on L-broth agar plates which contained 200µg/ml ampicillin. The plate had been previously prepared by spreading 200µl of L-broth on its surface which contained 50µl of 2% X-gal, 20µl of 2% IPTG, and 200µg/ml ampicillin. The solution was allowed to absorb into the agar plate before plating of the transformed cells for growth at 37C overnight. Bacterial colonies which contained recombinant plasmid appeared white since the lac-alpha peptide was disrupted by ligation of the DNA fragment of interest into the multiple cloning site of pGEM. As a result, ß-galactosidase activity could not be induced by isopropyl-thiogalactoside (IPTG) to catalyze the chromogenic substrate X-gal to a blue color.

Usually five to ten white colonies (putative subclones) were picked with sterile toothpicks to innoculate individual 3ml aliquots of L-broth plus 200µg/ml ampicillin. The subclones were grown overnight in a shaking incubator at 37C and plasmid DNA was isolated following the alkaline lysis method described (290). The mini-prep DNA was dissolved in 25µl TE pH 7.5 and 5-10µl of this solution was digested with the appropriate enzymes to release the subcloned DNA fragment. The digested DNA was analyzed by agarose gel electrophoresis as described above.

DNA Labelling

During the initial stages of this work purified DNA fragments and intact recombinant plasmids were labelled with [^{32}P] by nick-translation (292). Usually 100-

200ng of DNA was used per reaction. Nick-translations were performed as described by the supplier of the kit which contained a solution of deoxyribonucleotide triphosphates and an enzyme solution consisting of DNA polymerase and DNase I. Usually 70-100μCi of 3000Ci/mmole [alpha-^{32}P]dCTP was added to each reaction. The unincorporated label was removed by chromatography of the nick-translation reaction over a 3.5ml column of G100 equilibrated with TE pH 7.5. The specific activity of the resultant probe ranged from 1-5 x 10^8cpm/μg DNA.

The second and preferred method of DNA labelling was by random-primer labelling kit (293). This method required only 25ng of DNA and 50μCi of 3,000Ci/mmole [alpha-^{32}P]dCTP per reaction. Moreover, the DNA could be labelled while still in a solution of low-melting point agarose. This form of labelling is based on polymerization from a single stranded template primed with a random-sequence 6-base oligodeoxyribonucleotide. Priming and synthesis of DNA by DNA polymerase I (Klenow fragment) occurs along the entire length of the DNA fragment of interest. Unincorporated label was removed as described above. Routinely a specific activity of 1-5 x 10^9cpm/μg was obtained.

Isolation of Chromosomal DNA

Total cellular DNA was isolated from cell lines and human tissues essentially as described by Davis *et al* (294). Tissues were stored frozen at -70C. To facilitate the extraction of DNA, the frozen tissue was immersed in liquid nitrogen while the stainless steel receptable of a small Waring blender was also cooled down with liquid nitrogen. Then the frozen tissue plus a small volume of liquid nitrogen, sufficient to keep the tissue frozen, was poured into the blender receptacle and the rubber top was held on with an asbestos-gloved band. The tissue was reduced to a powder with a few short bursts of blender

action. With a spoon, cooled by liquid nitrogen, the powdered tissue was removed and placed in a 15ml snap-top Falcon tube which contained room temperature lysis buffer (0.1M Tris-HCl pH 8.0, 0.1M NaCl, 0.005M EDTA, 1% SDS and 100µg/ml proteinase K) at a ratio of 1ml lysis buffer per 0.5g of tissue. This mixture was incubated overnight in a 37C waterbath. Subsequently, RNaseA at 10mg/ml was added and incubated at 65C for 30 min. Then 1ml of sterile TE pH 7.5 was added followed by 2ml of 0.01M Tris-HCl pH 8.0 equilibrated phenol. The mixture was gently mixed and then centrifuged at 2500x g (4C) for 10 min. The agueous phase was removed with a P1000 pipetman (Gilson Medical Electronics, France) such that the end of the plastic pipet tip was cut off to increase the diameter of the opening. This was done to facilitate uptake of the very viscous aqueous phase and to avoid shearing of the high molecular weight DNA. Because the upper phase was often extremely viscous, uptake of the aqueous-organic interface was unavoidable but this was removed in subsequent organic extractions. The aqueous phase was then extracted with an equivalent volume of phenol:chloroform followed by a chloroform extraction. The DNA was then ethanol precipitated at -20C, centrifuged at 10,000 x g (4C) for 10 min; the pellet washed with 70% EtOH, briefly dessicated and dissolved in an appropriate volume of TE pH 8.2 at 4C overnight. Chromosomal DNA was isolated from fresh or frozen cell pellets using this protocol at a ratio of 1 x 10^9 cells/ml of lysis solution. The cell pellets were placed directly into the appropriate volume of lysis solution and gently swirled to resuspend the cells since mechanical disruption was not necessary.

Southern Transfer and Hybridization

Plasmid DNA fractionated by agarose gel electrophoresis was transferred to nitrocellulose as described by Maniatis *et al* (290). The nitrocellulose filters were hybridized with [^{32}P]-labelled DNA prepared as described above in an aqueous

hybridized with [^{32}P]-labelled DNA prepared as described above in an aqueous hybridization solution which consisted of 6 x SSC (SSC=0.15M NaCl-0.015M sodium citrate), 0.1% SDS, 1 x Denhardt's solution (1x=0.2% each of BSA, Ficoll, and polyvinylpyrrolidone) and 0.1mg/ml of sheared, denatured salmon sperm DNA. The hybridization reaction was carried out in plastic "heat-sealed" bags cut to dimensions slightly larger than those of the nitrocellulose filter and containing 100µl of hybridization solution per cm^2 of nitrocellulose. The hybridization bag was then incubated in a shaking waterbath at 65C ovenright. Plasmid Southern hybridizations were also performed in a solution composed of 50% deionized formamide, 5 x SSC, 1 x Denhardt's solution, 0.1mg/ml sheared, denatured salmon DNA, 0.1% SDS, 0.05M sodium phosphate pH 6.5 and 0.002M EDTA. These hybridizations were carried out at 42C for 24 hr. In both cases the [^{32}P]-labelled DNA probes were denatured by boiling H_2O for at least 5 min and then plunged into ice for an equivalent period before transfer to the hybridization solution already in the "heat-sealed" bag with the nitrocellulose filter. Usually 1 x 10^{6}cpm/ml or less of labelled probe was added to each hybridization reaction.

The nitrocellulose filters were then washed at room temperature four times in 2 x SCC, 0.1% SDS for 10 min intervals, followed by two washes at 60-65C in 0.1x SSC, 0.1% SDS for 30 min intervals. Blots were exposed to Kodak XAR film at -70C with an intensifying screen (Eastman Kodak, Rochester, NY) which constituted optimal autoradiographic conditions.

For genomic Southerns 15-20µg of DNA was digested according to the suppliers instructions with regard to optimum reaction buffer requirements. Two to three units of enzyme per microgram of DNA was used and digestion overnight was done to ensure complete cutting of the DNA in a volume of 40-60µl. The DNA was then fractionated on 0.8% agarose gels at 28 volts and constant current overnight using the TBE (290) gel running buffer system. The gels were then stained in 1-2 litres of 1 x TBE plus 0.5µg/ml EtBr for about 45 min and photographed by ultraviolet transillumination. The

gels were then treated 2 x 30 min in 1.5M NaCl, 0.5M NaOH followed by 2 x 30 min in 1M NH_4CH_3COO, 0.02M NaOH; the latter solution was used as the transfer medium. Southern transfer to nitrocellulose or nitroplus-2000 was allowed to proceed for at least 20 hr after which the blots were air-dried and baked for 1 hr at 80C under vacuum and then stored at 4C.

Prior to hybridization, the membrane was wet on 4 x SSC. The hybridization was performed at 42C for 24-48 hr in 50% deionized formamide, 6 x SCP (1xSCP=0.1M NaCl, 0.03M Na_2HPO_4, and 0.001M EDTA pH 6.2), 1% Sarkosyl, 200μg/ml sheared and denatured salmon DNA, 4 x Denhardt's solution and 10% dextran sulfate. The last ingredient was dissolved by vigorous agitation at medium heat on a stir-plate; the hybridization solution was then degased. Blots were prehybridized at 42C for a minimum of 1 hr in the previously described solution without dextran sulfate at a ratio of 100μl solution per cm^2 of nitrocellulose membrane. Genomic DNA blots were then hybridized with 3-7 x 10^6cpm/ml of random-primer labelled DNA fragments. The various genomic hPRL DNA probes described earlier were subcloned into pGEM-3 to facilitate gel-purification of large quantitites of the fragments of interest. A probe specific for the unique 5' untranslated sequence of IM-9-P/decidua PRL mRNA was constructed from an EcoRV-HindIII fragment of IM-9-P PRL cDNA #3. This 94bp fragment contained 72bp of unique 5' untranslated sequence extending 5' from the EcoRV site and contiguous with 22bp of 3' untranslated sequence of hPRL cDNA. The 3' non-coding region corresponded to the sequence between nucleotide 41 and 63 3' of the ochre codon. This unusual construction was due to a cloning artifact which occurred during cDNA synthesis and described in the "Results". This 94bp fragment was gel-purified and the HindIII terminus made flush with DNA polymerase (290). A large excess of this fragment was ligated into the SmaI site of pGEM-3 as described above in order to optimize the formation of 94bp concatomers. A subclone was isolated which contained a direct repeating tetramer of this fragment and thus provided a template of sufficient length (311bp when excised by XbaI-EcoRI) for efficient

were hybridization signals detected which were attributed to the 22bp 3' UTR sequence of this probe. This was confirmed by hybridization of the genomic DNA blot in question with a 3' end-specific hPRL genomic probe described earlier.

After hybridization, washes were carried out at 65C for two 15 min intervals in 6.6 x SCP-1% Sarkosyl followed by two 1.5 hr washes in 1 x SCP-1% Sarkosyl. If the background radioactivity remained high as judged with a hand-held radioactivity monitor, the blot was washed further in 0.1 x SCP-1% Sarkosyl at 65C until the background signal was reduced to an acceptable level. Genomic DNA blots underwent multiple separate hybridizations with different probes and were stripped of residual radioactive signal between experiments by 2-3 min incubations in boiling, sterile, distilled water. Blots were subjected to 24hr autoradiography to ensure removal of radioactive bands from previous hybridizations. To determine if common fragments were detected using different probes, the autoradiograms were aligned at the wells with the aid of radioactive orientation marks placed alongside the blots with radioactive ink. A hybridizing band was considered to be common to different probes if the band was directly superimposable over several different blots and hybridizations.

Isolation of RNA

Over the course of these studies, three different but related RNA extraction procedures were used. Initially the method of choice was the guanidine-HCl protocol which was developed by Protter *et al* (295) and described in detail elsewhere (291). Briefly, this method was based on the extraction of RNA from an aqueous-organic insoluble interface with a buffer containing 0.5% SDS, 0.1M NaCl, 0.01M EDTA, 0.05M Tris-HCl pH 9.0. The key ingredient in the extraction buffer was SDS since its inadvertent exclusion resulted in practically no RNA recovery. The second procedure used extensively

in this work was the guanidinium isothiocyanate-cesium chloride cushion method of Chirgwin *et al* (296). The third method utilized for RNA extraction was a modified version of that of Chromczynski and Sacchi (297). In this method the frozen tissue was homogenized using a polytron in a solution of 4M guanidinium isothiocyanate, 0.005% Sarkosyl, 0.025M sodium citrate and 0.007% vol/vol ß-mercaptoethanol at a ratio of 0.5-1.0g tissue/ml. After a brief homogenization (2 x 45 sec), 0.5ml of 2M $NaCH_3COO$ pH4.7 + 5ml equilibrated phenol + 1.0ml chloroform/isoamyl alcohol was added and the solution mixed vigorously. To separate the phases, the mixture was centrifuged at 4000 x g (4C) for 30 min. The nucleic acid in the aqueous phase was precipitated with an equivalent volume of isopropanol at -20C for approximately 45 min. The precipitate was centrifuged for 15 min at 10,000 x g (10C). The resultant pellet was then resuspended in 3ml TE pH 7.5 and extracted with 2ml equilibrated phenol, 0.3ml 2M $NaCH_3COO$ pH 4.6, 0.5ml chloroform/isoamyl alcohol and centrifuged for 15 min, 10,000 x g (10C). The aqueous phase was then extracted with an equivalent volume of chloroform/isoamyl alcohol and centrifuged as above. The RNA was precipitated from the aqueous phase with an equivalent volume of isopropanol at -20C for 30 min. The RNA was pelleted by centrifugation at 10,000 x g (4C) for 10 min, washed with 70% EtOH, dessicated and dissolved in sterile distilled water or TE pH 7.5. The yield of RNA was determined by A_{260}.

Poly(A)$^+$-enriched mRNA was isolated from total RNA by one cycle of oligo(dT) cellulose chromatography (298) or by batch absorption. In the latter technique, approximately 1ml of oligo(dT) cellulose slurry was treated with 0.1M NaOH by gentle vortexing and centrifugation at maximum speed in an IEC HN-S benchtop centrifuge for 2 min. The supernatant was poured off and the oligo(dT) cellulose was gently resuspended in 10ml of sterile 1x TKE (0.01M Tris-HCl pH 7.5, 0.001M EDTA, 0.5M KCl) and centrifuged as above to remove all traces of NaOH. The oligo(dT) cellulose was resuspended and approximately 0.25ml of the slurry was transferred to a sterile 1.5ml

microcentrifuge tube. Then 1-2mg total RNA in 0.75ml of TE pH 7.5 was denatured in a boiling water bath for about 5 min and plunged in ice. When the RNA had cooled down, 0.25ml of cold 2M KCl was added to give a final concentration of 0.5M KCl. The RNA was added to the oligo(dT) cellulose slurry and mixed by manual agitation for about 5 min. This was then centrifuged at maximum speed in a benchtop IEC Centra-M centrifuge for 1 min at room temperature and the supernatant containing free RNA was removed. Another 0.75ml of 1 x TKE was added to the oligo(dT) cellulose slurry, mixed gently and centrifuged as above. This washing step was repeated once more to ensure removal of all unbound, presumably poly(A)$^-$ RNA. To remove the bound poly(A)$^+$ mRNA, 0.2ml of room temperature sterile TE pH 7.5 was added to the oligo(dT) cellulose, mixed, centrifuged as above and the supernatant placed on ice. This was repeated and the supernatants were pooled and ethanol precipitated at -20C. The quantity of poly(A)$^+$ mRNA obtained ranged from 3-5% of the total RNA.

Northern Transfer

The RNA was fractionated on 1.3-1.5% agarose, 2.2M formaldehyde, horizontal slab gels containing 0.5μg/ml EtBr as described (299). The RNA gel was then photographed by ultraviolet transillumination and transferred to nitrocellulose, Nitroplus-2000 or Hybond-N matrix by capillary action for 24 hours (299). The Northern blot was then air dried and ultraviolet transilluminated to visualize the EtBR stained RNA; the 18S and 28S ribosomal RNA were easily visible and their locations marked on the nitrocellulose with a ballpoint ink pen. The blots were baked for at least 30 min at 80C under vacuum and stored at 4C until probed.

cRNA Synthesis

IM-9-P PRL cDNA fragments containing portions of the 5' untranslated sequence were subcloned into pGEM-3 and [^{32}P]-labelled antisense or sense cRNA was synthesized using Sp6 or T7 RNA polymerase (300) as outlined by the vendor of the *in vitro* transcription kit. Complementary RNA was synthesized from a blunt-ended EcoRV-EcoRI fragment of IM-9-P PRL cDNA IV cloned into the SmaI site of pGEM-3. This fragment contained the entire 93bp unique 5' UTR of IM-9-P/decidua PRL mRNA and was sequenced on both strands. Antisense cRNA was synthesized by T7 RNA polymerase on the HindIII digested template described above. Sense cRNA was also made using Sp6 RNA polymerase on a EcoRI digested template. The unique 5' UTR of IM-9-P PRL cDNA I-1 was subcloned as a HincII-EcoRI fragment into the corresponding restriction sites of pGEM-3. The sequence of the 143bp fragment was obtained from both strands and antisense cRNA was synthesized from an EcoRI digested template using Sp6 RNA polymerase. The sense cRNA was generated by T7 RNA polymerase on PstI digested template. The size and integrity of each cRNA product was analyzed by electrophoresis on a 6% polyacrylamide sequencing gel adjacent to a sequencing ladder.

Northern Hybridization

Northern blots were prehybridized in a 42C shaking waterbath for at least 2 hr in the hybridization solution of choice. Two similar hybridization solutions were used, one was that described by Thomas (299) and the other consisted of 50% deionized formamide, 0.1% sodium pyrophosphate, 0.025M sodium phosphate pH 6.5, 10 x Denhardt's solution, 0.2% SDS and 4 x SET (1 x SET=0.15M NaCl, 0.02M Tris-HCl pH 7.8, 0.001M EDTA). Hybridization was performed in "heat-seal" plastic bags containing 100µl

of solution per cm^2 of blotted membrane. The cDNA probes were denatured in boiling water for at least 5 min and plunged in ice; the cRNA probes were denatured for 1-2 min. The hybridization reaction at 42C proceeded for at least 20 hr with the addition of [^{32}P]-labelled probe at 10^6-10^7cpm/ml to the hybridization medium. All Northern hybridizations carried out with cRNA probes were done using the 50% formamide/SET buffer described above at 60-65C. Low stringency hybridization with cRNA probes was done by lowering the incubation temperature to 40C.

After hybridization the membrane was washed at room temperature four times in 2 x SSC, 0.1% SDS for 10 min intervals, followed by two high stringency washes at 65-70C in 0.1 x SSC, 0.1% SDS for 30 min intervals. Blots were then exposed to X-ray film as described above.

RNase Digestion of Poly(A) Tracts

RNaseH will selectively degrade poly(A) tracts of mRNA when it is in a double stranded form in the presence of oligo(dT) (301). Fifty micrograms of IM-9-P total RNA and 7μg human pituitary total RNA were hybridized to oligo(dT) 12-18 (Pharmacia) at a ratio of 8:1 in 0.1M KCl at 37C for 30 min, followed by denaturation at 65C for 2 min. Two units of RNase H (Pharmacia) were then added plus one equivalent volume 2 x Pharmacia RNase H digestion buffer (0.08M Tris-HCl pH 8.0, 0.008M $MgCl_2$, 0.002M DTT, 60μg/ml BSA), and the reaction was carried on for 20 min at 37C. This digestion reaction was then extracted with an equivalent volume of phenol-chloroform, ethanol precipitated, and subjected to electrophoresis on denaturing gels, as described above.

Construction and Screening of cDNA Libraries

Two cDNA libraries were generated from 10μg of IM-9-P lymphoblast poly(A)$^+$ mRNA template in lambda GT10 using conventional methods (302). Oligo(dT) was the first strand primer and the second strand was generated by the RNase H procedure (303). Approximately 1μg of the cDNA lambda GT10 ligation reaction was packaged and recombinant phage were plated on E.coli MA150. Both IM-9-P cDNA libraries contained approximately 8.5 x 10^5 recombinant phage/μg of DNA. Amplified and unamplified portions of these cDNA libraries were blotted onto nitrocellulose (304) for screening with either nick-translated or random-primer labelled, gel-purified fragments of pituitary hPRL cDNA (10). Hybridizations were carried out at 42C in 50% deionized formamide buffer (299) as described above. Positive clones were purified through three rounds of screening. A total of 150,000 recombinant clones were screened from the two unamplified libraries. In addition 100,000 recombinant phage were screened from amplified fractions of each library in the same manner. Approximately 300,000 phage were screened from an amplified human placental library (Clontech) in E.coli 803 using random-primer labelled rat prolactin-like protein A (28) and B (29) cDNAs under moderately stringent conditions of 60C in 6 x SSC, 1 x Denhardt's solution, 0.1mg/ml denatured, sheared salmon testes DNA, and 0.1% SDS. These probes were used in an effort to identify human homologues of the rat genes. Positive clones were then rescreened with random-primer labelled pituitary hPRL cDNA fragments under high stringency conditions (68C) to identify decidual PRL cDNAs. An amplified human decidual lambda GTII library (280) in E.coli 803 was screened with IM-9-P PRL cDNA IV using high stringency conditions identical to those described for analysis of the IM-9-P lymphoblast libraries. Approximately 500,000 phage from this library were screened and a final screening of purified decidual PRL cDNA clones was carried out to identify those which contained 5' untranslated sequence. For those isolated from the human placenta cDNA library, a cRNA probe was generated by

Sp6 RNA polymerase from a recombinant pGEM-3 template which contained an EcoRV-EcoRI fragment of IM-9-P PRL cDNA IV as described above. This hybridization was performed at 42C in 50% deionized formamide solution (299). For the human decidua cDNA library, 5' specific dPRL cDNA clones were selected from the initial positive plaques by hybridization with a 311bp tetramer of unique 5' untranslated sequence from IM-9-P PRL cDNA #3 described above.

Lambda phage DNA of the positive clones was isolated from confluently lysed plates overlayed with 5ml SM buffer (290) and shaken at room temperature for 2-24 hours. The eluted phage was collected from the surface of the plate in SM and incubated at 37C for at least 1 hr with DNase I (6μg/ml) and RNase A (3μg/ml). To remove the bacterial debris the above mixture was centrifuged at 2500 x g (4C) for 30 min. The supernatant was saved and the phage particles precipitated by the addition of an equivalent volume of 20% polyethylene glycol (M.W. 8000), 2M NaCl in SM. The solutions were mixed and placed on ice overnight. The phage were pelleted by centrifugation as above, the supernatant discarded, the wall of the tube wiped dry of residual supernatant and the phage resuspended in 0.5ml SM. RNase A to 400μg/ml was added to the phage and incubated for 30 min at 37C; this was followed by 10μl of freshly prepared 20mg/ml proteinase K which required incubation at 65C for 1 hr. The phage were lysed by making the mixture 5μM EDTA, 0.02% SDS and incubating an additional 30-40 min at 65C. Subsequently the lysed phage solution was extracted with an equal volume of phenol, phenol-chloroform and lastly with chloroform. The aqueous phase was recovered and the residual polysaccharide precipitated by ammonium acetate to 2.5M overnight on ice. The precipitate was removed by centrifugation as above and the lambda phage DNA in the supernatant was precipitated with an equal volume of isopropanol. The phage DNA was pelleted by centrifugation, washed with 70% ethanol, dessicated and dissolved in 50-100μl TE pH 8.2.

The purified lambda phage clone DNA was digested with EcoRI for size fractionation and subcloning of the cDNA insert into pGEM-3. The EcoRI digest consisted

of 25µl of phage DNA incubated in a total volume of 50µl which contained restriction buffer, 1µl of 10mg/ml RNase A, spermidine to 0.005M and 1µl of high concentration EcoRI incubated overnight at 37C. The size of the cDNA insert was determined by electrophoresis on 1.3% agarose gels as described above. Southern blots were generated from these gels and probed with pituitary hPRL cDNA.

The EcoRI fragments of IM-9-P and decidual PRL cDNA were subcloned into pGEM-3 as described earlier using 200ng of EcoRI digested pGEM-3 and up to 600ng of EcoRI digested lambda clone DNA in a volume of 20µl. Subclones containing PRL cDNA inserts were identified and the recombinant plasmids amplified and purified as described earlier.

DNA Sequencing

All nucleotide sequencing of DNA was done from pGEM-3 by the dideoxy chain termination method (305) in the presence of [alpha-^{35}S]dATP according to the Promega Biotech sequencing kit protocol. Samples were analyzed in parallel by denaturing 6% polyacrylamide-7M urea gel electrophoresis at approximately 30 watts per gel for 1.75 to 3.75 hours. Sequencing gels were then fixed for 20 min in 10% methanol-10% glacial acetic acid and dried using a Biorad model 1125B slab gel dryer. Autoradiography was performed at room temperature for 12-24 hr.

Primer Extension Analysis

A 21-base oligodeoxynucleotide (5'TGCCAGAGCGTGGCCCCCTTG3') complementary to amino acids +1 to -6 of the human preprolactin molecule was end-

labelled using [gamma-^{32}P]ATP and T4 polynucleotide kinase (306). The primer was annealed to samples of total or poly(A)$^+$ mRNA up to 40µg in quantity and primer extension reactions were carried out essentially as described by Duckworth *et al* (28). The end-labelled antisense primer was separated from unincorporated [gamma-^{32}P]ATP by chromatography over a 5ml Sephadex G-25 column equilibrated with 0.05M ammonium bicarbonate. The peak fractions were dried in a Savant Speed Vac and the labelled oligonucleotide was resuspended in 80µl of 0.4M KCl to a final concentration of 0.25 picomoles/µl. The RNA samples were ethanol precipitated, air dried and resuspended in 4µl of end-labelled antisense primer by repeated pipetting and subsequently hybridized at 37C for one hour after denaturation in boiling water for 1 min.

Synthesized cDNAs were resolved on a denaturing 6% polyacrylamide gel described above. A set of DNA sequencing reactions and end-labelled HpaII digested pAT153 plasmid DNA were run in parallel lanes to serve as size markers.

RESULTS

Characterization of Putative PRL-like RNAs in Rat

It had been observed by a number of investigators in Dr. H.G. Friesen's laboratory that rPRL cDNA (274) under high stringency conditions hybridized to a ubiquitously expressed RNA of approximately 1.7kb. As shown in Figure 1, in those rat tissues where the 1.7kb RNA was abundant, the presence of a number of other smaller RNAs was also detected. The most intense or abundant RNA species which hybridized to rPRL cDNA comigrated with the 1.6kb DNA marker band in good agreement with previous studies done by other investigators. The less abundant and smaller RNAs which hybridized were sized at 1.2, 1.1, 0.7, 0.57, 0.5 and 0.3kb. These putative rPRL RNAs did not represent non-specific hybridization to 18S rRNA as there was no signal obtained with 5μg of rat testes total RNA in Figure 1. The EtBr stained gel prior to Northern transfer to nitrocellulose showed that the RNA samples were intact and verified the presence of RNA in the testes lane.

The rPRL-like RNA was detected in all rat tissues examined using 30μg of total RNA by Northern blot hybridization. This appeared to be an all or none phenomenon since one group of rats tested did not possess the 1.6kb RNA of interest in any of the many tissues tested (Fig.2). The seven different non-hybridizing rat tissue RNAs analyzed were obtained from a group of intact, approximately 350gm males which had not been subjected to any experimental procedure. These results confirmed that the 1.6kb RNA species was not 18S rRNA since the EtBr-stained gel clearly showed the presence of a strongly staining 18S rRNA band in every lane. Moreover, these results were not due to an artifact of the RNA isolation protocol since different procedures were used on portions of the same tissue.

Oligo(dT) cellulose chromatography of total RNA was done to determine whether the putative PRL-related 1.6kb RNA contained a 3' poly(A) tail typical of eukaryotic mRNAs. Two sets of Northern blot experiments were done in which rat RNA enriched in polyadenylated mRNA was isolated and fractionated alongside total RNA and the oligo(dT) column flow through (poly(A)$^-$ RNA) from the same tissue. Ten different tissue RNAs were treated in this manner and hybridized with rPRL cDNA under high stringency conditions. One set of RNA blots contained 1.5-2.0μg poly(A)$^+$ RNA from various rat tissues which is equivalent to 30μg of total RNA since it is generally accepted that mRNA comprises 3-5% of total RNA in eukaryotic cells. Thus the putative PRL-like 1.6kb mRNA hybridization signal theoretically should be of equivalent intensity in poly(A)$^+$ and total RNA lanes with no signal in the poly(A)$^-$ RNA fraction. Another set of blots were made containing 10μg of each poly(A)$^+$ RNA fraction. The results of two of these experiments is shown in Figure 3. In those RNA blots containing 10μg of poly(A)$^+$ mRNA a signal corresponding to the 1.6kb RNA of interest was barely discernible compared to the strong and equivalent signals in poly(A)$^-$ and total RNA lanes (Fig.3a). That the poly(A)$^+$ RNAs were highly enriched in mRNA was proven when the same blots were probed with pRP54 cDNA (Fig.3b), an abundant clone isolated from a rat placenta lambda GT10 cDNA library by Dr. Mary Lynn Duckworth (Department of Physiology, University of Manitoba) and found to be ubiquitously expressed in rat tissues. The approximately 1.0kb pRP54 hybridizing mRNA was present in the total RNA lanes and enriched in the poly(A)$^+$ RNA samples as expected for a cDNA isolated from an oligo(dT) primed cDNA library. These data strongly suggested that the putative PRL-related 1.6kb RNA was not polyadenylated. The weak signal seen in the poly(A)$^+$ RNA lanes (Fig.3a) was most likely due to carry over of non-polyadenylated RNA since enrichment for mRNA was done by single passage of total RNA over an oligo(dT) cellulose column. In Figure 3c strong and equivalent signals at 1.6kb in total and poly (A)$^-$ RNA samples were obtained

with virtually no detectable band in 2μg of poly(A)$^+$ RNA which confirmed the above result.

In order to localize the segment of rPRL cDNA which conferred cross-hybridization with the 1.6kb RNA species, purified subfragments of rPRL cDNA were used as hybridization probes. The rPRL cDNA was divided into three segments: a 5' 229bp fragment, an internal 336bp piece and a 3' fragment of 256bp. Of these fragments only the internal fragment encompassing 51% of contiguous coding region did not hybridize to the non-polyadenylated 1.6kb RNA (Fig.4). Vector DNA, pBR322, also did not hybridize. It was curious that the non-contiguous 3' and 5' end fragments which contain only about 25% of coding sequence each, could detect the 1.6kb RNA. These fragments, however, have in common the oligopolymeric tract of GC residues (15bp at the 3' and 5' end) which facilitated cloning into the PstI site of pBR322 (274).

The possiblity was tested that hybridization to the 1.6kb RNA was through the GC-tails and not specific to the rPRL coding sequence. A series of identical Northern blots were probed with various cDNAs, some of which were GC-tailed. The results of some of these experiments are shown in Figure 5. Only the GC-tailed cDNAs of rPRL, probasin or M-40 (279) and one of the rat placental PRL-like protein-A (rPLP-A,18) cDNAs, pRP6-1, hybridized to the 1.6kb non-polyadenylated RNA. In other experiments done in the same manner, pRP52 and 52A cDNAs of rat placental lactogen II (rPLII,26) and pRP9 cDNA of rat placental PRL-like protein B (rPLP-B,29) also detected the 1.6kb RNA. These cDNAs are GC-linked as they were isolated from a plasmid cDNA library. The EcoRI linked rPLP-A cDNA, pRP6-5, did not hybridize nor did the rGH cDNA (275), the rPLII cDNA, pRP52B or pRP9A, an rPLP-B cDNA. These cDNAs are either HindIII or EcoRI linked to plasmid DNA (data not shown). These results proved that hybridization to the 1.6kb non-polyadenylated RNA was not due to homology with rPRL sequence or a related sequence. Only those cDNAs which contained GC-tails, despite identical overlapping cDNA sequence (ie. pRP6-1 vs pRP6-5) hybridized, which strongly indicated that this

cloning by-product was responsible for hybridization to the ubiquitous 1.6kb non-polyadenylated RNA.

Origin of the IM-9-P Cell Lines

During the screening of a number of different human cell lines for the presence of PRL or PRL-related molecules, the human IM-9 lymphoid cell line in our laboratory was found to transcribe an mRNA that hybridized to hPRL cDNA under high stringency conditions (Fig.6). The IM-9 line was first described by Fahey and co-workers in 1971 (307) as a B-lymphoblastoid line generated from the bone marrow of a female patient with multiple myeloma. Over the years this line has been of particular interest to endocrinologists because it contains a large complement of insulin and GH receptors and has been an important tool in the development of radioreceptor assays for these hormones (308,309). A single mRNA species of approximately 1.0kb was detectable in both total and poly(A)$^+$ enriched mRNA samples isolated from IM-9 cells. A difference in the electrophoretic mobility between IM-9 and human pituitary PRL transcripts was evident with the lymphoid transcript an estimated 150 nucleotides longer than that of the human pituitary. Since the hybridizations had been carried out at high stringency, it was assumed that the band detected represented authentic hPRL mRNA. Northern blots containing IM-9, rat placenta and human placenta total RNAs were hybridized under conditions of low stringency (37C/50% formamide hybridization solution) with cDNAs of rPLP-A (28), rPLP-B (29), rPLII (26) and hPL (276) in an effort to determine whether IM-9 cells also expressed other members of the PRL-GH gene family. No specific hybridization with these probes to IM-9 RNA was detected (data not shown). The species specificity of the Northern blot analysis in Figure 6 was demonstrated by the failure of hPRL cDNA to cross-hybridize with rat pituitary total RNA. This result eliminated the possibility that the

IM-9 line in our laboratory was contaminated with a rat pituitary cell line producing PRL. Interestingly, RNA from IM-9 cells obtained from different sources did not hybridize with hPRL cDNA. The integrity of these RNAs was demonstrated by the detection of intact transcript for the oncogene, c-myc (310) in those lanes in Figure 6. The PRL-producing IM-9 cells from our laboratory were designated IM-9-P to distinguish them from the non-PRL-producing IM-9 lines.

This observation prompted further investigation as to the identity of the IM-9-P line. A cloned member of the human interspersed AluI repeat sequence family (277) hybridized specifically, as a smear, to IM-9-P DNA but not rat testes DNA which confirmed the human origin of the line (Fig.7a). A fetal sex test probe, pY3.4 which hybridizes to a chromosome Y-specific repetitive element of approximately 3.5kb in EcoRI digested human DNA (278) did not hybridize to IM-9-P DNA (Fig.7b) proving that the cell lines were derived from a human female, in agreement with the original derivation of the IM-9 line from a female patient.

The original IM-9 line is known to synthesize immunoglobulins (311). Conditioned media from IM-9-P cells and classical IM-9 cells were assayed for human IgG by ELISA. Both lines were found to secrete 760-880ng/10^6 cells/24hr, consistent with IM-9-P cells being B-lymphoblastoid in derivation. The PRL-producing IM-9-P cells were tested for the presence of mycoplasma contamination in November 1986 using a commercially available assay based on hybridization of mycoplasma ribosomal RNA in cell culture medium to a specific [^{3}H]-labelled DNA probe. A positive control gave 50% hybridization to the labelled mycoplasma rRNA probe whereas the negative control was less than 2%. Duplicate IM-9-P cell samples gave approximately 26% hybridization which indicated the cells were contaminated. It was not known whether the cells had been contaminated when aliquots had been initially placed in frozen storage or when the line was thawed and cultured for these studies. The contaminated IM-9-P cells were treated over a period of 4 weeks with a series of antibiotics provided in a commercially available kit. The

IM-9-P cells were analyzed by liquid hybridization for the presence of mycoplasma after the treatment and tested negative on two separate occasions. However, after approximately 7 months of continuous culture, the ability of IM-9-P cells to secrete IgG was lost, whereas there was no change in this phenotype for classical IM-9 cells. Concomitant with the loss of IgG secretory ability was a diminished capacity of IM-9-P cells to synthesize PRL, as measured by Northern analysis.

To recapture a high PRL-production phenotype, the IM-9-P line was cloned by limiting dilution. Approximately 20% of the seeded wells developed IM-9-P colonies of which 56 were selected for further expansion and testing. Two clones were picked for future experimentation; a high PRL producer, IM-9-P3, and a non-PRL producer, IM-9-P6. These IM-9-P clones did not secrete IgG as measured by ELISA.

To confirm the B-lymphoblastoid origin of the IM-9-P cell line and the clonal lines derived from it, analysis of Ig gene rearrangement, a unique characteristic of B-cells, was performed. Genomic Southern analysis was done using a 5.6kb human DNA fragment encompassing the joining regions of the heavy chain Ig genes. In an ordered, step-wise fashion, rearrangement and expression of Ig genes includes at the earliest point the joining of a diversity segment to one of the six joining region genes (312). As can be seen in Figure 8a, the original IM-9 line has undergone rearrangement of the joining region germline 5.6kb band in both alleles to generate two fragments of approximately 9.2 and 4.2kb in BamHI/HindIII digested genomic DNA. The PRL producing IM-9-P3 clone, as well as the parent IM-9-P line, and the non-PRL-producing IM-9-P6 clone have conserved only the rearranged 4.2kb fragment with an apparent deletion of the 9.2kb fragment. A T-cell lineage for these cell lines was eliminated in a similar experiment in which genomic Southern analysis using a DNA probe to the T-cell receptor, ß-chain constant region detected no rearrangement from the germline configuration (Fig.8b).

A panel of 15 monoclonal antibodies (described in Materials and Methods) to various human leukocyte cell surface antigens were used to delineate the immunophenotype

characteristics of the original IM-9 cell line and the clonal IM-9-P3 line. The classical IM-9 cell line tested positive for the monoclonal antibodies B1, kappa light chain, IgG, I2, and negative for all other antibodies (Table 1). These four antigens are characteristic of the B-cell lineage. The clonal IM-9-P3 cell line exhibited reactivity with the monoclonal antibody I2 only (Table 1), which recognizes an HLA-D/DR-related, Ia-like antigen. The lack of reactivity with cell surface immunoglobulins corresponded to the previous finding that cell lines of the IM-9-P class no longer secrete IgG. As a further means of demonstrating or defining the relatedness of the classical IM-9 line and the PRL-producing IM-9-P3 clone, the cells were subjected to tissue-typing. By histocompatibility antigen testing the classical IM-9 line and the PRL-producing IM-9-P3 clonal line were found to possess identical haplotypes (Table I) which indicated a common line of parentage.

IM-9-P PRL Immunoreactivity

Human PRL had been previously detected in the cell lysates of various malignant cell lines but secretion into the culture medium was not detectable (122). To determine if the atypical PRL mRNA present in IM-9-P cells was translated into a secreted product, the presence of hPRL was assessed in conditioned medium. IM-9-P cells were cultured in serum-free medium for four days to eliminate the possibility of cross-reactivity with bovine PRL and the conditioned medium was analyzed by radioimmunoassay (RIA). IM-9-P cells were found to secrete immunoreactive hPRL, which exhibited a displacement curve essentially identical to that obtained with pituitary hPRL by RIA (Fig.9). The conditioned media from six other human lymphoid cell lines showed no cross-reactivity in the RIA.

Bioactivity of IM-9-P PRL

To demonstrate the biological activity of IM-9-P PRL, we utilized the rat Nb2 lymphoma bioassay. The Nb2 cell line is a rat T-cell lymphoma that is exquisitely dependent on the presence of lactogenic hormones (PRL, hGH, PL) or interleukin-2 for mitogenesis and shows a dose-dependent increase in cell number upon addition of these mitogens. Two fractions of immunoaffinity purified IM-9-P PRL as well as crude conditioned media obtained after four days of serum-free culture conditions and pituitary hPRL standard were serially diluted and added to growth-arrested Nb2 cells. The results shown in Figure 10a indicated that immunoreactive PRL secreted by IM-9-P cells was biologically active in the Nb2 lymphoma bioassay.

To confirm that no other factor in IM-9-P conditioned medium or co-purifying with IM-9-P PRL was responsible for the proliferative response of Nb2 cells a neutralization experiment was carried out. Two different amounts of IM-9-P conditioned medium were added to Nb2 cells in the presence or absence of monoclonal hPRL antibody 9C3. Figure 10b shows the results of these experiments. The stimulatory effect of IM-9-P conditioned medium on Nb2 cell growth was completely and specifically blocked by monoclonal antibody to hPRL. The concentration of monoclonal antibody 9C3 was sufficient to completely neutralize the activity of maximally stimulating doses of pituitary hPRL standard. The monoclonal antibody itself had no deleterious or non-specific toxic effects on the growth of Nb2 cells and was specific for hPRL as shown by the results in the presence of ovine PRL (Fig.10a).

hPRL Secretion by the Clonal IM-9-P3 Cell Line

The rate of hPRL secretion by the clonal IM-9-P3 cell line was measured over a four-day period. IM-9-P3 cells in stationary phase of growth were diluted to 1 x 10^5

cells/ml in RPMI/FCS and seeded in triplicate $25cm^2$ flasks. After 24, 48, 72, and 96h, 1ml of cell suspension was removed for determination of cell number. The remaining cells were pelleted at 250 x g for 4 min, and the supernatants were saved for analysis of hPRL content by RIA. The IM-9-P3 cell pellets were washed free of PRL with RPMI 1640 and resuspended in a reduced volume of fresh medium so as to keep the cell density unchanged.

As shown in Figure 11a, the secretion of hPRL paralleled the growth curve. The secretion rate was calculated to range from 40-50ng hPRL/10^6 cells/24 hr based on the assumption of exponential growth of the cells within the 24 hr observation periods. Extraction of logarithmically growing cells with PBS-0.1% Triton X-100 yielded an intracellular hPRL content of approximately 2.0ng/10^6 cells, as measured by RIA.

In a parallel experiment the level of IM-9-P3 PRL mRNA was examined. Total RNA was isolated from 1.5 x 10^7 cells on each day of the four-day cell density experiment and subjected to Northern hybridization analysis. It was apparent from the PRL transcript hybridization signal in Figure 11b that the abundance of this mRNA was unaffected and correlated with the constant level of PRL secretion by these cells over the same four-day growth period.

The Importance of PRL for IM-9-P Cell Viability

To determine the importance of PRL for IM-9-P cell growth a neutralizing agent, monoclonal anti-hPRL antibody 9C3, was added over a four-day growth period. This monoclonal antibody had previously been shown to completely block the mitogenic activity of hPRL on Nb2 lymphoma cells (Fig.10b). As can be seen in Figure12, the presence of the monoclonal antibody had no effect on the growth of IM-9-P cells (Fig.12). The successful neutralization of secreted hPRL was examined in a control experiment

where the IM-9-P conditioned media collected on day 4 were tested in the Nb2 bioassay (Fig.12, right panel). A significant stimulation of Nb2 cell growth was obtained using conditioned media from IM-9-P cells grown in the absence of antibody to hPRL. However, conditioned media from parallel cultures to which monoclonal antibody 9C3 had been added exhibited no mitogenic activity above control media from IM-9-P cells on the day-0 point of the experiment. Thus the PRL secreted by IM-9-P3 cells was completely neutralized and this had no deleterious effect on the growth of these cells over a 96 hr period.

Initial Analysis of IM-9-P PRL Protein, mRNA and Gene Structure

From Figure 6 it was clear that the IM-9-P PRL transcript was larger than its pituitary counterpart, yet experiments described above indicated that the protein was biologically active and immunoreactive. IM-9-P PRL was immunoaffinity purified and subjected to SDS-PAGE to compare the size of the IM-9-P secreted hormone to that of the human pituitary. The results in Figure 13 showed no difference in the mobility of the principal band in each of the lanes which meant that IM-9-P and pituitary PRL were identical in size despite the size difference between the corresponding mRNAs.

The difference in mRNA size could have been due to varying degrees of 3' end polyadenylation in the different cell types. This possibility was tested by comparing the electrophoretic mobility of the PRL mRNAs subsequent to removal of the poly(A) tail by RNase H. As can be seen in Figure 14, digestion of mRNA poly(A) tails did not eliminate the size differences between IM-9-P and pituitary hPRL transcripts.

Since the IM-9-P PRL protein was identical in size to the human pituitary protein, it was assumed that the protein coding region of the IM-9-P PRL mRNA was unaltered. It was thus not unreasonable to postulate that the intron/exon structure of the

PRL gene had not undergone a gross rearrangement in the IM-9-P3 genome even though the protein was produced ectopically and its transcript was atypical. To test this hypothesis genomic Southern hybridization analysis was performed on DNA isolated from a normal human placenta, classical IM-9 cells, and the PRL-producing IM-9-P3 line digested with six different restriction enzymes. These DNA blots were probed with pituitary hPRL cDNA (10) and DNA fragments of the hPRL gene (36). A cloned 557bp BglII/EcoRI genomic DNA fragment covering 165bp of exon 5 and 392bp of 3' flanking DNA was included in these studies to identify 3' end-specific fragments in the banding pattern and thus corroborate the experimental data with the published restriction map of the hPRL gene (see Fig.26). The same rationale applied to the utilization of a 5' end-specific genomic fragment encompassing 978bp of 5' flanking DNA, exon 1 and about 120bp of intron A. Moreover, in preliminary studies, and in the literature (35,36), it was evident that the pituitary hPRL cDNA appeared unable to detect genomic fragments containing only exon 1. Exon 1, including the 5' untranslated sequence is about 85bp in length; however, the pituitary hPRL cDNA used as a hybridization probe contains only 31bp of this sequence. Presumably there was insufficient complementary sequence length to allow stable hybrid formation between the random-primer labelled hPRL cDNA PstI subfragment and exon 1, under the stringent hybridization conditions employed in these studies.

In Figure 15a, DNAs had been cleaved with EcoRI, HindIII, and PstI and probed simultaneously in "a" with the pituitary hPRL cDNA and the 5' end-specific genomic fragment. This blot was then stripped of the radioactive signal and re-hybridized with the 5' specific probe alone, the result of which is shown in Figure 15b. This was done to identify those fragments in each digest which contained the 5' flanking region of the hPRL gene. The restriction fragment patterns were identical for all three DNAs in each digest. The additional under-represented low molecular weight band found in the human placenta DNA lanes of Figure 15 was unique to that particular DNA preparation. The

intensity of some of the bands in a particular set of digests varied because the overlapping complementary sequence between the hybridizing DNA species was short.

In other hybridization experiments with different DNA blots the pituitary hPRL cDNA alone was used as the probe. The hPRL gene restriction fragmentation pattern obtained with HindIII in Figure 15 implied that hPRL cDNA hybridized strongly to only one fragment which comigrated with the 9.4kb marker. This was confirmed in later experiments and a very weak signal was also reproducibly observed at approximately 5.0kb. DNA blots probed with the 3' end-specific genomic fragment hybridized strongly to the 9.4kb Hind III fragment as well as to the 5.0kb band seen only faintly with the hPRL cDNA. The 5.0kb HindIII fragment represented DNA extending into the 3' flanking region from a HindIII site located in the hPRL 3' UTR. Experiments performed with PstI and EcoRI digested DNAs probed with hPRL cDNA alone revealed a similar trend to those described for HindIII. That is, the 5' end-specific hPRL genomic probe detected DNA fragments not seen with pituitary hPRL cDNA and these 5' end genomic fragments corresponded to the bands seen in Figure 15b. As alluded to above, the strongest hybridizing band in each set of digested DNAs in Figure 15b contained exon 1 as the only coding region of the hPRL gene. The weaker band in HindIII and PstI digested DNAs represented further 5' flanking DNA, the origin of which will be discussed in greater detail in a later section. In EcoRI digested DNA, the 5' specific genomic probe detected one strong band of approximately 2750bp which contained exon 1 as the only coding sequence; this band was not observed when the pituitary hPRL cDNA alone was used as a probe. The 3'-end specific genomic probe did not detect fragments different from those seen with pituitary hPRL cDNA as the probe in PstI and EcoRI digested DNA. This genomic probe identified the approximately 4.1kb EcoRI fragment and the 1.2kb PstI band as the 3' end of the hPRL gene. In all experiments utilizing hPRL genomic probes, no bands unique to IM-9-P3 DNA were detected. Moreover, these experiments confirmed that pituitary hPRL

cDNA could not hybridize to 5' flanking DNA or genomic fragments containing only exon 1.

In Figure 15 "c" and "d" the results of similar experiments done using the enzymes NcoI and XbaI are shown. The nitrocellulose filter was initially hybridized with the pituitary hPRL cDNA alone. The blot was then sequentially stripped and reprobed separately with the 5' end-specific genomic fragment and 3' end genomic fragment. In NcoI digested DNAs, two extra bands were detected in the 7-8kb range in the human placenta DNA sample using the 5' specific genomic probe only, otherwise the NcoI banding pattern amongst the DNAs using the three hPRL DNA probes were identical (Fig.15c). The extra bands in the placental DNA sample were not reproducible. A new high molecular weight band of <11kb was detected by the 5' end-specific genomic probe which appeared identical in size in all three DNAs tested. The hPRL gene restriction map (36) indicated that this new high molecular weight band contained exon 1 yet it was not detected with the pituitary hPRL cDNA as seen in other digests above. The approximately 2.9kb NcoI fragment observed with both hPRL probes contains exon 2 and 6 codons of exon 3. Identical restriction fragment patterns were observed with XbaI among the DNAs tested (Fig.15d) and it was evident that the 5' and 3' end-specific XbaI fragments of the PRL gene comigrated at approximately 4.4kb. As a result, it was not possible to conclusively state that the pituitary hPRL cDNA did not hybridize to the 5' end XbaI genomic fragments of the hPRL gene. One might expect a very strong hybridizing signal in the 4.7kb range, indicative of a doublet, if the 5' and 3' end genomic fragments were both detected by the pituitary hPRL cDNA. From Figure 15d and other experiments it was clear that a doublet was not detected by pituitary hPRL cDNA which agreed with the results described above.

For the enzyme BglII, the results were not as straightforward as those described for the other digests. Non-uniform restriction fragment patterns were obtained (Fig. 16a) with the pituitary hPRL cDNA probe such that the lowest molecular weight band was

missing from the human placenta pattern, and the approximately 3.5kb band was very weak in intensity in DNA from the classical IM-9 line obtained from M. Lesniak (NIH). If these results were due to incomplete digestion of the DNAs then one would have expected to detect larger molecular weight precursor bands; however, none were observed. The 5' and 3' end-specific hPRL genomic probes revealed no differences in banding pattern between the human placenta, IM-9-P3 and IM-9 cell DNAs. Thus, by elimination, the BglII restriction sites responsible for the differences seen with the pituitary hPRL cDNA must lie in the internal regions of the gene. The 5' end-specific genomic probe identified two new fragments. The 1.9kb band in each lane, according to the published hPRL gene restriction map (36), contained only exon 1 coding sequence and was not detectable with pituitary hPRL cDNA as observed in other digests. The larger molecular weight band comigrated with the 23kb marker band and represented further 5' flanking DNA of the hPRL gene. These results reinforced the importance of including the 5' end genomic probe in such studies not only to identify the location of a specific fragment in the hPRL gene, but also to obtain a complete picture of the structure of the hPRL gene in IM-9-P3 cells.

Hybridization of a number of different human DNAs revealed that absence of the smallest molecular weight BglII fragment could have been due to a restriction fragment length polymorphism (RFLP). As can be seen in Figure 16b, peripheral blood lymphocyte DNA from another individual also lacked the smallest molecular weight BglII fragment, a pattern identical to the human placenta DNA seen in Figure 16a. In contrast, DNA isolated from a human breast cancer cell line and from another normal individual yielded a BglII restriction fragment pattern identical to that seen in the PRL-producing IM-9-P3 line. Consequently it was unlikely that the non-uniform BglII banding pattern of the hPRL gene was due to a gene rearrangement. Moreover, no rearrangement or deletion of the hPRL gene was observed using the five different restriction enzymes described in Figure 15. By elimination then, it was assumed that the varying BglII banding pattern of the hPRL gene

was due to restriction fragment length polymorphism. Further studies, including pedigree analysis, are required to confirm this hypothesis.

Weak hybridization to the 3.5kb BglII fragment in the DNA of IM-9 cells from M. Lesniak (NIH) was reproducible and unique since that fragment was strongly represented in DNA of IM-9 cells from two other sources (Fig.16b). This phenomenon may have been related to the putative BglII RFLP.

It was important to these genomic DNA hybridization studies to identify the bands detected in each digest in order to ensure that the entire hPRL gene was represented in the fragmentation pattern. Only in this way could one confidently state that the hPRL gene in IM-9-P3 cells was not rearranged internally or in flanking regions given the resolving power of this technique. Unfortunately sequence pertaining to large intronic segments of the hPRL gene are not available and the location of some restriction sites relevant to the above studies remain to be determined. It was possible, however, to identify the origin of most bands in the various digests by comparison to the restriction map of the gene (Fig.26) and the use of 5' and 3' end-specific genomic probes. The fragmentation patterns of hPRL gene described above were consistent with the presence of an intact gene in the IM-9-P3 genome.

Comparison of IM-9-P3 PRL mRNA to that of Uterine Decidua

It has been assumed since the work of Clements *et al* (74) that human decidual PRL mRNA is identical to the pituitary transcript. To confirm this data and compare the atypical IM-9-P3 PRL message to that found in the human decidua, a series of Northern blot hybridization experiments were done. Gestational decidua was obtained from 9-12 weeks of pregnancy and RNA was isolated. The results of one of these experiments is shown in Figure 17. Surprisingly, the PRL mRNA found in normal human decidual cells

was larger than the pituitary hPRL transcript and essentially identical in size to the hPRL mRNA seen in IM-9-P3 cells. In total, 27 different decidual RNA samples were analyzed in this manner, the results of which were identical to Figure 17.

Isolation and Characterization of PRL cDNA Clones

To localize precisely the source of elongation in IM-9-P3 and decidual PRL mRNAs relative to the pituitary hPRL mRNA, cDNA libraries were constructed from poly $(A)^+$ mRNA isolated from the original PRL-producing line, IM-9-P. Both IM-9-P cDNA libraries (I and II) contained approximately 8.5 x 10^5 recombinant phage/μg of DNA. From unamplified portions of these cDNA libraries, approximately 150,000 recombinant phage were screened and a total of 8 cDNAs were isolated (numbers 3,6,10,7,9,11,IV and C) and purified by high stringency hybridization to PstI subfragments of pituitary hPRL cDNA. Subsequent screening of amplified portions of both libraries (100,000 recombinant phage) yielded 55 strongly hybridizing plaques from library I and 19 from library II. All 74 positive phage underwent secondary screening with a 5' untranslated region-specific cDNA probe in order to further purify and analyze primarily full-length cDNAs. Three such cDNAs (1A,9A and I-1) were identified in this manner for a final yield of 11 putative IM-9-P PRL cDNAs.

Human decidual PRL cDNA clones were isolated and purified from two different amplified lambda GT11 cDNA libraries; one constructed from human placenta mRNA (Clontech Laboratories Inc., Palo Alto, CA) and the other from human decidua mRNA (281) provided by Dr. Olli Janne (Population Council and the Rockefeller University, N.Y.). A total of 300,000 phage from the human placenta cDNA library were screened with rat prolactin-like protein A (28) and B (29) cDNAs. This screening had been done as part of earlier studies attempting to identify human homologues of these rat genes. Since the human placenta at delivery contains decidual tissue on the maternal side due to the

invasive nature of placental trophoblasts; a cDNA library constructed from such tissue would likely contain decidual PRL cDNA clones. Rat PRL-like protein A cDNA contains regions of high sequence homology with hPRL cDNA (28). Using rat prolactin-like protein A cDNA, under moderately stringent conditions, seven strongly hybridizing clones were identified of which three were chosen (F1-a,4a,O) for sequence analysis based on the amount of 5' sequence present. A human decidua lambda GT11 cDNA library, when screened with IM-9-P PRL cDNA IV which appeared to contain the longest 5' non-coding sequence, resulted in 94 positive plaques which were subsequently narrowed down to 15 clones which contained 5' untranslated sequence. Restriction analysis (EcoRI) of these cDNAs revealed that only five (19A,13B,8B,2A,15A) appeared to possess elongated 5' ends.

All nineteen hPRL cDNAs were subcloned and determined to be of independent origin by differences in the 3' and 5' termini. The majority of PRL cDNAs isolated from the IM-9-P libraries were not intact because of inefficient methylation-protection of cDNA EcoRI sites prior to cleavage of concatinated EcoRI linkers ligated to the cDNA. Human PRL cDNA contains a single EcoRI site at amino acids glutamine 39-phenylalanine 40; as a result one end of almost every cloned hPRL cDNA isolated from these IM-9-P lymphoblast libraries was generated from this internal EcoRI site. A preliminary comparison of the IM-9-P and decidual PRL cDNAs was performed by EcoRI restriction analysis and hybridization with 5' and 3' end-specific pituitary hPRL cDNA probes. From digestion of a full-length pituitary hPRL cDNA one would expect two fragments; a 5' cleavage product of 258bp, including the 5' untranslated region (UTR), and a 3' fragment of 628bp plus an unspecified poly(A) tail. When this form of restriction endonuclease analysis was applied to the nineteen hPRL cDNAs described above, eleven were found to contain significantly larger 5' EcoRI fragments without a major difference in the individual 3' EcoRI fragments. Thus, the elongation of IM-9-P and decidual PRL mRNA resided in the 5' untranslated sequence relative to the 57 nucleotide 5' non-coding region of the

pituitary PRL mRNA (36). These analyses also indicated that three of the IM-9-P PRL cDNA clones possessed an unusual restriction map which implied that these cDNA were chimeric in nature. This was confirmed by nucleotide sequencing. The structure of the nineteen IM-9-P and decidual PRL cDNAs compared to that of a full-length pituitary PRL cDNA is depicted in Figure 18.

Nucleotide Sequence Analysis of IM-9-P and Decidual PRL cDNAs

Previously we had shown IM-9-P lymphoblast PRL protein to be essentially identical to human pituitary derived PRL based on a number of physicochemical criteria. To confirm these data, all eleven putative IM-9-P PRL cDNAs were subjected to various degrees of nucleotide sequence analysis, the strategy for which is shown in Figure 18. IM-9-P PRL cDNA #3 was the largest clone and was sequenced fully on the mRNA strand and 64% on the opposite strand. The portion that was not sequenced on the cDNA strand was included in the sequence analysis of the other ten IM-9-P PRL clones and all were identical. The nucleotide sequence corresponding to the protein coding portion of IM-9-P PRL was found to be essentially identical (Fig.19) to that previously published for human pituitary cDNA (10). Two silent substitutions at the third base of the codons for leucine -8 and glutamate 162 have been reported in dPRL mRNA (75); data herein did not agree with these substitutions.

Sequence analysis confirmed that three of the IM-9-P PRL cDNAs were chimeric. These were rather unusual chimaera in that the independent cDNAs were both derived from PRL mRNA. The generation of these chimeric structures occurred during construction of the cDNA library since atypical cDNA restriction fragments were observed on digestion of the original lambda clones. All three chimeric cDNAs (C, 3 and 1A) contained an inverted portion of hPRL 3' untranslated sequence ligated to the 5' end of the

cDNAs. This peculiar cDNA cloning artifact appears to have occurred via an 11bp sequence (5' GAAGGTCCTTA 3') beginning 31 nucleotides 3' of the terminal cysteine codon which is 90% homologous to sequence located between -157 and -167 of the IM-9-P PRL 5' untranslated region. This 11bp sequence is palindromic and could form an intramolecular stem-loop structure in the 3' UTR. Since the junction point in all three chimeric cDNA clones occurred within this 11bp region it was conceivable that sequence complementarity facilitated the annealing and ligation of independent hPRL cDNAs. There is no other sequence homology between IM-9-P PRL 5' and 3' untranslated sequences. The 5' end of IM-9-P PRL cDNA clones C and #3 were ligated to an adenine residue located 42 nucleotides 3' of cysteine 199 in an inverted fashion such that 106bp of hPRL 3' untranslated sequence plus poly (A) tail were present at the 5' end of these cDNAs. IM-9-P PRL cDNA 1A was of a similar structure except that the junction point occurred at a cytosine residue located 37 nucleotides 3' of the terminal cysteine codon. These chimeric hPRL cDNA were isolated from IM-9-P cDNA library I only.

A similar nucleotide sequencing strategy was applied to the decidual PRL cDNAs to compare its sequence to that of IM-9-P PRL mRNA. The dPRL cDNAs purified from two different libraries were found to be intact and not chimeric. The 3' and 5' non-coding regions of each dPRL cDNA was sequenced as well as selected portions of the protein coding region of each clone. Interestingly, the coding region of three of the four dPRL cDNAs analyzed contained an extra in-frame alanine codon in the hydrophobic leader sequence between lysine-20 and glycine-19 resulting in a signal sequence of 29 amino acids.

Eight of the IM-9-P PRL cDNAs contained 3' untranslated sequence which was essentially identical to that reported for pituitary PRL cDNA (10). Takahashi and co-workers (75) reported two differences in the 3' untranslated sequence of dPRL of which only one was also found in all IM-9-P and decidual PRL cDNAs; these differences most likely represent allelic variants. Only one IM-9-P cDNA possessed a 3' end identical to that

of the pituitary PRL cDNA reported by Cooke and colleagues (10) while seven cDNAs contained a 7-14 nucleotide elongation (Fig.20). Poly(A) tails were present on ten of the nineteen PRL cDNAs purified from the four different cDNA libraries and averaged 18bp in length. Other essentially full-length IM-9-P and decidual PRL cDNAs were truncated somewhere in the 3' UTR. IM-9-P PRL cDNA 9 was truncated at the adenine residue 118 bases 3' of the termination codon, dPRL cDNA O ended 26bp 3' of the ochre codon, and dPRL cDNA 4a ended 107 bases downstream of the terminal cysteine codon. Decidual PRL cDNA 13B contained a 3' end identical to the pituitary PRL cDNA, but did not possess a poly(A) tail and therefore may have represented a truncated cDNA. The 3' end heterogeneity shown in Figure 20 could not account for the approximately 150 nucleotide difference between IM-9-P PRL mRNAs and the pituitary message.

Five of the IM-9-P PRL cDNAs (IV,3,C,1A,I-1) contained a 5' non-coding region in which the sequence differed dramatically from that reported for the pituitary mRNA (Fig.21). Truong and colleagues (36) determined that the 5' UTR of pituitary hPRL mRNA is 57 nucleotides in length. The 5' non-coding sequence shown in Figure 21 is that of IM-9-P PRL cDNA IV which had the longest 5' untranslated sequence at 191 nucleotides. IM-9-P PRL cDNAs 1A, 3 and C contained 5' non-coding information identical to that seen in cDNA IV but shorter in length presumably due to premature termination of reverse transcription during cDNA synthesis. The length of the 5' UTR in IM-9-P PRL cDNAs 1A, 3, and C is denoted by the negative number at the 5' end of each cDNA in Figure18. The 5' non-coding region of IM-9-P PRL cDNAs contained an extra 41 nucleotides upstream of the normal transcription start-site for the human pituitary PRL gene (Fig.21). The additional 5' flanking sequence to nucleotide -98 includes the putative TATA box at position -85. In IM-9-P cDNA IV the extra 41 nucleotides were immediately preceded by an additional 93 nucleotides which were not homologous to the published DNA sequence found 5' to the human pituitary PRL gene cap site. The identical 5' untranslated sequence was found in IM-9-P PRL cDNAs 1A, 3 and C (data not shown).

Moreover the junction point sequence between the unique 5' UTR and the previously published genomic sequence at nucleotide -98 was identical. Clearly this consistent unique 5' non-coding structure could easily account for the size difference between lymphoblast and pituitary PRL transcripts.

IM-9-P PRL cDNA I-1 possessed a 5' UTR very similar to that described above with some differences. Initially IM-9-P PRL cDNA I-1 was thought to possess the longest authentic 5' UTR of 248bp (Fig.22). This sequence differed from that described for the other IM-9-P PRL cDNA 5' UTRs in that IM-9-P PRL cDNA I-1 contained an extra 45bp at its 5' end relative to cDNA IV and an extra 12bp at the -98 junction point with the known 5' flanking region of the PRL gene. The unique sequence between these regions was identical to that seen in the other IM-9-P PRL cDNAs as shown in Figure 22. Since the extra 45bp at the 5' end of this clone was unique, its authenticity as a region of IM-9-P PRL mRNA was assessed by Northern blot hybridization. Sense and antisense cRNA generated from the unique 5' UTR upstream of -98 in IM-9-P PRL clone I-1 was hybridized to human decidual and IM-9-P RNA. The antisense probe detected a single band of approximately 1.0kb which comigrated with hPRL mRNA. Surprisingly the sense cRNA also hybridized to an abundant, approximately 500bp mRNA in all RNA samples (data not shown). This hybridization was due to the extra 45bp at the 5' end of IM-9-P PRL cDNA I-1 which indicated that this cDNA was chimeric. As will be discussed in the next section, sense cRNA generated from the shorter unique 5' UTR of other IM-9-P cDNAs did not hybridize to any RNA.

An analysis of the 5' ends of several overlapping dPRL cDNAs revealed differences in the extent of 5' non-coding information (Fig.18) but showed complete sequence homology to each other in overlapping regions. The structure and sequence of the dPRL 5' untranslated region was identical to that described above for the IM-9-P lymphoblast mRNA (see Fig.21) and thus provided the basis for the mRNA size difference seen when compared with the pituitary transcript on denaturing formaldehyde gel

electrophoresis. Decidual PRL cDNA 15A represented a distinct, although minor, second class of elongated PRL mRNA with a 5' untranslated region of 300bp. As shown in Figure 21, clone 15A contained an additional 148 nucleotides beyond -98 which was homologous to the 5' flanking DNA of the hPRL gene to -246. This extended 5' flanking sequence was preceded by 54 nucleotides of unique non-coding information which was identical to that seen in all of the IM-9-P and decidual PRL cDNAs. This cDNA would appear to have represented a scarce member of the total dPRL mRNA population since a hybridizing PRL transcript 300 nucleotides or more longer than the pituitary mRNA had not been detected on Northerns and clone 15A was the only cDNA of this unique structure represented amongst the original 55 positive plaques.

Strand-Specific RNA Hybridization Analysis

To confirm that the unique 5' untranslated sequence was indeed an authentic component of PRL mRNA *in vivo*, RNA hybridization experiments were performed. Total RNA from IM-9-P3 cells, individual human decidual samples and human pituitaries were electrophoresed on denaturing formaldehyde-agarose gels and blotted onto a nitrocellulose based membrane. These blots were probed with an antisense cRNA synthesized from a template generated from the unique 5' non-coding region of IM-9-P PRL cDNA IV which was contained in an EcoRV-EcoRI fragment subcloned into pGEM-3. High stringency hybridization of this cRNA probe to RNA blots containing various sources of hPRL mRNA showed a single band which exactly comigrated in decidua and IM-9-P3 RNA at approximately 1.1kb (see Fig.23). No hybridization was seen to pituitary mRNA. This RNA blot was then stripped and rehybridized with a random primer labelled hPRL cDNA probe consisting of only coding region sequence. The latter probe detected PRL mRNA in pituitary total RNA as expected as well as the slightly larger transcript

which had been seen exclusively in decidual and IM-9-P3 RNA using the unique 5' UTR probe. These results illustrated that the new 5' UT sequence was not present in pituitary PRL mRNA. The differing intensity of the PRL mRNA signal in the decidua lanes, despite equal gel loading, was due to the inherent variability associated with individual tissue sampling and was seen over the 27 different decidual RNAs tested in this manner. Control experiments performed under low stringency conditions of hybridization (42C, 40% formamide hybridization buffer) utilizing the sense cRNA transcribed from the same template did not result in any form of consistent, specific hybridization signal.

Primer Extension Analysis of hPRL mRNA 5' Termini

Primer extension analysis was performed to compare the transcription start site in IM-9-P3, decidua and pituitary PRL mRNAs (Fig.24). A 21-nucleotide primer corresponding to leucine 1 through cysteine -6 of the signal sequence was annealed to mRNA isolated from human pituitaries, pooled decidua tissue samples, IM-9-P3 cells and as a negative control, the parental IM-9 line which does not produce PRL. The cDNA products consistently obtained using many different pituitary RNA preparations are illustrated in Figure 24 by a broad signal extending from 120-128 bases upstream of the priming site. On shorter autoradiographic exposure this wide strong signal was discernable as a series of 6 closely positioned bands of varying intensities which corresponded to 5' untranslated regions of 55-63 nucleotides in length in pituitary hPRL mRNA. These data were in good agreement with the results obtained by Truong and co-workers (36) in which a single distinct fragment corresponding to a 57bp 5' non-coding region was resolved after S1 nuclease protection by human pituitary tumour RNA. The pituitary-specific pattern of cDNA products suggested that transcription initiation of the hPRL gene occurred over a cluster of 9 nucleotides located 16bp downstream of the putative TATA box. Over 5

separate experiments the most abundant primer extended products represented a 5' UTR of 55-57 nucleotides. Additional exposures did not reveal longer cDNA products consistent with the utilization of start sites further upstream.

The pattern of bands obtained with decidual and IM-9-P3 RNA were identical to one another and markedly different from the pituitary products. In four separate experiments no primer-extended cDNAs co-migrated with those obtained using pituitary RNA, even on long autoradiographic exposure. Using IM-9-P3 or decidual RNA as the template, a series of seven bands were reproducibly resolved resulting from the primer having been extended to various positions between 204 and 244bp upstream of the priming site. These cDNA products were generally stronger in intensity in decidual RNA (Fig.24) as compared to the IM-9-P3 products because of the greater abundance of PRL mRNA in decidua total RNA. Of the consistent primer extended products, the largest were the predominant bands at 265 ± 3 nucleotides, indicating a 5' untranslated region of 178 ± 3 nucleotides. Two bands were commonly present around 265bp after primer extension and may have represented the inefficiency of reverse transcriptase rather than the presence of two separate 5' ends. The sizes of the remaining six somewhat weaker signals are provided on the left of the autoradiogram in Figure 24; these bands represented 5' untranslated regions of 140,154,160 and 162 bases. None of these primer extended products were generated when RNA of parental IM-9 cells was utilized. Various minor extension fragments of lower molecular weight were inconsistently generated, but these likely represented premature stops due to the secondary structure of IM-9-P3/decidua PRL mRNA.

It appeared that a rather complex series of transcription start positions were used on the hPRL gene which is present in single copy in the human genome. The locations of these cap sites could be deduced from the sizes of the various primer extended products. The position of these new IM-9-P3/decidua-specific start sites are labelled on IM-9-P PRL cDNA IV (Fig.21). The 5' ends of the IM-9-P and dPRL clones as shown in Figure 18

were very close to these putative start sites. The somewhat shorter transcripts seen with the clones probably resulted from premature termination of reverse transcription. Such DNAs were clearly present on prolonged autoradiographic exposure as seen in the lanes on the right in Figure 24. The largest of these minor products extended 386 bases upstream of the primer and denoted a 5' untranslated region estimated as 320 bases in decidua and IM-9-P3 RNA. The size of this cDNA correlated well with the unique structure of IM-9-P PRL clone 15A (see Fig.21) in which the 3' splice junction between the unique 5' non-coding sequence and the known flanking region of the pituitary PRL gene was at position -246, rather than at position -98 as was seen with the other cDNA clones.

The presence of multiple longer primer extended products generated with IM-9-P3 or decidual PRL mRNA as compared to those derived from the pituitary mRNA was entirely consistent with the existence of a considerably extended 5' non-coding region found in IM-9-P and decidual PRL cDNA clones. The discovery that a segment of unique sequence up to 93 bases in length, contributed to this 5' extension, in large part accounted for the elongated size of decidua and IM-9-P3 PRL message. Initially, the estimated size difference between the IM-9-P3/decidua PRL message and the pituitary transcript was 150 bases. Collectively, the above experiments defined that difference as 83 to 134 nucleotides, a size range easily represented within the broad hybridizing band of decidual and IM-9-P3 PRL mRNA on Northern blots.

Genomic Localization of the Unique 5' Segment of IM-9-P3/Decidua PRL mRNA Untranslated Sequence

A series of human genomic DNA hybridization experiments were undertaken to try to determine the length of the putative intervening sequence between the pituitary-specific cap-site and the new non-coding exon of the PRL gene utilized in IM-9-P3 and

decidual cells. It was also to compare the organization of the DNA surrounding the new 5' non-coding sequence of the ectopically activated PRL gene in IM-9-P3 lymphoblasts to that found in normal cells. These studies would potentially detect a transposition or recombination event which may have led to the inappropriate activation of the PRL gene.

In the first set of experiments, DNA samples were digested with EcoRV-HindIII. These restriction enzymes were chosen because an EcoRV site was present at -96 of the IM-9-P3/decidual PRL cDNA 5' non-coding sequence (see Fig.19). The upstream HindIII site of interest was located at position -776 relative to exon 1 of the hPRL gene and together these enzymes released a genomic fragment of 680bp from phPRLg2750. A series of 5-7 human DNAs isolated from the general population was included in each genomic DNA hybridization experiment to distinguish between restriction fragment length polymorphism of the hPRL gene and legitimate recombinatorial events which may have been detected in the IM-9-P3 DNA. The Southern blot was initially probed with the homologous, gel-purified 680bp EcoRV-HindIII fragment from the cloned hPRL gene 5' flanking DNA shown schematically in Figure 25. A single band/lane of 680bp was detected for all DNAs (Fig.25a) which implied that the IM-9-P3 PRL gene structure had not been rearranged in the region of the pituitary-specific transcription start site. This region also included the putative 3' splice acceptor sequence for the processing of the new 5' non-coding sequence. This Southern blot was rehybridized with a DNA fragment containing 3 copies of 72bp of unique 5' non-coding sequence from IM-9-P PRL cDNA #3. As can be seen in Figure 25b, a signal estimated at 1.1kb was present in every lane which suggested that the IM-9-P3 gene structure surrounding the new 5' non-coding exon did not differ from normal human DNA. More importantly these results graphically demonstrated that the unique 5' untranslated sequence of IM-9-P3/decidua PRL mRNA was not colinear with the immediate 5' flanking DNA of the hPRL gene and thus located at some distance from the pituitary-specific PRL RNA start site.

The location of this putative 5' non-coding exon of the hPRL gene was further confirmed and the length of the intervening sequence more precisely determined by a series of genomic Southern blots constructed in a manner identical to that described above. These blots were first probed with a previously described 5' end-specific fragment of 1132bp excised from phPRLg2750 by EcoRI-XbaI digestion (see Fig.26). The DNA blots were then stripped and rehybridized with a tetramer of 72bp of IM-9-P3/decidua-specific PRL 5' UTR. By comparing the resultant hybridization pattern obtained with each probe on the same blot it was possible to estimate the distance between the tissue-specific PRL cap sites. Experiments utilizing EcoRI, HindIII, and HincII digested human DNAs did not demonstrate linkage between the new 5' UTR and exon 1 of the hPRL gene since common hybridizing DNA fragments were not identified using the above probes (Fig.27). In EcoRI digested DNAs (Fig.27a) the 5' end-specific genomic probe hybridized as predicted by the restriction map (Fig.26) to a 2750bp fragment. When stripped and rehybridized with the unique 5'UTR probe a fragment of approximately 7.0kb was observed in every lane. In retrospect, this hybridization could not have shown linkage between the sequences of interest without a genomic DNA probe 5' to the EcoRI site at -978 relative to the hPRL coding region. Experiments with such a probe will be discussed below.

In HindIII digests the 5' end-specific hPRL genomic probe hybridized to two fragments (Fig.27c); a strong band of approximately 2.2kb and a less intense signal at 4.2kb. The 2.2kb band represented the DNA sequence extending 3' of the HindIII site at -758 in the 5' flanking region of hPRL exon 1 and the 4.2kb band corresponded to the DNA located to the next unknown upstream HindIII site (see Fig.26). Therefore the 4.2kb fragment reached approximately 5.0kb into the 5' flanking DNA of the pituitary PRL RNA start site. When this blot was hybridized with the unique IM-9-P3/decidua 5' UTR probe a single band of 1.1kb was seen in each lane (Fig.27d). Therefore the new 5' non-coding exon was not located within 5.0kb of the pituitary PRL cap site. DNAs digested with HincII and hybridized with the 5' end-specific genomic fragment resulted in detection of a

single band of approximately 5.7kb in all lanes (Fig.27e). The same blot probed with the new 5' non-coding sequence probe yielded a band of about 2.1kb in each DNA sample (Fig.27f). These data confirmed those described above with HindIII digested DNA. The first HincII site of the hPRL gene is located in intron A approximately 2.39kb downstream of the 5' end of the hPRL genomic clone (see Fig.26). Therefore about 3310bp of the 5.7kb HincII framgent was composed of DNA between the 5' end of the hPRL genomic clone at -978 and the next unknown upstream HincII site. Consequently, the new 5' non-coding exon could not have been located within approximately 4.3kb of exon 1 of the hPRL gene (i.e. 3310bp + 978bp).

The next series of genomic blots defined the outer boundary for the location of the new 5' non-coding exon of IM-9-P3/decidua PRL mRNA. DNAs digested with PstI demonstrated linkage between the pituitary specific exon 1 and the IM-9-P3/decidua-specific 5' non-coding sequence (Fig.28). Using the hPRL 5' end-specific genomic probe, two bands were resolved, an intense 3.2kb fragment and a weaker band which migrated at approximately 7.4kb (Fig.28e). The strong 3.2kb fragment represented sequence extending in a 3' direction from the 5' PstI site at -866 to the PstI site in intron A (see Fig.26). The 7.4kb band consisted of DNA 5' to the PstI site at -886. This band was relatively weak in intensity because there was only 111bp of overlap with the 5' end of the random-primer labelled, 5' end-specific hPRL genomic probe (1132bp EcoRI-XbaI fragment) and thus this fragment encompassed about 8.2kb of DNA 5' to the pituitary PRL gene cap site. Subsequent hybridization of this Southern blot with the unique 5' UTR cDNA fragment revealed strong hybridization to a single band which exactly comigrated with the 7.4kb fragment detected with the hPRL genomic probe (Fig.28f). This provided strong evidence for the localization of the new 5' non-coding exon within 8.2kb of the hPRL exon 1. Similar data was obtained using Southern blots which contained NcoI or BglII digested DNAs. In Figure 28a, two NcoI digested human DNA fragments hybridized to the 5' end-specific hPRL genomic probe in all samples. The smaller more

intense fragment was sized at approximately 3.6kb and extended from the NcoI site in exon 1 to the next downstream site in exon 3 of the hPRL gene (see Fig.26). The other hybridizing fragment migrated slightly smaller than the 23kb marker band and corresponded to DNA upstream of the exon 1 NcoI site to the next unknown NcoI site. This latter NcoI fragment was also detected when the same blot was stripped and reprobed with the new 5' non-coding segment of IM-9-P3/decidua PRL mRNA (Fig.28c). In blots which contained human DNAs digested with BglII, the 5' end-specific hPRL genomic probe hybridized to two fragments (Fig.28b). As in the NcoI digests, the smaller BglII fragment of about 2.1kb represented DNA which extended downstream from the BglII site at -43 of the hPRL gene 5' flanking region to a site in intron A (see Fig.26). The much larger hybridizing BglII fragment comigrated with the 23kb marker in all lanes and was the only fragment detected when this blot was rehybridized with the unique 5' UTR of IM-9-P3/decidua PRL mRNA (Fig.28d).

The BglII and NcoI genomic Southern hybridization experiments confirmed the data obtained with PstI digested DNAs but did not further delineate the outer limit of the intervening sequence between the IM-9-P3/decidua-specific hPRL cap sites and the pituitary PRL mRNA start site. These studies demonstrated no major rearrangement in the DNA surrounding the new 5' non-coding exon in the IM-9-P3 lymphoblast genome and strongly suggested that it was located 5-8kb upstream of the PRL gene exon 1.

In order to more precisely delineate the size of the intron separating the tissue-specific RNA start sites of the hPRL gene additional 5' flanking DNA to hPRL exon 1 was required. Such a genomic clone (phPRLg4800) was generously provided by Dr. J. Martial (University of Liege, Belgium); the restriction map for which is provided in Figure 29. This hPRL genomic clone provided in total, about 5.8kb of DNA upstream of hPRL exon 1 and the locations of the HindIII, HincII and XbaI restriction sites agreed very well with the corresponding genomic fragments detected by Southern hybridization above. To determine if the new 5' non-coding exon was located in the 5' region of phPRLg4800,

Northern and Southern hybridization experiments were done with the gel purified, 830bp SalI-HindIII fragment (see Fig.29). Genomic Southern hybridization experiments described above (HindIII and HincII digests) proved that the new 5' non-coding exon could not have been situated in DNA 3' to the 830bp SalI-HindIII fragment. No hybridization to dPRL mRNA or to IM-9-P PRL cDNA clones was observed, therefore the new 5' non-coding exon was located beyond 5.8kb upstream of the pituitary specific PRL cap site (data not shown).

Next a series of genomic DNA blots were sequentially probed with the 5' 830bp SalI-HindIII fragment of phPRLg4800, the 1132bp EcoRI-XbaI fragment of phPRLg2750 used in the previous studies and the unique 5' non-coding exon. The results of one such experiment are presented in Figure 30. IM-9-P33 cell DNA, digested with a variety of enzymes, was hybridized with the unique 5' UTR fragment of IM-9-P3/decidua PRL cDNA. The banding pattern obtained was identical to that obtained in previous experiments (see Fig.27) with an approximately 1.1kb and 2.1kb band resolved in HindIII and HincII digests respectively (Fig.30b). On this blot double digests including these enzymes each with XbaI, did not result in cleavage of the above fragments. The same blot was then stripped and reprobed with the 5' end-specific genomic fragment (Fig.30a) encompassing only 978bp of immediate 5' flanking DNA of the hPRL gene and resulted in the identification of bands which were expected based on the published restriction map (see Fig.26) and experiments described above (see Fig.15 and 27). When this genomic DNA blot was subsequently hybridized with the 830bp SalI-HindIII 5' fragment of phPRLg4800, the restriction fragmentation pattern obtained in all lanes was identical and directly superimposable with that seen with the unique 5' UTR sequence probe of IM-9-P3/decidua PRL mRNA (Fig.30c). This short step along the chromosome in a 5' direction from exon 1 of the hPRL gene demonstrated linkage between exon 1 and the new 5' non-coding exon. This had not been previously observed in HincIII or HindIII digests using

the 1132bp EcoRI-XbaI fragment which encompassed only 978bp of immediate 5' flanking DNA as a probe.

In other experiments, the 830bp SalI-HindIII genomic fragment was hybridized to DNA blots which had been previously probed with the unique 5' untranslated sequence of IM-9-P3/decidua PRL message or the 1132bp 5' end-specific fragment described above. This was done to corroborate the data presented in Figure 30. An example of one such experiment is shown in Figure 31 where the DNA blot in Figure 15 was re-probed with the most 5' fragment of DNA flanking the hPRL gene (830bp SalI-HindIII). In each set of digests identical bands were observed for all DNAs as seen previously (Fig.31a). In PstI digested DNAs the 830bp SalI-HindIII probe detected a 7.4kb fragment which was directly superimposeable with the largest molecular weight band resolved (in Fig.15) in which the 1132bp 5' end-specific fragment of phPRLg2750 was used as a hybridization probe. This confirmed the origin and contiguous nature of the two probes. As in Figure 30, the 830bp SalI-HindIII genomic probe hybridized to a 1.1kb HindIII fragment which had been seen previously only with the unique 5' UTR probe derived from IM-9-P3/decidua PRL mRNA (Fig.31a). To illustrate this point and for easy comparison the parallel autoradiogram is provided (Fig.31b). The same DNA blot had been probed with IM-9-P PRL cDNA #3which contained 72bp of unique 5' UTR which specifically hybridized to the 1.1kb HindIII band and the 7.4kb PstI band described above. The EcoRI digests in Figure 31a released a fragment of approximately 7.0kb which hybridized with the 830bp SalI-HindIII genomic probe. This EcoRI band was also present in the parallel autoradiogram probed with IM-9-P PRL cDNA #3; its detection was attributed, from previous studies (see Fig.15 and 27), to the unique 5' UTR of the cDNA. Similar studies were performed with NcoI, HincII and XbaI digested genomic DNA and the trend described above was corroborated (data not shown), that trend being the identification of identical genomic DNA fragments (released with six different restriction enzymes) by the unique 5' UTR of IM-9-P3/decidua

PRL mRNA and the 830bp SalI-HindIII genomic fragment which corresponded to DNA 5.8kb upstream of hPRL exon 1.

Collectively these data, along with the restriction map of the hPRL gene 5' flanking DNA in Figure 29, allowed a more accurate positioning of the new 5' non-coding exon relative to exon 1 of the hPRL gene. One of the genomic HindIII sites which constituted the 1.1kb HindIII fragment, to which both above probes hybridized, must have been the same one which released the 830bp 5' fragment along with SalI from phPRLg4800 (Fig.29). The other HindIII site which released the 1.1kb fragment of interest must be located 5', approximately 270bp beyond the SalI site which demarcates the 5' end of phPRLg4800. The same rationale could be applied to any of the restriction sites analyzed in the experiments described above. This line of reasoning in the case of HindIII, predicted the location of the 93 or more basepair 5' non-coding exon to the 270bp segment just 5' of the SalI site of phPRLg4800. Thus the intervening sequence between the IM-9-P3/decidua-specific 5'UTR and the pituitary-specific cap site was approximately 5.9kb. These results necessitate an alternative designation for the first two exons of the hPRL gene with the new 5' non-coding exon expressed in decidua and IM-9-P3 cells as "1a", the intervening sequence as "A-1" and the pituitary-specific 5' exon "1b" (Fig.32).

DISCUSSION

Putative PRL RNA Ubiquitously Expressed in Rat

Rat PRL cDNA was used to investigate the possibility of the expression of PRL-like genes in a variety of adult rat tissues. The analysis of total cellular RNA from these different tissues by Northern hybridization detected multiple, distinct RNAs ranging from 1.6kb to 0.3kb, with the 1.6kb RNA having been the most abundant (Fig.1). These RNAs appeared to have been ubiquitously expressed and lacked the typical poly(A) tail of eukaryotic mRNAs since they were not preferentially enriched by oligo(dT) cellulose chromatography (Fig.3). This was an unexpected result even though poly(A)$^-$ mRNA has been described previously (313-315). Further investigation revealed that hybridization to this group of nonpolyadenylated RNAs was not specific to the rPRL cDNA and in fact could be demonstrated with a totally unrelated cDNA, probasin (275) (Fig.5). Moreover within a group of different overlapping cDNAs of the same mRNA (i.e., rPRP-A) only those that had been isolated from a plasmid library were capable of hybridizing to the 1.6kb nonpolyadenylated RNA. The common feature among these hybridizing cDNAs was that they all contained GC-tails which were necessary for ligation of the cDNA into the original plasmid vector. Consequently, it was concluded that the 1.6 to 0.3kb family of nonpolyadenylated RNAs did not possess nucleotide sequence homology to rPRL mRNA. Although not conclusively demonstrated it appeared likely that hybridization to these nonpolyadenylated RNAs was conferred by the GC-tails on both ends of the unrelated cDNAs. The identity of these nonpolyadenylated RNAs was not pursued even though it was clear that the 1.6kb species was not 18S ribosomal RNA (Fig.2). The contribution of GC-tails to this artifactual hybridization could have been directly demonstrated by the use of GC homopolymeric oligodeoxyribonucleotides as a hybridization probe or the placement

of GC tails onto those cDNAs which did not hybridize to the nonpolyadenylated RNAs of interest.

This phenomenon occurred under highly stringent conditions and therefore could be misconstrued at first glance as the identification of authentic rPRL-related mRNAs. There have been three recent reports (abstracts) which claim the identification of a rPRL mRNA in rodent lymphocytes. Shah and co-workers (316) simply stated that a 1.4kb mRNA was detectable in murine lymphocytes using rPRL cDNA. Woldeyesus and colleagues (317) claim that murine spleenocytes contain an mRNA of approximately 1.6kb which can be resolved using a mouse PRL cDNA or an oligonucleotide encoding a portion of the PRL signal sequence and that the PRL-like mRNA in murine spleenocytes is even more abundant than the pituitary PRL transcript. The lymphocyte 1.6kb PRL-related mRNA they observe is constitutively expressed in a number of cell lines including the rat Nb2 lymphoma cell line described previously. Clevenger *et al* (318) reported the presence of a PRL-like 2.1kb mRNA that was detectable in an undescribed rodent cell line using an undescribed probe.

These results from various laboratories possess characteristics very similar to the artifactual hybridization using rPRL cDNA described here. In various experiments it was found that the 1.6kb nonpolyadenylated RNA was constitutively expressed in Nb2 cells, in serum stimulated normal rat kidney fibroblasts and in estrogen treated rat uterus (data not shown). Woldeyesus *et al* (317) also found a 1.6kb RNA to be constitutively expressed in a number of different cell lines; their probe is the mouse PRL cDNA which is also GC-tailed (319). In the abstract from Clevenger and co-workers (318) a 2.1kb PRL-like mRNA would place the band slightly greater in size than 18S ribosomal RNA which is approximately 1950 bases in rat (320). In each abstract the putative PRL-like mRNA varies rather significantly in size, from 1.4 to 1.6 to 2.1kb despite the fact that rodent lymphocytes were used in each case. Given these rather curious characteristics it would

seem reasonable to suggest that the 1.6kb nonpolyadenylated RNA described here may account for the hybridization signal observed by the other investigators described above.

Identification and Characterization of hPRL Producing IM-9-P Lymphoblast Cells

The synthesis of PRL by the human B-lymphoblastoid cell line, IM-9, in our laboratory was initially discovered by Northern blot hybridization (Fig.6). The original objective had been the identification of the human equivalent to the putative 1.6kb nonpolyadenylated RNA described in the previous section prior to its classification as an artifact of hybridization. A number of other leukocyte cell lines (RAJI, HL-60, K562, MOLT-4) as well as normal human peripheral blood lymphocytes were analyzed in the same manner and no PRL mRNA was detected. Curiously, the PRL-producing phenotype was not a general characteristic reflected in the IM-9 RNA from cells obtained through three different laboratories (Fig.6). This unexpected result prompted closer scrutiny as to the origin of the IM-9 line in our laboratory. Initially the IM-9 line in our laboratory, designated IM-9-P, synthesized and secreted IgG in quantities similar to the IM-9 line from other laboratories which was clearly indicative of a differentiated B-lymphocyte origin. The IM-9-P cells were shown to be of human female origin as were the classical IM-9 cells by genomic Southern hybridization (Fig.7). The B-cell lineage of the IM-9-P line was then confirmed by assessment of rearrangement of immunoglobulin genes and T-cell receptor genes, only the latter revealed a germline restriction fragmentation pattern (Fig.8). Moreover it appeared that one of the rearranged Ig bands in IM-9-P cells was lost when compared to the pattern obtained from the classical IM-9 cells (Fig.8) and this correlated with the loss of IgG secretion by IM-9-P cells after continuous culture for 7 months. This observation suggested that the genome of the PRL-producing IM-9-P cells was in an

unstable state with the loss of DNA. At that time the IM-9-P line was found to be contaminated with mycoplasma. It has been well-documented that these small prokaryotes can promote chromosomal alterations in cultured mammalian cells (321) and therefore could have contributed to the loss of IgG secretory ability by IM-9-P cells. The IM-9-P cells were subsequently cured of this contamination and immunoglobulin secretion was not regained.

Immunophenotyping revealed differences between the classical IM-9 line from the ATCC and the cloned PRL-producing line, IM-9-P3. As stated above, those morphogenetic changes that involved loss of IgG secretion may have continued to occur and resulted in the loss of cell-surface B-cell specific antigens on IM-9-P3 cells. These B-cell specific markers were prominently featured on the cell membrane of the classical IM-9 line which was still producing IgG. The loss of the common B-cell marker, B1, on IM-9-P3 cells was surprising because the B1 associated antigen, a 35,000 M.W. integral membrane protein, is not found on normal T-cells, monocytes, and granulocytes or tumours of these cellular lineages. The only marker held in common was I2, which according to the supplier, is present on normal B-cells, monocytes and activated T-lymphocytes. The monoclonal antibody I2 recognizes a cell surface antigen with an estimated M.W. of 29,000/34,000 and is a member of a set of closely linked genetic loci playing a central role in transplant immunology, namely the major histocompatability complex. This epitope has the rather complex definition of an HLA-D/DR related Ia-like antigen. This means that the antigen to which I2 binds is encoded by a group of genes located on the centromeric side of HLA-B genes on chromosome 6 (322). These molecules are heterodimeric transmembrane glycoproteins involved in the binding and presentation of foreign peptide antigens for recognition by T-lymphocytes (322). This particular HLA antigen is found on a number of different bone-marrow derived cells and thus did not aid in more precisely delineating the identity of IM-9-P3 cells.

It was not possible from these studies to assign IM-9-P3 cells to a specific stage along the B-cell differentiation pathway. The loss of the B1 marker and IgG secretion, however, could be indicative of retro or dedifferentiation of the classical IM-9 cells to give IM-9-P cells. One might then extrapolate and suggest that PRL production is a characteristic of B-cells somewhere very early in the ontogenic pathway in the pre-B-cell phase where mature B-cell features are not expressed (323). To accurately assess the stage of IM-9-P3 B-cell differentiation and thus the legitimacy of the above hypothesis, further study into the presence of cytoplasmic immunoglobulin and isotype switching is required.

As an additional measure of the relatedness of classical IM-9 cells and the PRL-producing IM-9-P3 clone the haplotype of these cells, commonly referred to as tissue-typing, was determined. This process involves the identification of a particular subset of cell surface antigens encoded by the major histocompatability complex and function in graft versus host reactions. Because so many genes are involved, most haplotypes are rare except within a specific line of inheritance. Mathematically, the chance for two persons to having a common phenotype is less than 1 in 20 million. The finding that the classical IM-9 line and PRL-producing IM-9-P3 clone possessed identical haplotypes strongly supported a common lineage (Table 1).

In the early stages of these studies a gradual loss of PRL expression by the IM-9-P line was observed over the latter part of a 7 month period in continuous culture. Prior to the gradual loss of this phenotype the IM-9-P cells had been cured of mycoplasma contamination as described in the "Results". The PRL synthesizing variant, IM-9-P3, regained by cloning exhibited a three times higher level of PRL secretion. Presumably, those cells not expressing the PRL gene in the parent IM-9-P population were gradually diluting the PRL-synthesizing subpopulation. Those morphogenetic changes that had activated the PRL gene locus were thus stably inherited within the IM-9-P3 subpopulation. The IM-9-P3 line was further cloned by limiting dilution and all subclones (i.e., IM-9-P33) produced PRL, albeit at different rates (324). When the classical IM-9 line from the ATCC

was cloned, no PRL-producing isolates were identified. Thus, in the unstimulated, steady-state population of classical IM-9 cells PRL production was probably not a normal phenotype expressed by even a small subgroup of IM-9 cells.

Karyotypic analysis confirmed that the IM-9-P line and its clonal derivatives were identical and had undergone rearrangements in 9 of its chromosomes leaving only 37 normal chromosomes (325). The classical IM-9 line was shown to exhibit a normal female karyotype, 46XX. Interestingly, chromosome 6 which contains the PRL gene appeared normal using the conventional C- and G-banding techniques in the PRL-producing cells of the IM-9-P class.

In summary, HLA-typing and the analysis of immunoglobulin gene rearrangement proved that the IM-9-P3 cells were of B-cell origin and strongly implied that these cells were a divergent subline of the classical IM-9 line. Immunophenotyping and karyotypic analysis revealed differences between the parental IM-9 line and the PRL-producing derivatives which could have been attributed to major chromosomal anomalies. These genotypic alterations included deletion(s) of the immunoglobulin genes which correlated with the loss of IM-9-P IgG secretion and corresponding cell surface markers. It is thus reasonable to postulate that the PRL-producing IM-9 line in this laboratory had undergone some physiological crisis and the corresponding alterations in chromosome structure allowed expression of the PRL gene and eventual loss of some B-cell specific characteristics. This hypothesis was supported by the karyotyping data and the fact that the parental and cloned classical IM-9 lines did not synthesize hPRL. Consequently, expression of PRL by cells of the IM-9-P class was considered abnormal or ectopic and likely activated by non-specific chromosomal aberrations which were initiated by seemingly deleterious environmental stimuli (i.e., mycoplasma). Such a conclusion was contrary to the possibility that PRL expression was a latent biological function of mature lymphocytes and required a specific physiological perturbation (i.e., immune cell growth factor) to which PRL gene expression was linked. It also precluded a retrodifferentiation theory

earlier where an unknown physiologic stimuli caused the IM-9 line in our laboratory to revert to a pre-B-cell phenotype which included PRL expression. Even though the data presented here tend not to support such an hypothesis it was not possible to rule out, given the cellular complexity of the immune system, that a small cell population of the bone marrow normally synthesized PRL during a particular stage of differentiation, much like the well-documented transient expression of certain cell surface antigens (326). Other reports in the literature, which are discussed below, tend to support the synthesis of PRL or PRL-like proteins by normal cells of the immune system.

Comparison to Background Literature

Two reports have recently appeared describing the induction of a PRL-like molecule from mitogen-stimulated rodent lymphocytes. Hiestand and co-workers (165) claim that proliferating rat spleenocytes contain a mRNA with homology to both rat GH and rat PRL and is detectable under high stringency hybridization conditions. The majority of this data was obtained by RNA dot-blot analysis using relatively small quantities of total RNA and the GC-tailed rat PRL cDNA as a probe (274). The actual autoradiographic results of these experiments was not shown, therefore it was not possible to assess the intensity of the hybridization signals above background. Instead, only graphic representations of arbitrary densitometric scanning units is provided. It is entirely possible that the signal observed by these workers on RNA dot blots reflected non-specific hybridization of the GC-tails of rPRL cDNA to the 1.6kb nonpolyadenylated RNA described in an earlier section of this thesis. It is unusual that on Northern hybridization, Hiestand and colleagues (165) required 25μg of poly(A)$^+$ enriched spleenocyte mRNA to detect a faint, smeared signal for this putative PRL-like mRNA whereas by RNA dot blot analysis the signal was, presumably, clearly measurable with total RNA from 10^6 cells. It

is also somewhat peculiar that the putative mRNA for this PRL-like molecule is at least ten times larger than that of rat PRL or rat GH, whereas the authentic PRL-like mRNAs so far cloned are all in the 1.0kb range (26-33). Hiestand's data (165), although provocative, is somewhat contradictory.

Montgomery *et al* (166) using Con-A stimulated murine spleenocytes, identified a PRL-like protein based on bioactivity in the Nb2 rat lymphoma mitogenic assay and Western dot-blot detection of PRL-like immunoreactivity in spleenocyte conditioned medium. The molecular weight of this PRL-like molecule is 46,000 according to Russell (327). This has only been alluded to since confirmatory experimental data was not presented and to date no report supporting the existence of the 46,000 M.W. PRL-like protein has been published. In any case sequence analysis of the putative <10kb PRL-like mRNA detected by Hiestand et al (165) will be of great interest in light of the fact that the putative protein product has a M.W. of 46,000.

The only other indications of a PRL-like mRNA produced by lymphocytes come as brief statements in three abstracts discussed in the first section of the "Discussion". No complete reports presenting the data to support these claims has occurred in the literature at this time. Moreover, the size of the lymphocyte PRL-like mRNA in the abstracts varies considerably and with the inclusion of Hiestand's work (165) ranges from more than 10kb to 1.4kb. Given the controversial and incomplete nature of the work on a putative lymphocyte PRL-like message, it is reasonable to conclude that the only conclusive published report of immune cells producing PRL or a PRL-like molecule is described herein (328-330). Another important difference between IM-9-P3 lymphoblast production of PRL and the work of investigators described above was that the IM-9-P3 lymphoblast phenotype was not considered to be representative of normal lymphocyte physiology but rather the result of random mutation(s). In addition the existing reports of ectopic PRL production (119-122) do not include direct measurements of PRL synthesis by the tumours

in question, thus IM-9-P3 PRL expression is the only unequivocal report of ectopic or inappropriate PRL synthesis (328-330).

Biochemical and Physiological Characteristics of IM-9-P3 PRL

Prolactin synthesized by cells of the IM-9-P class was indistinguishable from pituitary hPRL. The conditioned medium of IM-9-P cells grown under serum free conditions contained immunoreactive hPRL, as measured by a standard hPRL RIA (Fig.9). In contrast, the only previous report of ectopic hPRL production by cell lines (122) found very small amounts of PRL in cell lysates and none in the culture medium. Further examination revealed that immunoreactive IM-9-P PRL was as active as immunoaffinity-purified pituitary hPRL in the rat Nb2 lymphoma mitogenic assay (Fig.10a). This biological activity was entirely due to IM-9-P PRL, since the proliferative response of Nb2 cells was completely and specifically abolished by the addition of monoclonal antibody to hPRL. An appropriate lactogenic control (oPRL) was included in these studies (Fig.10b) to ensure that the monoclonal hPRL antibody did not have non-specific toxic effects on these cells. Such a phenomenon may have been a factor in the studies reported by Russell's group (166) where polyclonal rat PRL antibodies severely reduced Nb2 lymphocyte proliferation stimulated with ConA, PRL or alloantigen.

The secretory rate of hPRL from the original IM-9-P cells was approximately 30ng/10^6 cells/day (324). The IM-9-P3 clone secreted hPRL at a rate of approximately 50ng/10^6 cells/day. This secretion was constitutive over the IM-9-P3 growth curve and was paralleled by a constant level of PRL mRNA (Fig.11). The human pituitary tumour cell line, HPA, described in 1985, produced 5-10ng PRL/ml over a 3 day period in which 5 x 10^5 cells had been cultured (270). The secretion rate had fallen from a level of 756ng/ml when the pituitary tumour cells were initially dispersed in culture. After about

130 days in culture, the production of PRL by HPA cells dropped dramatically to 1ng/ml and subsequently rose to approximately 10ng/ml after 270 days in culture. To date no other publications describing the use of HPA cells has appeared. Another human pituitary cell line (HPT1) established from a pituitary adenoma was described by Prysor-Jones and co-workers in 1987 (271). The quantity of PRL secreted by HPT1 cells over a defined growth period was not provided; however, 5 x 10^5 cells in a 30 minute incubation produced an average of 400mU of PRL per ml which is equivalent to 12.3μg/ml based on 32.5 international units = 1mg PRL. More recently these investigators reported the acquisition of a second human pituitary cell line, HPT2, from a prolactin-secreting pituitary tumour (331); however, no indication was given whether these cells secreted pituitary hormones. There have been no other reports regarding the establishment of a rapidly growing continuous culture of human cells which produce PRL or GH. To date IM-9-P3 cells and HPT1 cells remain the only stable hPRL secreting cell lines.

With regard to primary cell culture, the level of PRL secreted by human decidual cells obtained from term placenta at 50-70ng/10^6 cells/day (332) is similar to that secreted by IM-9-P3 cells which can range from 40-80ng/10^6 cells/day (324). The majority of studies on uterine PRL synthesis and regulation involved short-term explant cultures of endometria from different days of the menstrual cycle and therefore a direct comparison of the PRL secretory rate with that of IM-9-P3 cells is not credible. Under serum-free conditions Irwin and colleagues (71) cultured dispersed endometrial stromal cells from proliferative and secretory phases of the menstrual cycle. No PRL secretion was observed in serum-free medium; however, the addition of progesterone and estrogen stimulated PRL secretion up to 95ng/10^7 cells/day. This is slightly less than that seen from IM-9-P3 cells but the uterine cells cultured by Irwin (71) were probably lacking key factors present in FCS which normally maintain the cells in a healthier physiological state. Relatively few reports exist regarding the release of hormone from human pituitary cells in primary culture as opposed to organ culture. In general, organ culture of fetal pituitaries or

tumours of the pituitary can remain viable for as long as 1 year (266-268) and secrete an enormous amount of PRL ranging from 0.5 to 200μg/pituitary/day during the early stages *in vitro* (333,334,266-268). In dispersed primary cultures of human pituitary cells the quantity of PRL secreted is at least two orders of magnitude greater than that produced by IM-9-P3 cells (335,336), but is eventually lost due to slow growth of the hormone producing cells.

The intracellular storage of PRL in decidual and pituitary cells is different. Firstly, the amount stored differs dramatically with 20-50μg PRL/100mg of pituitary tissue (337) and only 0.05-0.10μg PRL/100mg decidua (338). Prolactin in the pituitary lactotrope is stored in large secretory granules whereas PRL in decidual cells is found, upon subcellular fractionation, in the post-microsomal supernatant implying that decidual PRL is not localized to granules (338). The IM-9-P3 intracellular concentration of PRL was between 2 and 4ng/10^6 cells (329) which was comparable to the storage of PRL by decidual cells at about 10ng PRL/10^6 cells (339). Clearly these characteristics indicate that steady-state PRL production by IM-9-P3 cells most closely resembles that of the normal human decidual cell.

Significance of Secreted PRL to IM-9-P Cell Viability

As reviewed previously, PRL is purported to exert immunomodulatory influences. The data is controversial yet some investigators believe that PRL can act directly on lymphocytes via specific cell surface receptors (163-166). It is well established that prolactin also serves as a growth factor for the Nb2 lymphoma T-cell line that expresses 12,000 PRL receptors/cell and is widely used for measuring the bioactivity of lactogenic hormones (169). In view of these reports and a report on the production of autostimulatory growth factors by human myeloma cells, including IM-9 cells (340) it

seemed important to consider a possible autocrine function for PRL secreted by IM-9-P cells. IM-9-P cells cultured in the presence of monoclonal hPRL antibody were not altered in their growth characteristics over a four day period (Fig.12). That the hPRL secreted from IM-9-P cells was completely neutralized was demonstrated by the inability of the conditioned medium containing antibody to stimulate proliferation of Nb2 cells. Similar and more extensive experiments were carried out on the IM-9-P3 clonal line (330). Neutralization of IM-9-P3 PRL did not affect the PRL secretion rate or the growth of these cells over a 96 hr period. Even the addition of excess hPRL to IM-9-P3 cultures had no effect on cell viability (324). Based on these parameters it was concluded that hPRL played no trophic role in the physiology of cells of the IM-9-P class. This was not a surprising result because repeated attempts to identify PRL receptors on IM-9-P3 cells have yielded negative results (324). IM-9 cells are well known as a source of hGH receptors, carrying approximately 3500 high affinity binding sites/cell (341). The presence of hGH receptors on IM-9-P3 cells on the order of 2500 sites/cell having an affinity similar to that of the parental IM-9 line has been confirmed (324). One might then hypothesize that hPRL secreted by IM-9-P3 cells could, as a nonpreferential ligand of lower affinity, bind to the IM-9-P3 GH receptor. This situation would be analogous to the actions of insulin at the insulin-like growth factor type 1 receptor (342) and based on the high degree of amino acid sequence homology betwen hPRL and hGH. Lesniak *et al* (343) has demonstrated, however, that this is an unlikely possibility because the EC_{50} for hGH binding to IM-9 cells is 10^{-9}M whereas displacement by one of three preparations of hPRL was approximately 10^{-5}M and may have been due to hGH contamination. Competition binding studies performed with IM-9-P3 cells as the substrate containing hGH receptors found that a 500 molar excess of unlabelled hPRL is required to displace the homologous hormone from its receptor (324). Collectively these data strongly suggested that IM-9-P3 PRL was not acting in an autocrine fashion on these lymphoblasts. This also tended to support the

notion that the production of PRL by IM-9-P3 lymphoblasts was an innocuous by-product of the mutational events which transformed the classical IM-9 line into IM-9-P3 cells.

Analysis of IM-9-P PRL mRNA and Gene Structure

IM-9-P PRL appeared identical to pituitary PRL based on immunological reactivity and bioactivity, yet the IM-9-P PRL transcript appeared approximately 150 bases longer than that of the pituitary (Fig.6). To determine whether this elongation was present in the protein coding portion of the transcript, the size of immunoaffinity purified pituitary and IM-9-P PRL was compared by SDS-PAGE (Fig.13). The lymphoblast PRL comigrated with its pituitary counterpart at approximately 24,000 daltons which implied that the mature PRL proteins were identical. Slightly higher molecular weight bands were present in both pituitary and IM-9-P immuno-purified PRL samples and may have represented the glycosylated form of hPRL. Attempts to purify a glycosylated IM-9-P PRL from conditioned medium by lectin affinity chromatography were unsuccessful.

Since the ectopically produced IM-9-P PRL protein was identical in size to its eutopic counterpart in the pituitary, the variation in length of the IM-9-P mRNA could have resided in the length of the poly(A) tract and/or 5' and 3' UTRs. Variation in poly(A) length may contribute to mRNA stability (344) and has been associated with developmental progression (345), circadian rhythm (346), serum or growth factor stimulation (347,348) and cell-type (349). After RNase H digestion of mRNA poly(A) tail, the IM-9-P PRL mRNA comigrated with intact pituitary hPRL mRNA, but remained larger than RNase H-treated pituitary PRL mRNA (Fig.14). Consequently the difference between IM-9-P and pituitary PRL transcript size was largely due to different 5' and/or 3' UTRs.

With regard to PRL produced by the human uterine decidua, other investigators have shown it to be essentially identical to that synthesized in the pituitary and therefore not different from the IM-9-P3 PRL. In the first demonstration of hPRL mRNA in decidua,

Clements *et al* (74) used ovine pituitary total RNA as the positive control and the mRNA signal in human decidua-chorion appeared to comigrate with the ovine PRL transcript. Variation in migration of these transcripts was present but was probably due to smearing of the bands and as a result an accurate size comparison could not be made. Huang and colleagues (70) studied decidual PRL mRNA levels in response to various hormones by Northern blot hybridization. In that report it appeared that uterine PRL mRNA was slightly larger than the pituitary transcript but this was not noted by the authors. There was no indication in the literature that decidual PRL mRNA was different from the pituitary message and therefore should be smaller than the ectopically produced IM-9-P PRL transcript. To test this hypothesis total RNA was extracted from a number of different gestational decidual tissue samples and subjected to Northern blot analysis along with pituitary and IM-9-P3 RNA. Unexpectedly, the decidual and IM-9-P3 PRL transcripts appeared identical in size, both being larger than the pituitary PRL message (Fig.17) (350). This result did not, however, prove that IM-9-P3 and decidual PRL mRNAs were identical in structure. In other words, the comigration of these hPRL mRNAs may have been coincidental with the source(s) of the elongation (ie. poly(A) tail, UTRs) having been different in each mRNA.

There had been no evidence to indicate the use of alternative transcription initiation or polyadenylation sites in the hPRL gene (10,36,75) which could have been responsible for the presence of elongated PRL transcripts in decidual and IM-9-P3 cells.Takahashi and co-workers (75) analyzed three cloned decidual PRL cDNA, the longest of which was truncated in the signal sequence. These investigators found essentially no difference between decidual and pituitary PRL sequence including the 3' UTR. To investigate the possibility of poly(A) tail length heterogeneity as the cause of the size difference, samples of RNA from pituitary, IM-9-P3 cells and decidua were subjected to RNase H digestion as described in Fig.14. The elimination of 3' poly(A) tails did not reduce the size difference between decidual and IM-9-P3 PRL mRNA relative to the

pituitary transcript (350). The poly(A)$^-$ PRL transcripts from decidual and IM-9-P3 cells comigrated indicating no significant difference in the degree of polyadenylation of hPRL mRNA in these distinct tissue types. Consequently the length of the poly(A) tract, the 3' UTR and protein coding region could not account for the larger size of the human decidual PRL transcript. By elimination, the source of elongation in the dPRL transcript was probably the 5' UTR.

One might therefore expect to find that the IM-9-P3 PRL gene had undergone rearrangement, a phenomenon known to activate genes inappropriately (351-355). As discussed earlier, karyotypic analysis of cell lines of the IM-9-P class indicated the presence of a number of chromosomal abnormalities. Translocation-type rearrangements could affect the 5' or 3' flanking regions of the IM-9-P3 PRL gene and alter the size of the 5' and 3' UTR which in turn could derepress or activate transcription of the PRL gene. Such rearrangements are characteristic in several types of malignancy of the hematopoietic system such as the c-myc translocation to the immunoglobulin heavy chain locus in most human Burkitt lymphomas (356), the BCR-ABL gene fusion in chronic myeloid leukemia (357), and the BCL2 and BCL1 fusion to the immunoglobulin joining region genes in B-cell lymphoma (358,359). In multiple myeloma, the B-cell neoplasia from which the classical IM-9 cells were derived, karyotypic abnormalities have been documented; however, these abnormalities are not consistent (360). In a recent abstract, Shadle and colleagues (361) reported a genomic alteration in the first c-myc exon in 8 of 13 multiple myeloma patients. Although the karyotypic markers of a c-myc translocation have not been described in multiple myeloma, other chromosomal changes beyond the resolution of karyotypic analysis may occur at the c-myc locus in some cases of multiple myeloma. This is relevant to the discussion here because conventional karyotypic analysis of IM-9-P cell lines revealed no gross changes of chromosome 6 which contains the hPRL gene (325). Obviously this does not preclude the possibility of smaller deletions or insertions in

and around the IM-9-P3 PRL gene which could be detected as a loss, gain or alteration of hPRL gene fragments by genomic Southern hybridization analysis.

The IM-9-P3 hPRL gene restriction fragment pattern was compared to that of a normal human placenta and the classical, non-PRL-producing IM-9 cells using 6 different restriction enzymes (Figs.15 and 16). No deviant restriction fragment patterns compared to the other human DNAs were detected with five of the six restriction endonucleases. The enzyme which did show a difference in fragmentation pattern of the hPRL gene was BglII. This difference was probably due to a RFLP of the hPRL gene since the BglII pattern seen in IM-9-P3 DNA was also observed in brain DNA from a normal individual and DNA from the T47D breast cancer cell line (Fig.16b). The alternative BglII pattern seen in the human placenta DNA contained an extra low molecular weight band of about 2.9kb and was also observed in DNA from another normal individual (Fig.16b). According to the published hPRL gene sequence and restriction map (Fig.26) there are five BglII sites in the gene. The 5' BglII fragments corresponded to the 23kb and 1.9kb fragments (Fig.16a); the fragment which encompassed a portion of exon 5 and 3' flanking DNA was the 4.1kb band which was the largest molecular weight fragment seen with pituitary hPRL cDNA as the probe. The BglII fragment extending from the site in intron C to the site in exon 5 (Fig.26) is approximately 2940bp according to the map of Truong *et al* (36). This, however, is only an estimate since a large portion of intron D (2040bp) has not been sequenced and presumably sized only by gel mobility. The remaining BglII fragment of the hPRL gene extends from the restriction site in intron A to the site approximately 6.0kb downstream in intron C according to the published sequence; yet a BglII fragment of that size was not detected in any of the DNA samples tested. Conversely the 3.5kb BglII fragment seen in Fig.16 was not predicted by the reported hPRL gene sequence. Thus it is conceivable that the 3.5kb and other BglII fragments were generated from the non-existent 6.0kb band by polymorphic restriction sites located in the unsequenced regions of introns B and C. Such

a hypothesis was not tested since cloned hPRL gene fragments which corresponded to the intronic regions of interest were not available at that time.

In summary, these genomic Southern hybridization studies identified new hPRL 5' flanking DNA fragments released by HindIII, NcoI, PstI and BglII. The exon/intron structure of the IM-9-P3 PRL gene appeared intact supporting the hypothesis that the elongation of IM-9-P3 PRL mRNA was not due to a change in the complexity of the protein coding region. Moreover the genomic fragments which corresponded to the immediate 5' and 3' flanking DNA of the IM-9-P3 PRL gene did not appear altered in size. This implied that putative alternative transcription initiation and/or polyadenylation sites of the IM-9-P3 PRL gene responsible for the atypical transcript size were not induced by a gross rearrangement of the flanking DNA.

Identification of an Elongated 5' UTR in IM-9-P and Decidual PRL mRNA

The observation that IM-9-P PRL mRNA was about 150 nucleotides longer than its eutopic counterpart was made even more intriguing when it was discovered that the normal decidual PRL transcript resembled the ectopically produced PRL mRNA. In order to obtain direct evidence for the presence of elongated untranslated regions, the IM-9-P and decidual PRL messages were isolated as cDNAs and sequenced. In this way unambiguous evidence was provided that the distinctly larger transcript detected by Northern hybridization studies in IM-9-P cells was authentic PRL and not a new member of the PRL-GH gene family analogous to the recently described PRL-like genes expressed in rat, mouse and bovine placenta (26-33).

A total of eleven IM-9-P cDNA clones from two different libraries were isolated and sequenced, showing a protein coding region identical to that previously described for the pituitary protein (Fig.19). The protein coding sequence of decidual PRL mRNA has

been published by Takahashi *et al* (75); no major differences were detected in comparison to the pituitary transcript. In the studies described here, eight distinct dPRL cDNAs were purified from two different libraries and partial sequence analysis confirmed the previously published data (75), although three of these cDNAs contained an extra alanine codon between lysine -20 and glycine -19 of the signal sequence. This region of the leader sequence constitutes the splice junction between exon 1 and 2 of the human pituitary PRL gene (36). The 3' end of exon 1 terminates in a G residue which normally combines with the 5' end of exon 2 to complete the GGG codon for glycine -19. It appeared from decidual PRL cDNAs 2A, 15A and O, that occasionally an alternative 3' splice junction at the intron A/exon 2 boundary is utilized such that an "AG" dinucleotide located two nucleotides 5' of the normal 3' splice acceptor site was recognized as the splice junction for exon 2. As a result the 3' G residue of exon 1 combined with the "CA" at the 3' end of intron A to generate the extra alanine codon "GCA". The adjacent G residue which usually formed the 3' terminus of intron A reads in-frame with the "GG" residues at the 5' end of exon 2 to form the glycine -19 codon. The hydrophobic nature of the signal sequence is likely not altered by the extra alanine residue which contains a nonpolar R group. This same phenomenon has been described for rat PRL-like protein B (29) and rat PRL mRNA.

The 3' untranslated sequence of decidual and IM-9-P PRL transcripts did not differ markedly from the pituitary transcript. The 7-14 nucleotide elongation relative to the pituitary transcript was probably not unique to decidual or IM-9-P3 PRL messages. The sequence of only one pituitary hPRL cDNA has been published (10). Had more cDNAs been analyzed it is likely that the pituitary hPRL mRNA possesses a 3' terminus identical to that of decidual/IM-9-P3 PRL transcript. With regard to the 3' end of these PRL transcripts, previous studies using RNase H degradation of poly(A) tails resulted in essentially the same size differential between pituitary and IM-9-P3/decidual PRL mRNAs. Thus there was no appreciable poly(A) tail length polymorphism which contributed to the transcript size difference.

The absence of an obvious cell-type specific difference at the 3' end of the hPRL mRNAs suggested that the atypical size of the IM-9-P3 and decidual mRNAs resulted from structural alterations elsewhere in the mRNA, namely the 5' end. The alignment of nucleotide sequences of IM-9-P PRL cDNA-IV, containing the longest 5' non-coding segment, with that of the PRL gene provided evidence for the unmistakable elongation of the ectopic PRL transcript (Fig.21). The 5' untranslated region of human pituitary PRL mRNA is colinear with the region of genomic DNA immediately upstream of the translation initiation codon. Clearly the IM-9-P PRL cDNA sequence diverged from the immediate 5' flanking sequence of the hPRL gene at a position 98bp upstream of the coding sequence. At this position the IM-9-P PRL cDNAs contained a common 5' untranslated region which extended for 93 bases in the longest clone and whose sequence was not colinear with the genomic PRL sequence 5' of -98. Five independent IM-9-P PRL cDNAs contained the identical 5' UTR structure. Furthermore, six distinct dPRL cDNAs from two different cDNA libraries also possessed a segment of unique 5' UTR completely homologous to that found in IM-9-P cDNAs. Hence the basis for the difference in electrophoretic mobility between the pituitary PRL message and that of IM-9-P3 and decidual cells lies in a unique 5' UTR structure found exclusively in the latter two species of PRL mRNA.

As further proof of the authentic nature of this unique 5' UTR, Northern blot hybridizations using antisense cRNA corresponding to this sequence identified only the larger IM-9-P3 and decidual PRL transcript (Fig.23). Primer extension studies confirmed these results by demonstrating that decidua and IM-9-P3 PRL mRNAs do not initiate at the same position as the pituitary gene but rather possess a somewhat complex set of predominant 5' ends ranging from 83 to 121 nucleotides longer than the pituitary-specific 5' UTR. The pattern of primer extended bands was identical using either decidual or IM-9-P3 RNA (Fig.24). That only a single broad band of hybridizing PRL mRNA was seen

with IM-9-P3 or decidual RNA on Northern blots would be explained by the lower resolving power of this technique.

In addition to the seven closely spaced principal 5' ends, the primer extension studies showed that the IM-9-P3 and decidual PRL mRNA population contained members which had even longer 5' UTRs. The largest of these minor members possessed a 5' non-coding region of 320 bases as determined by prolonged exposure of primer extension polyacrylamide gels (Fig.24). Interestingly the estimated size of the longest primer extended product correlated well with the 300bp 5' UTR of dPRL cDNA 15A (Fig.21). This decidual clone was unique among all of the PRL cDNAs isolated, in that the greater proportion of the 5' UTR was colinear with the genomic PRL sequence. At position -246 relative to methionine 1, the 5' UTR of clone 15A diverged into novel sequence identical to that seen at -98 of all of the other IM-9-P/decidua PRL cDNAs. This unique sequence extended for 54 nucleotides before terminating 20 bases short of the largest primer extended cDNA. This 20 base difference could have been easily accounted for by premature termination of reverse transcription since IM-9-P PRL cDNA IV contained an additional unique 39 nucleotide 5' extension relative to the 5' end of clone 15A. The existence of cDNA 15A and the multiple primer-extended cDNA products suggested that either PRL gene transcription began at multiple points in decidua and IM-9-P3 cells or that the new 5' non-coding exon was spliced to multiple acceptor sites. The latter possibility appears to occur, albeit at a very infrequent rate with clone 15A was the sole representative of this novel extended 5' UTR structure among the large population of IM-9-P/decidua PRL cDNAs isolated.

The structure of IM-9-P PRL cDNA I-1 was another potential example of the use of different splice sites. In this case, however, the variation was in the 5' splice donor. In all of the IM-9-P and decidual PRL cDNAs which contained the unique 5' non-coding information, the sequence at the junction with -98 of the known 5' flanking DNA of hPRL exon 1 or 5' splice donor was identical. In IM-9-P PRL cDNA I-1 this 5' splice junction

was different and contained an extra 12 nucleotides which may have constituted the use of an alternative 5' splice donor sequence. The unique 5' untranslated sequence upstream of the extra 12 nucleotides of interest in IM-9-P cDNA I-1 was identical to that seen in the other cDNAs (Fig.22). Therefore it was reasonable to suggest that the extra 12 nucleotides at the splice junction was colinear with the upstream novel sequence and not an insertional cDNA cloning artifact. Unfortunately, this latter possibility appeared somewhat tenable since clone I-1 was shown to have been chimeric and contained a 45bp 5' end sequence linked in the opposite orientation to the novel 5' UTR of IM-9-P/decidua PRL mRNA. Confirmation of the former explanation requires the isolation of genomic clones encompassing the novel 5' untranslated sequence of IM-9-P/decidua PRL message.

The predicted length of the 5' untranslated sequence of IM-9-P/decidua PRL mRNA from primer extension studies ranged from approximately 140 to 178 nucleotides of which 42 to 80 nucleotides was unique sequence. The outer limit on the size of the unique 5' untranslated sequence was extended by 13bp in IM-9-P PRL cDNA IV. This was not surprising since primer extended products beyond a 178 nucleotide 5' UTR were evident, but less abundant, by primer extension analysis (Fig.24). Collectively the IM-9-P and decidual cDNAs indicated that the 5' end of the hPRL message in these cell types were generated by multiple clustered transcription start sites and by the use of alternative splice sites, the latter having been a much less prominent mechanism given the scarcity of the exemplary cDNAs.

Localization of the New 5' Non-coding Exon of the hPRL Gene

As alluded to above, the segment of unique 5' UTR found in the decidua/IM-9-P3 PRL mRNA was not present in the immediate 5' flanking region to -978 of the PRL gene. The fact that this novel 5' UTR existed as a new 5' non-coding exon located at some distance upstream of the pituitary hPRL RNA start site was proven by genomic Southern

hybridization analysis (Fig.25). In an effort to define the length of this intron, designated A-1, a series of genomic Southern hybridizations were carried out. The objective was to identify common fragments using a DNA probe, described previously as the 5' end-specific genomic probe, which extended to position -978 relative to the translation initiation site (Fig.26) and a 72bp segment of the novel 5' non-coding exon. In this way the size of intron A-1 was estimated at 5-8kb (Figs.27 and 28). To bridge this distance genomic clones extending further upstream of the pituitary hPRL transcription start site were needed. Such a genomic clone, which overlapped with known hPRL sequences, was isolated by Dr. J. Martial (University of Liege, Belgium) (280) and extended approximately 5.8kb upstream of the origin of pituitary transcription. The most 5' 830bp SalI/HindIII fragment of phPRLg4800 was used in these studies. By sequential hybridization using this more 5' genomic DNA and the 72bps of novel 5' non-coding cDNA it was shown (Figs.30 and 31) that these diverse probes hybridized to the same sized genomic fragments over six different restriction digests. These experiments strongly suggested that the new 5' non-coding exon of the PRL gene was located just beyond the 5' end of phPRLg4800 which made intron A-1 approximately 5.9kb in length.

This form of gene analysis based on the demonstration of comigrating superimposable restriction fragments found upon hybridization with different regions of the hPRL gene, was open to two possible sources of misinterpretation. Firstly, a comigrating band identified by two distinct probes might be found simply because one of the probes happened to detect a restriction fragment of the same size in a particular digest. Secondly, identical sized restriction fragments in some cases could have been due to a coincidental genetic variation or genetic polymorphism (RFLP). This latter possibility was ruled out in these experiments because 5-7 DNAs from normal individuals was included in each set of restriction digests so that a DNA polymorphism would likely have been detected, as was the case for BglII. Furthermore the utilization of 6 different restriction enzymes in single and double digests made a coincidental demonstration of linkage between diverse segments

of the hPRL gene highly unlikely. Most importantly, the results of initial experiments (Figs.27 and 28) which utilized only 978bp of hPRL 5' flanking DNA were confirmed with another fragment of hPRL 5' flanking DNA located approximately 4.2kb upstream (ie. 830bp SalI/HindIII fragment).

In addition these studies indicated that no major rearrangement had occurred specifically in the DNA surrounding the novel 5' non-coding exon in the IM-9-P3 genome. Therefore, it was unlikely that a viral insertion event (353,362,365) or other major rearrangement in the immediate vicinity of the IM-9-P3 PRL gene could explain how it was activated such that a decidual-type mechanism of hPRL transcription was initiated.

Analysis of the IM-9-P/decidua PRL cDNA sequence provided two possible interpretations with regard to the use of consensus splice site sequences. At first glance it appeared that the processing of the new 5' non-coding exon 1a occurred via variant 3' splice acceptor sites. In the majority of IM-9-P and decidual PRL cDNAs the unique 5'UTR spliced into -98 relative to the translation start site and rarely at -246. The dinucleotides preceding this splice junction, as deduced from the published genomic sequence (36), are AA and TA respectively and thus do not obey the universal GT/AG rule (366). If, however, the guanosine nucleotide at -98 and -246 actually represented the last base of the new 5' non-coding exon rather than the first base of exon 1b, then the GT/AG rule would have applied to both sites. This latter placement would make the adenine at -97 or -245 (see Fig.21) the first base of exon 1b and allow the adjacent 5' AG dinucleotide to function as the canonical 3' splice acceptor. Whichever hypothesis proves correct, the polypyrimidine tract which constitutes the remainder of the consensus 3' splice acceptor site was essentially missing from the sequence preceding -98 and -246 of hPRL exon 1b.

A clue regarding the 5' splice site may have been gleaned from IM-9-P PRL cDNA I-1 which contained an extra twelve nucleotides 3' of the splice jucntion utilized in the remaining IM-9-P and decidual PRL cDNAs (Fig.22). If it was assumed that this extra 12bp was colinear with the preceding unique 5' untranslated sequence of IM-9-P/decidua

PRL mRNA then a GG dinucleotide occupied the "invariant" position of the consensus 5' splice donor sequence rather than the canonical GT. Alternatively the guanosine nucleotide at position -110 of IM-9-P PRL cDNA I-1 (see Fig.22) could have represented the last base of exon 1a in the majority of IM-9-P/decidua PRL cDNAs. This would designate the next 3' dinucleotide as the 5' splice donor sequence which was a GT and thus followed the GT/AG rule. As in the other cDNAs, the last base of the unique 5'UTR of IM-9-P PRL clone I-1 could have been the guanosine nucleotide at -98 (see Fig.22) which would then place the immediate upstream AG dinucleotide in the hPRL gene sequence in the invariant 3' splice acceptor site. The nucleotide sequence which surrounded the putative 5' splice site as deduced from clone I-1 did not resemble the consensus sequence (NAGGT$^{A}/_{G}$AGT) determined for mammalian genes (366). Shapiro and Senapathy's (366) analysis of 3700 splice sites resulted in the identification of only four non-confirming 3' splice acceptors of which one contained an AA or TA dinucleotide at the so-called "invariant" 3' end position of the intron (367,368). Another example, however, of a GG dinucleotide replacing the invariant GT at the 5' splice donor is found in the mouse immunoglobulin active heavy chain gene (366). The genomic sequence of the new upstream exon 1a is required to accurately identify the splice site sequences involved in its processing. A human genomic DNA library (ATCC number 37458) has been screened with the 311bp tetramer of unique 5'UTR described previously and several strong positive plaques were identified. Curiously however, these positive clones proved impossible to purify. It is possible that the DNA surrounding hPRL exon 1a contains sequences (i.e., inverted and/or direct repeats and palindromic sequences) which reduce the viability of the host bacterium and make the phage very difficult to propagate. Cosmid human DNA libraries will be screened in a attempt to circumvent this putative problem.

Significance of the IM-9-P3/Decidua PRL Novel 5' UTR Sequence

Several examples exist in the literature which suggest that the secondary and tertiary structures of a mRNA can play an important role in determining its translational efficiency (369-372). In some instances the effect on translation is inhibitory and can be correlated to the predicted secondary structure in the 5' UTR of the mRNA. Stretches of nucleotides possessing a high probability of forming stem-loop structures in the 5' non-coding region can lead to diminished mRNA translation *in vitro* (370) and can be the binding site of a cytosolic repressor protein (373). Translational regulation of decidual PRL has not been reported. Nevertheless the new 5' UTR sequence of IM-9-P3/decidua PRL mRNA was analyzed for the presence of palindromic sequences which might form stem-loop structures; no such obvious sequences were found.

Another mode of translational regulation afforded by the 5' UTR of a mRNA is the occurrence of initiator AUG codons which could be recognized by the ribosome upstream of the primary open reading frame. This results in the presence of short open reading frames which are postulated to impede the translational efficiency of the downstream region (374-376). A search of the unique 5' UTR of IM-9-P3/decidua PRL mRNA revealed no ATG triplets. On the other hand, the hPRL genomic sequence between -98 and -248 relative to the translation start site, contained nine ATG triplets. Recall that -98 is the splice site joining the unique 5' untranslated sequence of IM-9-P3/decidua PRL message to the flanking DNA of exon 1b. It is noteworthy that the extended 5' UTR of decidual PRL cDNA 15A (Fig.21) contained 5' flanking DNA of exon 1b up to -246. The nine ATG triplets within this region, which is GC-rich, were not found in the consensus sequence for initiation in higher eukaryotes (376). These ATG triplets were embedded in AT-rich sequence and it was therefore highly unlikely that they could act as preferential translational initiation sites on a decidual PRL mRNA having the extended 5' UTR of clone 15A. In any case, clone 15A was a very rare member of the PRL mRNA population and

therefore the significance of the nine potential ATG translation start codons was diminished even more.

It will be of interest to determine if the sequence of the optional exon is conserved in non-human primate decidual PRL mRNA. Conservation of nucleotide sequence and alternative splicing pattern of PRL exon 1a across species might suggest a functional role for this exon.

Implications of the Novel 5' UTR of IM-9-P3/Decidua PRL mRNA for Tissue-Specific PRL Gene Expression

As discussed in the "Introduction", there are numerous examples of alternative use of exons and promoters as a means to generate tissue-specific RNA diversity and new modes of control of gene expression. The now classical examples of such mechanisms include the mouse alpha-amylase 1 gene (230) and the Drosophila alcohol dehydrogenase gene (231). In both cases two different mRNA species are synthesized having different 5' UTRs transcribed from separate promoters which are active in different cell types. The studies presented here clearly demonstrated that the hPRL gene also subscribed to such a mode of tissue-specific regulatory flexibility. These data suggested that alternative *cis*-acting sequences and corresponding nuclear *trans*-acting factors constituted a novel mechanism which conferred decidual and lymphoblast PRL gene expression. This had evolved by the inclusion of a new 5' non-coding exon to the decidual and IM-9-P3 PRL gene. An analogous precedent for such an hypothesis is the gene for macrophage colony stimulating factor (c-fms) receptor. In human placental cells, transcription of the c-fms receptor gene begins at a series of clustered sites located approximately 25kb upstream of the monocyte-macrophage specific cap site (203). This results in a much extended 5' UTR by the inclusion of a 197bp 5' non-coding exon as defined by S1 nuclease and primer-

extension experiments using human placental RNA. As appears to be the case for the pituitary PRL transcript, the myeloid-specific transcription start site of the c-fms receptor gene occurs within the non-coding region of exon 2 which contains the translation initiation site (203).

To date several laboratories have confirmed that Pit-1, a POU-homeodomain *trans*-acting factor expressed exclusively in the anterior pituitary, is sufficient to allow tissue-specific expression of the rat PRL gene (253-258). The corresponding Pit-1 DNA binding sites are located within 1.8kb of the rat pituitary PRL RNA start site. The 5' flanking region of the human pituitary PRL gene is highly homologous to that of the rat gene and contains a cluster of sequences which fit the canonical Pit-1 recognition site (36). Lemaigre and colleagues (273) have demonstrated that these putative Pit-1 sequences in the hPRL gene will indeed interact in a functional manner with the rat Pit-1 molecule. The possibility that Pit-1 or a related protein may have evolved to activate decidual PRL gene expression at sites about 5.9kb upstream of the pituitary-specific cap site was an intriguing hypothesis. To this end human decidual poly(A)$^+$ mRNA was analyzed in collaboration with Dr. H.P. Elsholtz (Banting and Best Diabetes Centre, University of Toronto) for the presence of transcripts containing the highly conserved POU-homeodomain sequence (260). He *et al* (377) have been successful in isolating new members of the POU-homeodomain gene family of transcription factors using degenerate primers homologous to the POU-homeodomain boundary sequences in polymerase-chain amplification of hybridizing cDNAs from various rat tissues. The same highly sensitive technique was employed in the quest for Pit-1 like factors which might be implicated in decidual PRL gene expression. After thirty cycles a single distinct band was resolved, cloned and when sequenced found to have represented the POU-homeodomain of the ubiquitously expressed transcriptional activator, Oct-1 (378) (data not shown). Thus Pit-1 or other closely related POU-domain *trans*-activators may not have been involved in conferring decidual specific transcription of the hPRL gene. It cannot be ruled out, however, that the presence of a

decidual-specific *trans*-activator containing a more poorly conserved POU-homeodomain, such as that found in the liver-specific transcription factor LF-B1, was involved in hPRL gene expression (379).

It has been well-established that the classical regulators of pituitary PRL synthesis do not affect the expression of decidual PRL (91). Although there are a multiplicity of reasons to explain this fact, it seems that the explanation could reside in the structure of the PRL gene. It has been shown that those *cis*-acting elements responsible for transducing regulatory signals (i.e., epidermal growth factor) from the cell membrane to the rat pituitary PRL gene are located in the immediate vicinity of the transcription initiation site associated with Pit-1 response elements (380-382). Hence it seems unlikely that those *trans*-activators associated with the hormonal control of the human pituitary PRL mRNA synthesis could exert a controlling influence over the initiation or rate of PRL gene transcription initiated at the decidual specific site about 5.9kb upstream.

A large battery of potential regulators of PRL synthesis was tested on IM-9-P3 cells, the most significant response was obtained with the synthetic glucocorticoid, dexamethasone (329,330). Dexamethasone inhibited PRL release and mRNA abundance by significantly reducing the half-life of the mRNA from about 17hr to 3.6hr after 16hr exposure (330). Studies of this type are necessarily limited by the complement of receptors expressed by the cell of interest. In the case of IM-9 cells, the parental line of the IM-9-P3 variant, receptors for a number of different hormones, have been identified (cited in reference 330). It is not known whether the PRL-producing IM-9-P3 cell contained a similar complement of receptors, therefore, its usefulness as a model system to study decidual-specific PRL gene regulation is limited.

The initial objective, when the studies which constitute this thesis were undertaken, was to gain some insight into the mechanism by which the hPRL gene was ectopically activated in IM-9-P cells. The preponderence of evidence indicates that the inappropriate activation of the lymphoblast PRL gene occurred via a mechanism which

selected the decidual mode of transcription. The question remains as to whether this activation event was mediated by promiscuous lymphoid-specific nuclear transactivators or by decidual-specific factors which had also been ectopically generated in IM-9-P3 lymphoblast cells and then act on a receptive chromatin template. Other potential scenarios include point mutations in the 5' flanking region of the IM-9-P3/decidua specific hPRL exon 1a which could enhance binding of a promiscuous activating transcription factor (383). Conversely, point mutation(s) might have abolished the binding of a critical suppressor factor (384). Whatever the case, the IM-9-P3 lymphoblast cell line provides an easily manageable and unique resource with which to delineate the elements and regulatory mechanisms which confer cell-specific expression of the hPRL gene to decidual cells and pituitary lactotropes. It will be of considerable interest to identify the essential regulatory elements and critical *trans*-acting factors of the IM-9-P3 PRL gene to gain some understanding as to the nature of the recent evolutionary event by which primate uterine decidual cells acquired the ability to express PRL.

Future Prospects

The existence of the PRL-producing IM-9-P3 clonal cell line, the IM-9-P6 line which does not synthesize PRL and the IM-9 progenitor line constitute the first homologous experimental system with which to analyze the molecular mechanism of hPRL gene transcription. The expression of the rat PRL gene has received considerable attention in the field of cell-specific regulation, to the point where at least one of the pituitary-specific transcription factors (Pit-1/GHF-1) has been purified and cloned (253-259). Relatively little work has been done in this area on the human PRL gene largely because of a lack of a human cell system and the fact that the hPRL gene is also expressed in the decidualized endometrium has been virtually ignored. Reasonable evidence exists to suggest that human

pituitary PRL gene transcription follows a mechanism essentially identical to that described for the rat PRL gene (273). Clearly these data cannot be simply advanced to the human system in the case of the decidual PRL gene since work described here has shown that the 5' flanking DNA of interest is located at least 5kb upstream of the pituitary-specific *cis*-acting promoters. Given the identical structure and transcription start sites of the IM-9-P3 and normal human decidual PRL mRNAs described herein, it is plausible that the IM-9-P3 cell line contains unique *trans*-acting DNA binding proteins essential for decidual-like PRL gene expression. The putative DNA binding proteins would presumably be inactive or absent in the non-PRL-producing cell lines IM-9-P6 or the classical IM-9 line and these lines would therefore act as convenient negative controls. These assumptions are based on the fact that the rat PRL pituitary-specific transcription factor, Pit-1 is solely sufficient for transcription of a marker gene containing rat PRL tissue-specific enhancer elements in heterologous cell systems (256,258). Moreover, others have shown that loss of rat GH expression in somatic cell hybrids is strictly correlated with the loss of positive cell-specific transcription factors (385,386). There are also numerous examples where cell lines which ectopically synthesize a hormone, have been utilized to compare and contrast the transcriptional regulation of the ectopic hormone gene to its normal counterpart in the eutopic tissue source (387-390).

Lymphoblast specific PRL transcription could be demonstrated using gene-fusion plasmids containing 5' flanking DNA of the IM-9-P3/decidua PRL gene transfected transiently into IM-9-P3, P6 and classical IM-9 lines. In this way *cis*-acting DNA sequences will be identified which interact in trans with nuclear factors to stimulate the lymphoblast (and presumably the decidual-specific) expression of the hPRL gene. Having identified the cell-specific enhancers of lymphoblast PRL transcription, the IM-9-P3 cell line becomes a homogeneous source from which the corresponding *trans*-activator proteins could be purified and cloned. These DNA binding proteins could then be characterized for their ability to direct decidual-specific gene expression in various human endometrial

tumour cell lines which have not been shown to synthesize PRL. It may be that these cell lines contain decidual specific *trans*-acting factors necessary for hPRL gene transcription but fail to express the PRL gene due to epigenetic influences such as inappropriate methylation of DNA recognition elements. On the other hand, the endometrial cell lines may simply lack the appropriate *trans*-activating factors.

The synthesis of PRL by secretory endometrium has been shown to coincide with the initial appearance of decidual cells in late luteal phase of the menstrual cycle (66,67,70). Thus the initiation of decidual PRL gene expression could be considered a marker for the terminal differentiation of endometrial stromal cells to decidual cells. Since the specific complement of genes expressed by a cell dictates its phenotype, one could envisage the activation of decidual PRL gene transcription intimately linked to the endometrial differentiation process. Should the above studies indicate that IM-9-P3 PRL gene transcription is analogous to the mechanism operating in decidual cells, then the biological reagents (i.e., *trans*-acting positive transcription factors and their genes) obtained from IM-9-P3 cells could be used to analyze, in a retrograde fashion, the cascade of genomic events leading to the appearance of uterine decidual cells. By understanding the molecular regulation of decidual-specific transcription factors, a better understanding of the remarkably regular physiological trigger of endometrial turnover could be obtained.

Progesterone is the primary stimulator of decidual PRL release and can increase the relative abundance of the corresponding transcript (67,70). By transfecting IM-9-P3 cells with a plasmid expressing the progesterone receptor, one might demonstrate regulation of the endogenous PRL gene and identify the level of RNA biogenesis at which the progesterone receptors act to increase PRL transcript levels. If the progesterone receptor were found to regulate the rate of PRL transcription from the decidual-specific start sites, then one could determine if the regulation was direct by measuring the time course of induction in the presence and absence of protein synthesis inhibitors. The logical extension of this work would focus on the identification of progesterone response elements around

exon 1a of the hPRL gene by co-transfection of reporter fusion plasmids and a progesterone receptor expression vector. The cell lines of the IM-9-P class would then provide a model system with which to analyze the interaction of the progesterone receptor with cell-specific *trans*-activators and potentially provide some insight into the mechanism by which the decidual PRL gene is normally activated.

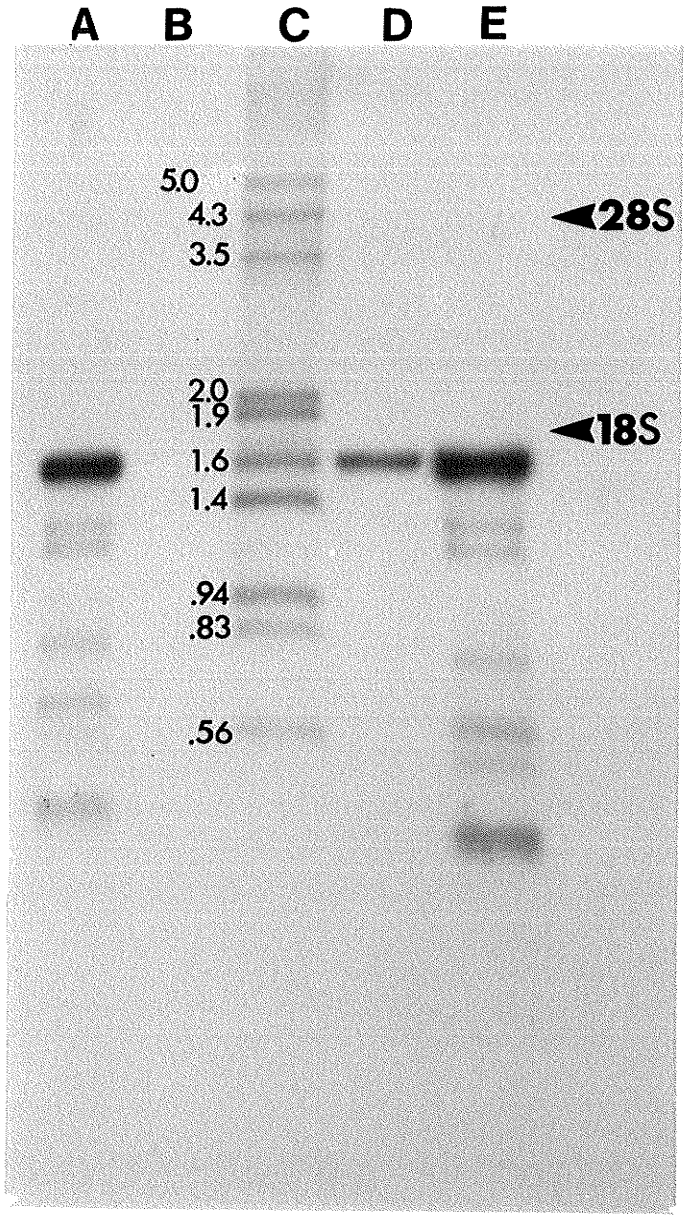
Another potentially interesting series of experiments would focus on determining whether lymphokines can modulate PRL gene expression in IM-9-P3 lymphoblasts. As discussed earlier there is growing support for PRL as an immunomodulatory factor; however, there is little direct evidence of immune cell factors regulating PRL expression. In recent years it has been reported that many of the various lymphokines are produced by the uterine decidua and/or placenta and the presence of corresponding receptors has also been documented (391-396). Thus it would seem worthwhile to test the ability of various lymphokines (i.e., colony stimulating factor-1) to modulate transcription of the IM-9-P3 PRL gene from the transcription start sites held in common with the dPRL gene. Such studies are limited by the complement of receptors expressed by IM-9-P3 cells. Nevertheless the identification of dramatic regulation of IM-9-P3 PRL gene expression by a single or group of lymphokines would prompt one to investigate whether a similar form of regulation occurs *in vivo* during gestation in decidual cells. This could be demonstrated by *in vitro* cell culture of gestational decidua (71). The function of dPRL is unknown, but evidence that lymphokines can directly regulate dPRL gene expression would imply that a role for PRL exists in maintaining the local immunological milieu during pregnancy.

The cloning of the pituitary-specific PRL transcriptional activator, Pit-1, opens the door to a number of unique experiments using IM-9-P3 cells. These experiments would be designed to examine the interaction of two different mechanisms of tissue-specific transcription on one gene in a particular cell type, in this case, IM-9-P3 cells. An expression plasmid containing the Pit-1 cDNA would be transfected into cell lines of the

IM-9-P class and primer extension experiments performed to determine if the pituitary cap site is utilized in transfected lines. In classical IM-9 cells and the non-PRL-producing clone IM-9-P6, one might expect to observe activation of the PRL gene from the pituitary specific RNA start site in IM-9-P6 cells and no activation in the progenitor cell line, IM-9. This hypothesis is based on the fact that IM-9-P6 cells are, for all intents and purposes, identical to the PRL-producing IM-9-P3 cells except for the ability to express the PRL gene. This suggests that the PRL gene in IM-9-P6 cells is in an open chromatin conformation but lacks the necessary factors for active transcription. The progenitor IM-9 cells would likely contain a repressed PRL gene inaccessible to modulation by Pit-1. A positive result would be the first demonstration in intact cells that Pit-1 is sufficient for expression of an endogenous human PRL gene in a lactotrope-specific manner. In IM-9-P3 cells the results of such an experiment are unpredictable and may depend on the strength of the upstream decidua/IM-9-P3 specific enhancers and the dose of Pit-1 cDNA transfected. Nevertheless, these experiments would be a lot of fun and isn't that what science is really all about!

Fig.1. Size determination of putative rPRL-like RNAs.

Twenty micrograms of total RNA isolated from various rat tissues was denatured and fractionated through a 1.5% agarose, 2.2M formaldehyde gel. A photograph of the EtBr-stained gel is shown on the right and the sample in each lane was as follows: A, kidney; B, testes 5μg total RNA; C, [^{32}P]-end-labelled lambda DNA digested with HindIII; D, uterus; E, heart. The RNA was capillary blotted to nitrocellulose prehybridized and probed with nick-translated [^{32}P]-labelled rPRL cDNA (3x10^{6}cpm/ml) at 42C in 50% formamide/5xSSC hybridization solution for 24 hours. Final washing of the blot was at 53C, twice for 30 minute intervals in 0.1xSSC, 0.1% SDS. The blot was exposed to X-ray film at -70C with an intensifying screen for 4 days. The sizes of the DNA marker bands are given in kilobases (kb) next to each band. The location of 28S and 18S ribosomal RNA is denoted by the arrows.


A
B
C
D
E
5.0
4.3
3.5
28S
2.0
1.9
18S
1.6
1.4
.94
.83
.56

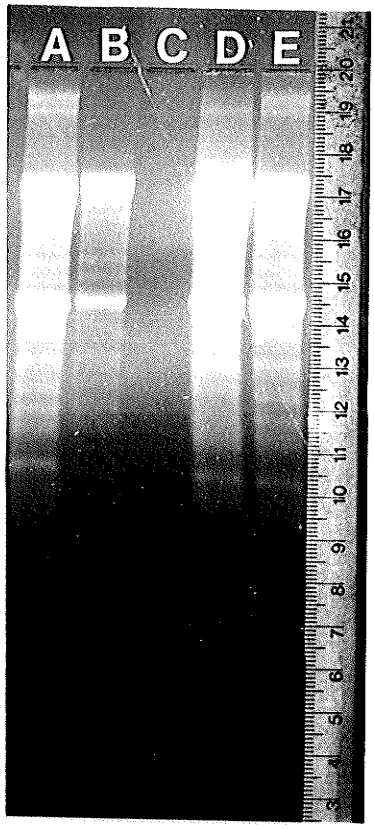

A B C D E

Fig. 2. Elimination of intact 18S ribosomal RNA as the molecule to which rPRL cDNA hybridized.

Total RNA (20μg) was isolated from lung and skeletal muscle of two separate groups of adult Sprague-Dawley rats. The RNA in lanes A and D were isolated from normal 150g female rats using the guanidinium isothiocynate/CsCl cushion method described in the "Materials and Methods". The remaining lanes contained RNA extracted from approximately 350g male Sprague-Dawley rats. RNA in lanes B, E and F was purified using the method described above while the RNAs in lanes C and G were isolated following the guanidine-HCl procedure as described in the "Materials and Methods". These RNAs were fractionated, blotted to nitrocellulose and hybridized with rPRL cDNA as described in Fig. 1. The photograph on the right shows the EtBr-stained gel prior to transfer to nitrocellulose. The location of 28S and 18S rRNA is provided. Autoradiography was for 4.5 days under optimal conditions defined as incubation at -70C with an intensifying screen.

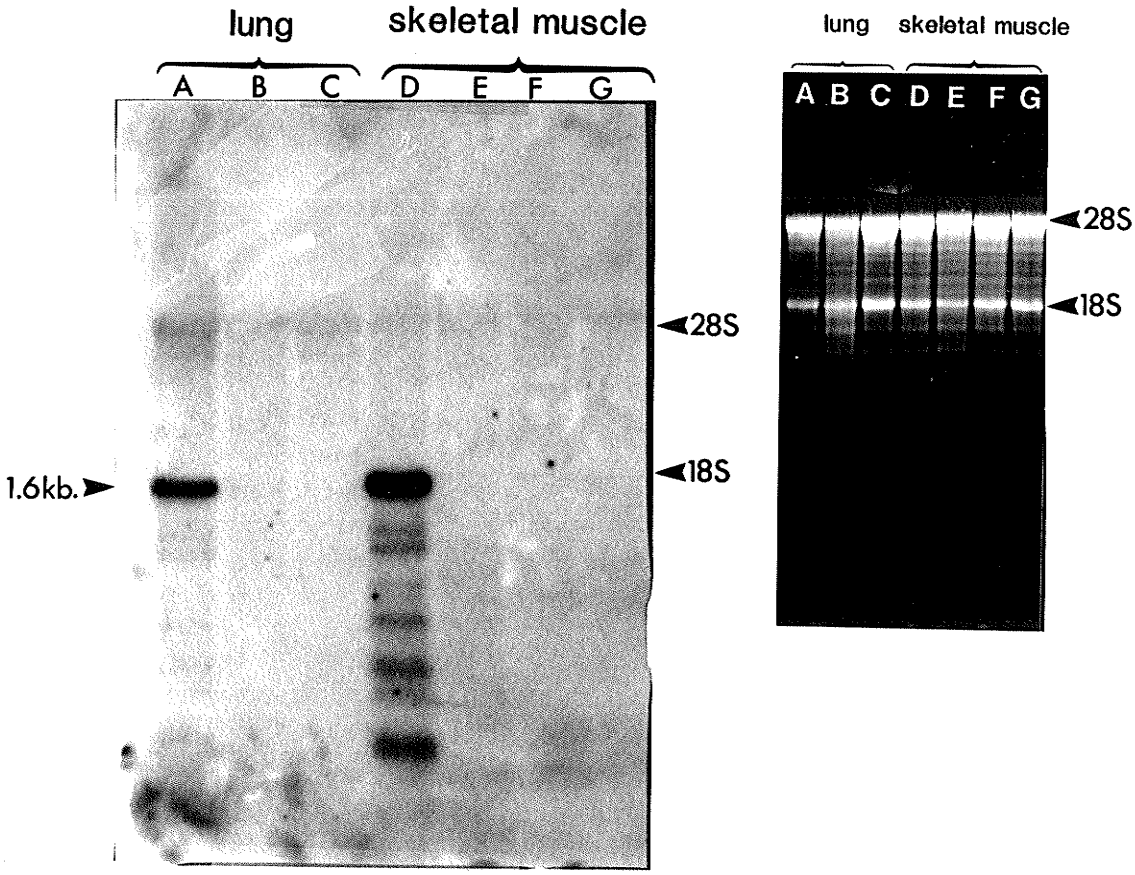

lung
skeletal muscle
A B C D E F G
28S
18S
1.6kb.
lung
skeletal muscle
A B C D E F G
28S
18S

Fig. 3. Identification of putative rPRL-like RNAs as non-polyadenylated species by Northern blot hybridization.

Lanes marked T contained 30μg of total RNA and those labelled A- contained the same amount of RNA which did not bind to oligo (dT) cellulose. In "a" the lanes labelled A^+ contained 10μg and in "c" approximately 2μg of RNA enriched in polyadenylated mRNA species by chromatography over oligo (dT) cellulose. The RNA blots in "a" and "c" were hybridized with 3.5 x 10^6cpm/ml and 2.5 x 10^6cpm/ml of nick-translated rPRL cDNA respectively at high stringency. Final wash of these blots was at 50C in 0.1xSSC, 0.1% SDS for two 30 min intervals. The blot in "a" was stripped and rehybridized at high stringency with nick-translated pRP54 cDNA ("b"). All blots underwent autoradiography for four days at optimal conditions. The migration of 18S and 28S ribosomal RNA is indicated by the arrows.

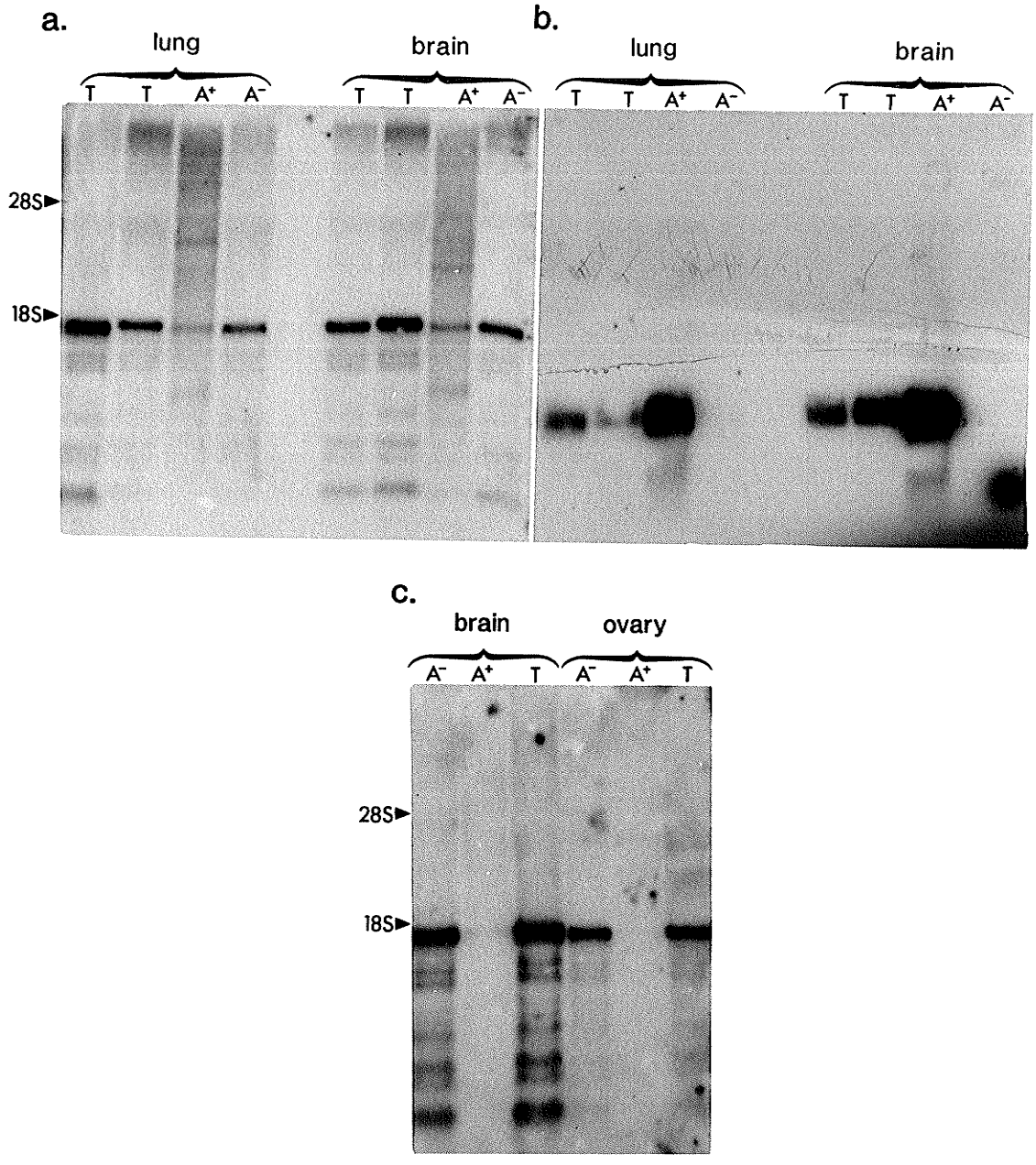
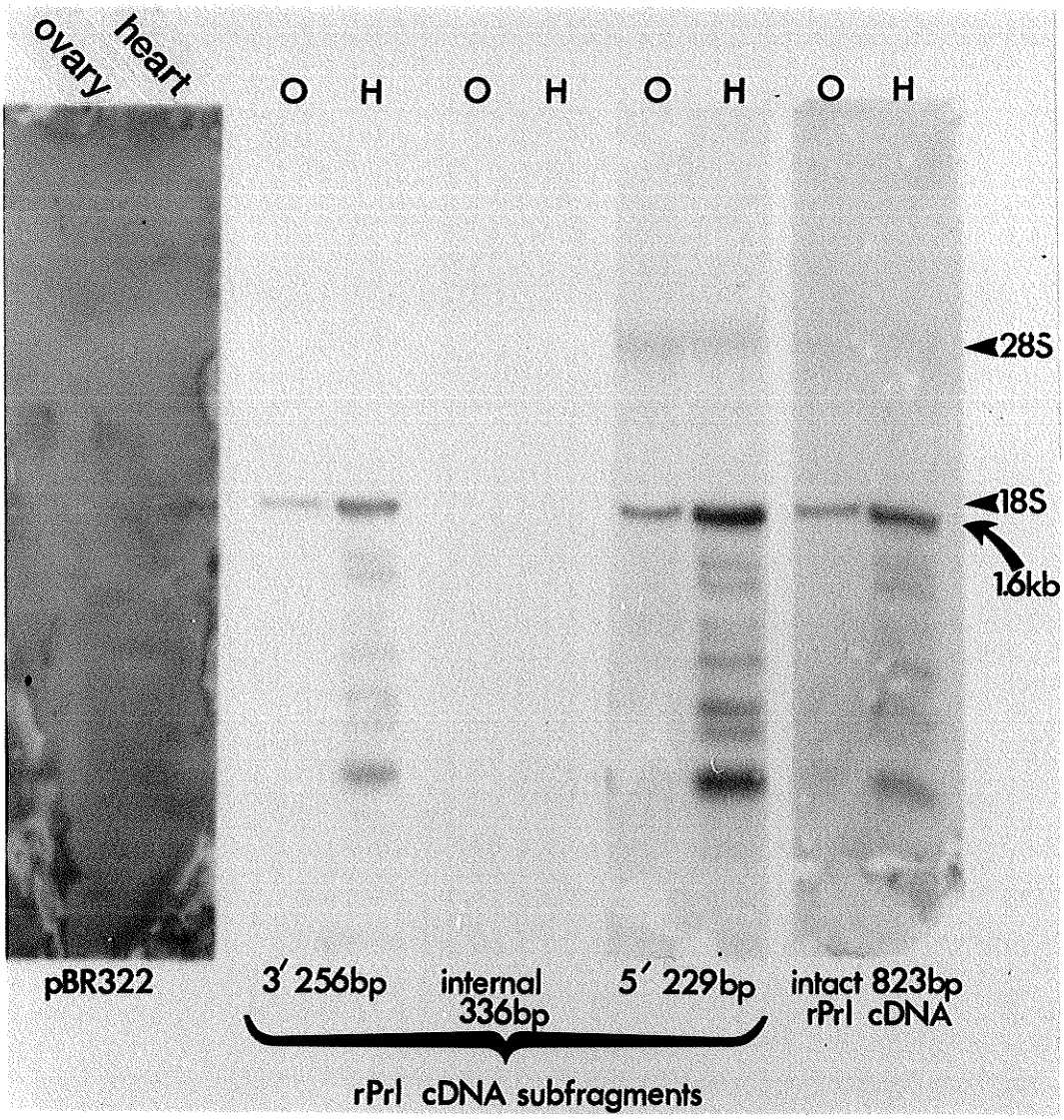

a.
lung
brain
T T A+ A-
T T A+ A-
28S
18S
b.
lung
brain
T T A+ A-
T T A+ A-
c.
brain
ovary
A- A+ T
A- A+ T
28S
18S

Fig. 4. Localization of the rPRL cDNA sequence responsible for hybridization to the 1.6kb family of non-polyadenylated RNAs.

A Northern blot containing multiple 20μg lanes of rat ovary (O) and heart (H) total RNA was cut into five strips containing a lane of each source of RNA. These strips were individually hybridized under high stringency conditions with gel-purified, nick-translated pBR322, the entire 823bp rPRL cDNA, and subfragments of rPRL cDNA: a 3' 256bp BglII/PstI fragment, an internal 336bp AccI/BglII product, and a 229bp AccI/PstI fragment. A partial restriction map of rPRL cDNA is shown schematically below indicating the restriction sites used to generate the above probes. The 5' and 3' untranslated regions are represented by the striped bar and the solid bar denotes the coding region. The GC-tails which facilitated ligation to plasmid DNA are shown at the 5' and 3' ends of the cDNA diagram. The probe used in each hybridization is given at the base of each strip. Each hybridization contained 4-9 x 10^6cpm/ml of probe. The final post-hybridization washes were at 55C in 0.1 x SSC, 0.1% SDS. The blots underwent autoradiography for three days at optimal conditions. The location of the 1.6kb RNA signal is given relative to 18S and 28S ribosomal RNA.


ovary
heart
O H O H O H O H
28S
18S
1.6kb
pBR322
3′ 256bp
internal 336bp
5′ 229bp
intact 823bp rPrl cDNA
rPrl cDNA subfragments

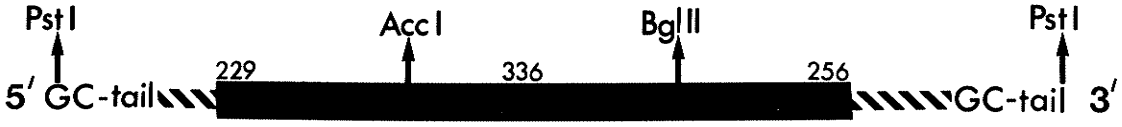

Pst I
Acc I
Bgl II
Pst I
5′ GC-tail
229
336
256
GC-tail 3′

Fig. 5. Identification of putative rPRL-like non-polyadenylated transcripts as artifactual hybridization.

Identical Northern blots containing 20μg each of rat heart (H) and kidney (K) or brain (B) total RNA were individually hybridized at high stringency with nick-translated plasmids of probasin (M-40), rPRL, and rPLP-A (pRP6-1 or pRP6-5). The amount of radiolabelled probe in each hybridization ranged from 5-9 x 10^6 cpm/ml. All blots were washed at 50-55C in 0.1 x SSC, 0.1% SDS for 1 hr. Autoradiography under optimal conditions was for 4 days. The position of the 1.6kb RNA signal is indicated relative to the location of 18S and 28S ribosomal RNA. The cDNAs of probasin, rPRL and pRP6-1 contained GC-tails.

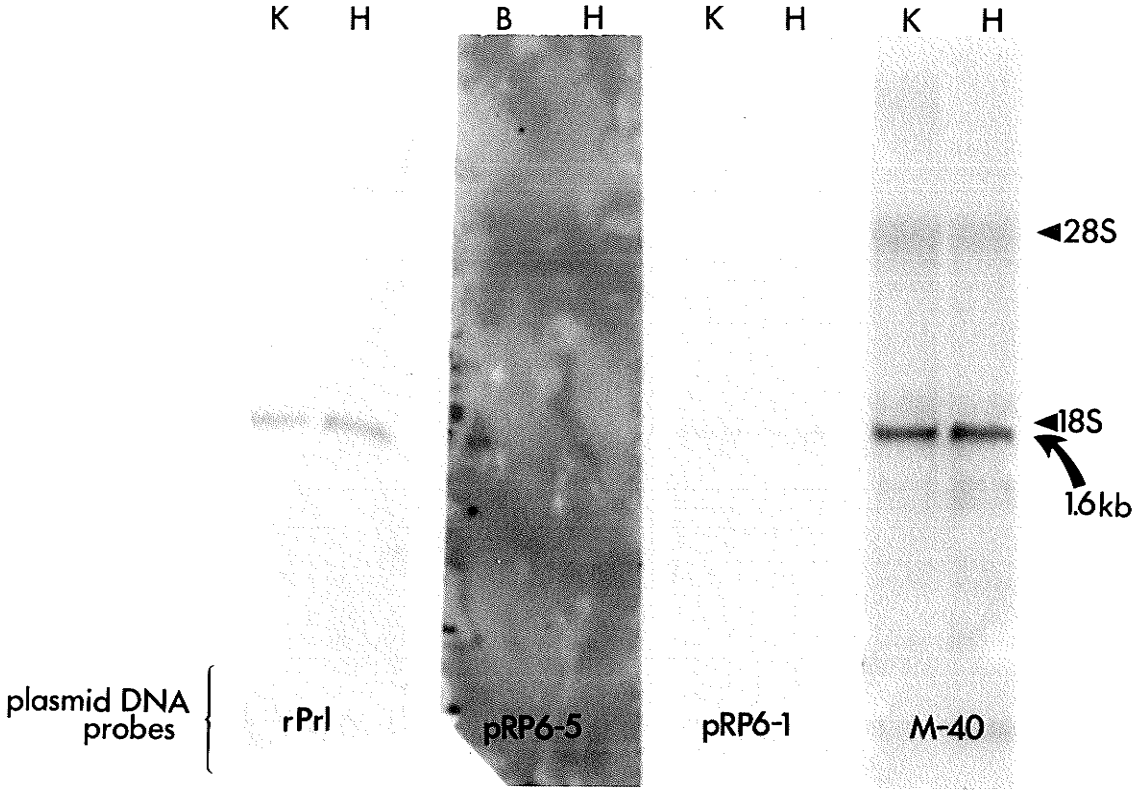

K
H
B
H
K
H
K
H
28S
18S
1.6kb
plasmid DNA probes
rPrl
pRP6-5
pRP6-1
M-40

Fig. 6. Detection of PRL mRNA in a human B-lymphoblastoid cell line, IM-9-P, by Northern blot hybridization.

A Northern blot was constructed containing 10μg of total RNA from rat pituitaries (lane rat pit.); and 50μg of total RNA from IM-9 cells obtained from each of the American Type Culture Collection (Rockville, MA) (lane ATCC), M. Lesniak (NIH) (lane LES.), and Dr. R. Rosenfeld (Stanford University) (lane R.R.); 50μg total RNA from IM-9 cells from our laboratory (lane IM-9-P); 3.0μg of human pituitary total RNA (lane hum.pit.) and 15μg of poly(A)$^+$ enriched mRNA obtained by single passage over an oligo(dT) cellulose column (lane IM-9-P*). Simultaneous hybridization with nick-translated hPRL cDNA and the human c-myc gene was done at high stringency. The blot was then exposed to X-ray film at optimal conditions for one day. The hPRL and c-myc mRNA bands are identified relative to the migration of 18S ribosomal RNA.

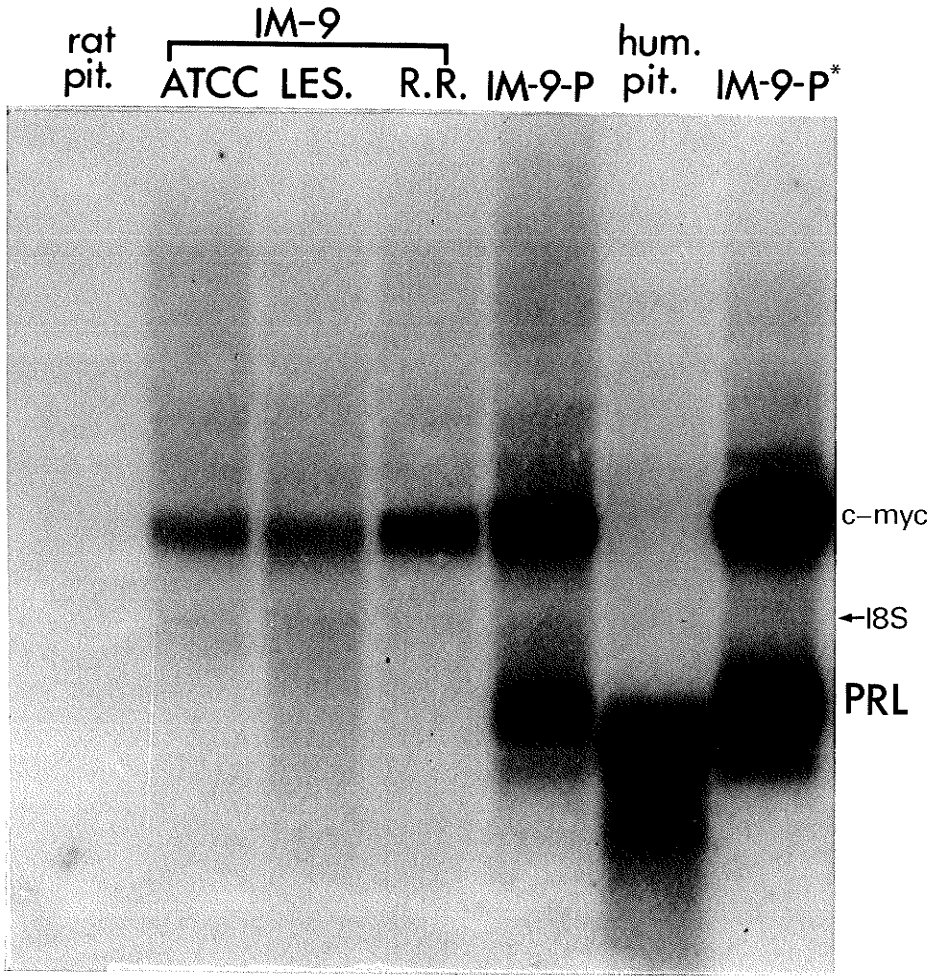

rat pit.
IM-9
ATCC
LES.
R.R.
IM-9-P
hum. pit.
IM-9-P*
c-myc
←18S
PRL

Fig. 7. Human female origin of IM-9-P cells determined by genomic Southern blot hybridization.

a. Five micrograms of rat testes DNA (rat), normal human peripheral blood lymphocyte DNA (HUM.PBL), ATCC IM-9 DNA (IM-9), and IM-9-P DNA (IM-9-P) were digested with AluI, blotted and probed under high stringency conditions as described in the "Materials and Methods" with a human AluI repetitive element DNA, pBLUR-8. Lambda phage DNA cleaved with HindIII and ØX174 digested with HaeIII served as DNA markers, the sizes of which in kilobases (kb) is indicated on the autoradiogram. Only the human DNA hybridized to this probe. The photograph of the EtBr-stained gel demonstrates the presence of rat testes DNA in lane (rat).

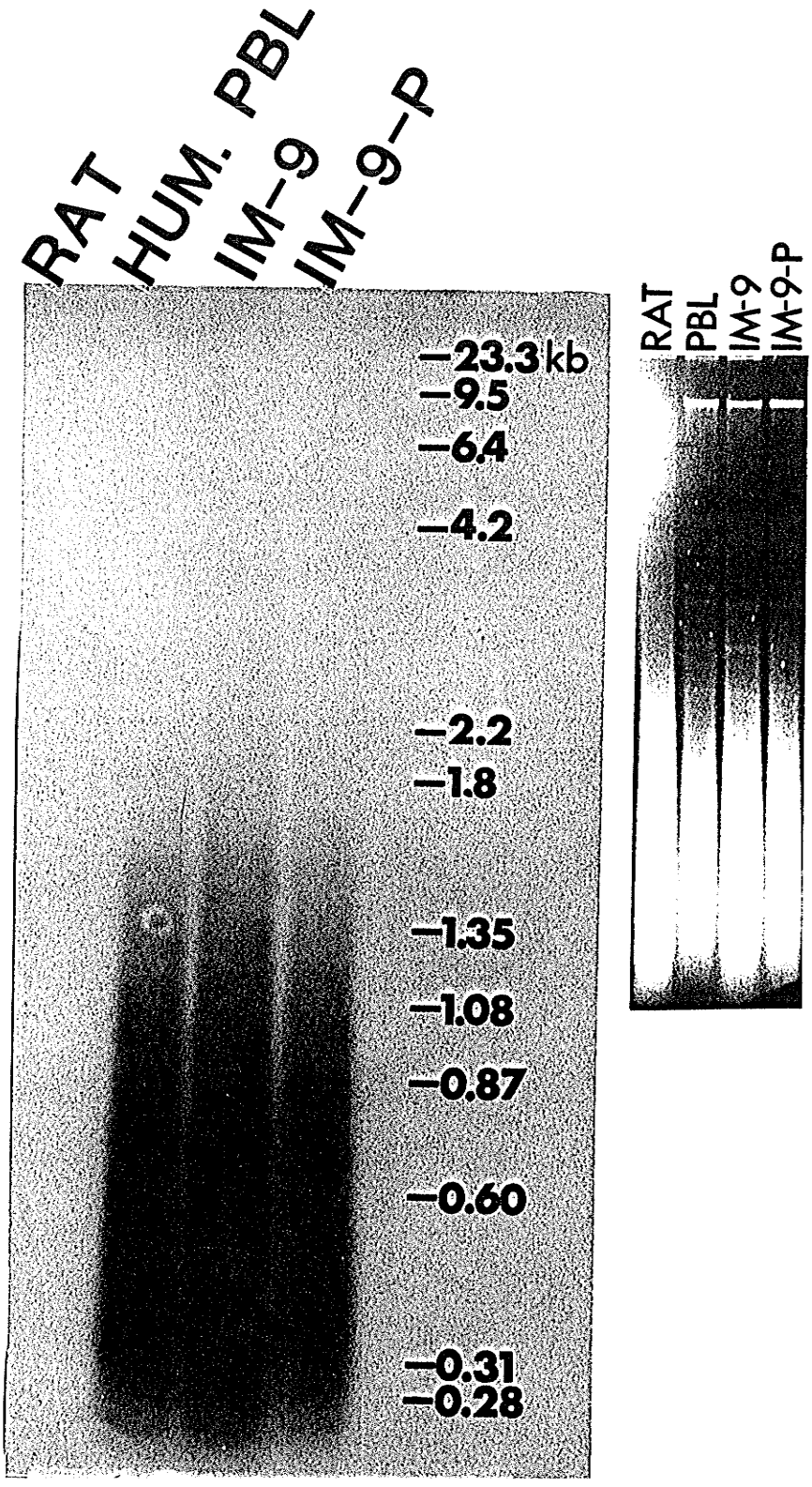

RAT
HUM. PBL
IM-9
IM-9-P
23.3 kb
9.5
6.4
4.2
2.2
1.8
1.35
1.08
0.87
0.60
0.31
0.28
RAT
PBL
IM-9
IM-9-P

Fig.7. Human female origin of IM-9-P cells determined by genomic Southern blot hybridization.

b. Ten microgram aliquots of IM-9-P DNA, ATCC IM-9 DNA, and human male peripheral blood lymphocyte (PBL) DNA were digested with EcoRI (E), HindIII (H) and BglII (B). The resultant Southern blot was hybridized under high stringency conditions with a human Y-chromosome-specific repeat element DNA, pY3.4. The sizes of HindIII digested lambda phage DNA in kilobases is provided as is the estimated size of the major hybridizing band in human male PBL DNA digested with EcoRI.

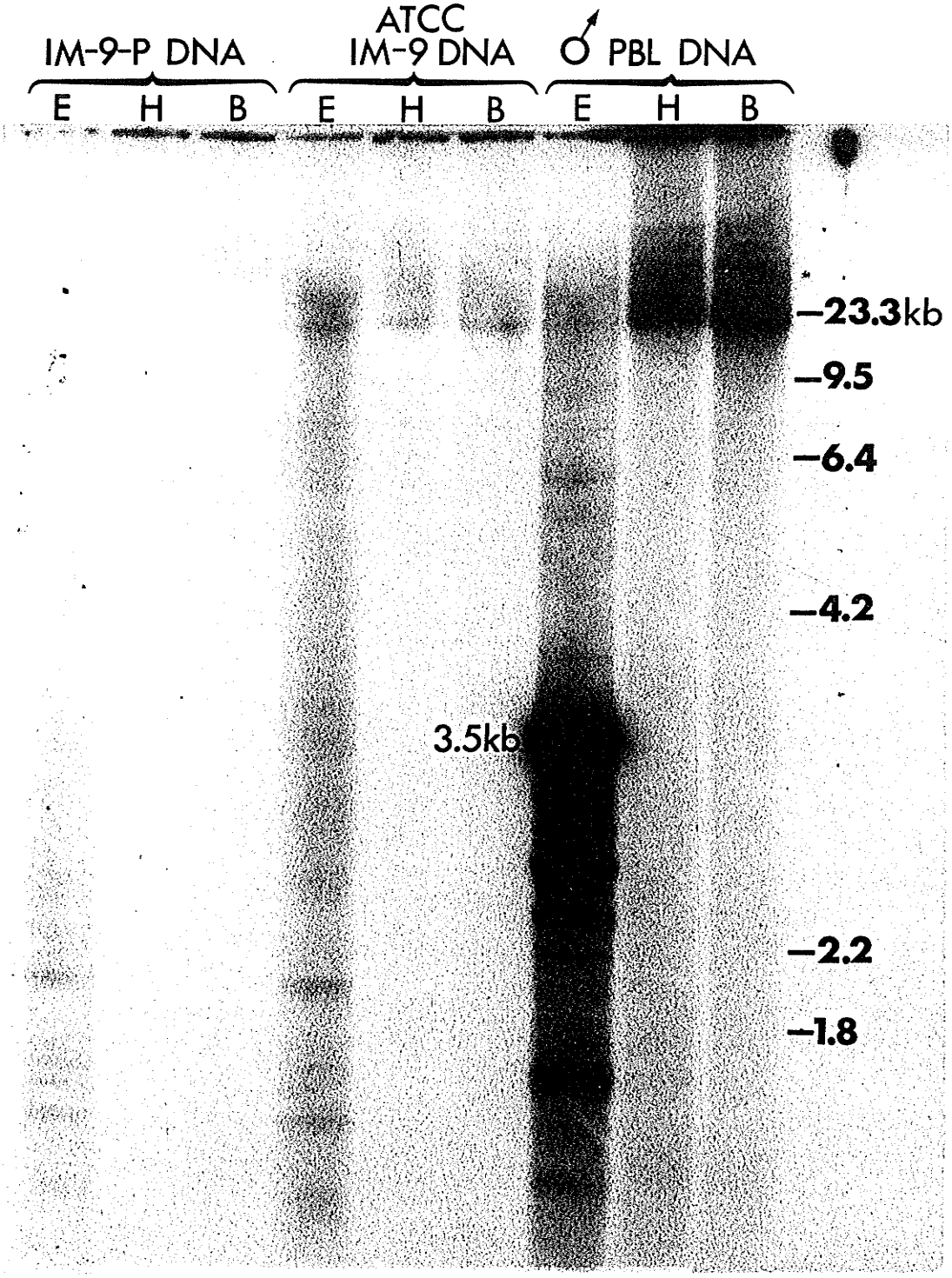
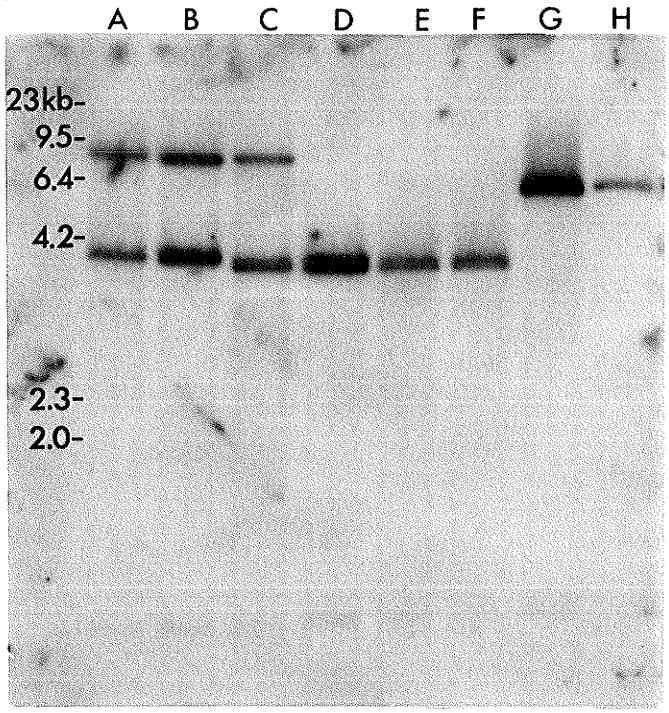

IM-9-P DNA
ATCC IM-9 DNA
♂ PBL DNA
E H B E H B E H B
23.3kb
9.5
6.4
4.2
3.5kb
2.2
1.8

Fig. 8. Confirmation of the B-cell lineage of IM-9-P cell lines.

a. Approximately 20μg samples of BamHI/HindIII-digested genomic DNA were transferred to nitrocellulose and subsequently probed with a 5.6kb DNA fragment encompassing the joining region of the human heavy chain immunoglobulin genes. Lanes A, B and C contained DNA isolated from the IM-9 line obtained from M. Lesniak (NIH), the ATCC (Rockville, MA) and Dr. R. Rosenfeld (Stanford University), respectively. Lane D contained DNA from the PRL-producing IM-9-P line, lane E from the PRL-producing IM-9-P3 clone and lane F from the non-PRL-producing IM-9-P6 clone. Lanes G and H show the 5.6kb germline band in human placenta and human peripheral blood lymphocyte DNA, respectively. The sizes of HindIII digested lambda DNA in kilobases (kb) is indicated.

b. Approximately 20μg samples of EcoRI-digested genomic DNA from sources described above were used to make a Southern blot which was hybridized under high stringency conditions with the human T-cell receptor beta-chain constant region genes. Lanes 1, 2 and 3 contained IM-9 cell DNA from the sources described above in lanes A, B and C. Lanes 4, 5 and 6 contained IM-9-P3, IM-9-P and IM-9-P6 DNA respectively and lanes 7 and 8 contained the control DNAs described above in lanes G and H. According to the restriction map of the human T-cell receptor ß-chain locus two non-rearranged EcoRI fragments, 12kb and 4.2kb should be detected using the constant region probes. DNA markers were those described above.

a.



b.

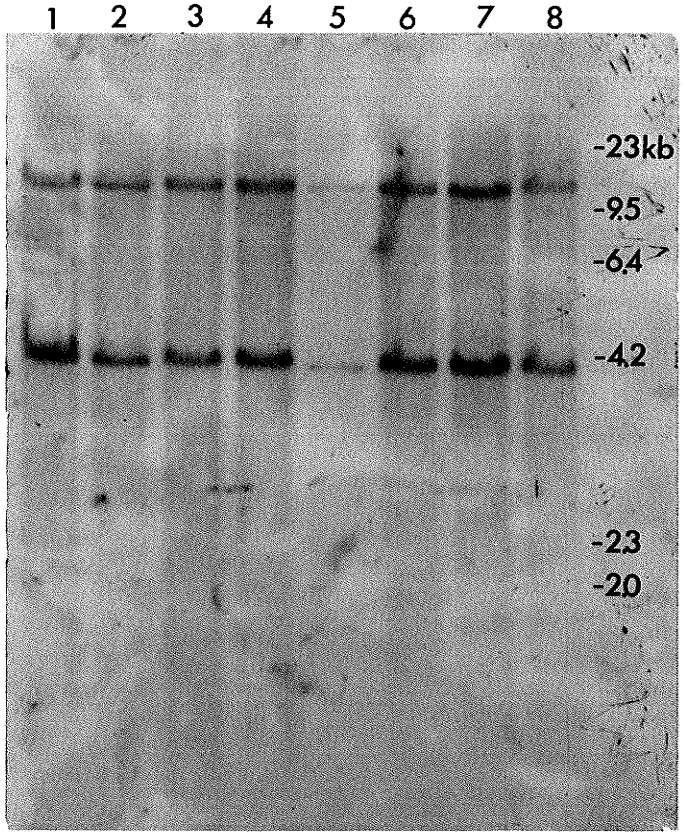


Fig.9. Detection of PRL in the conditioned medium of IM-9-P cells.

IM-9-P cells were cultured in RPMI 1640/0.1% BSA; 4 days later the medium was harvested, concentrated 5-fold, dialyzed, and assayed in a double antibody hPRL RIA. Immunoaffinity-purified pituitary hPRL was the standard. The conditioned media from six other human lymphoid cell lines (RPMI 7666, 6666, 8226, and 1788; CCRF-SB; and HS-Sultan) as well as that from IM-9 cells obtained from three different sources showed no cross-reactivity in the RIA. B/B_0, Bound to free ratio.

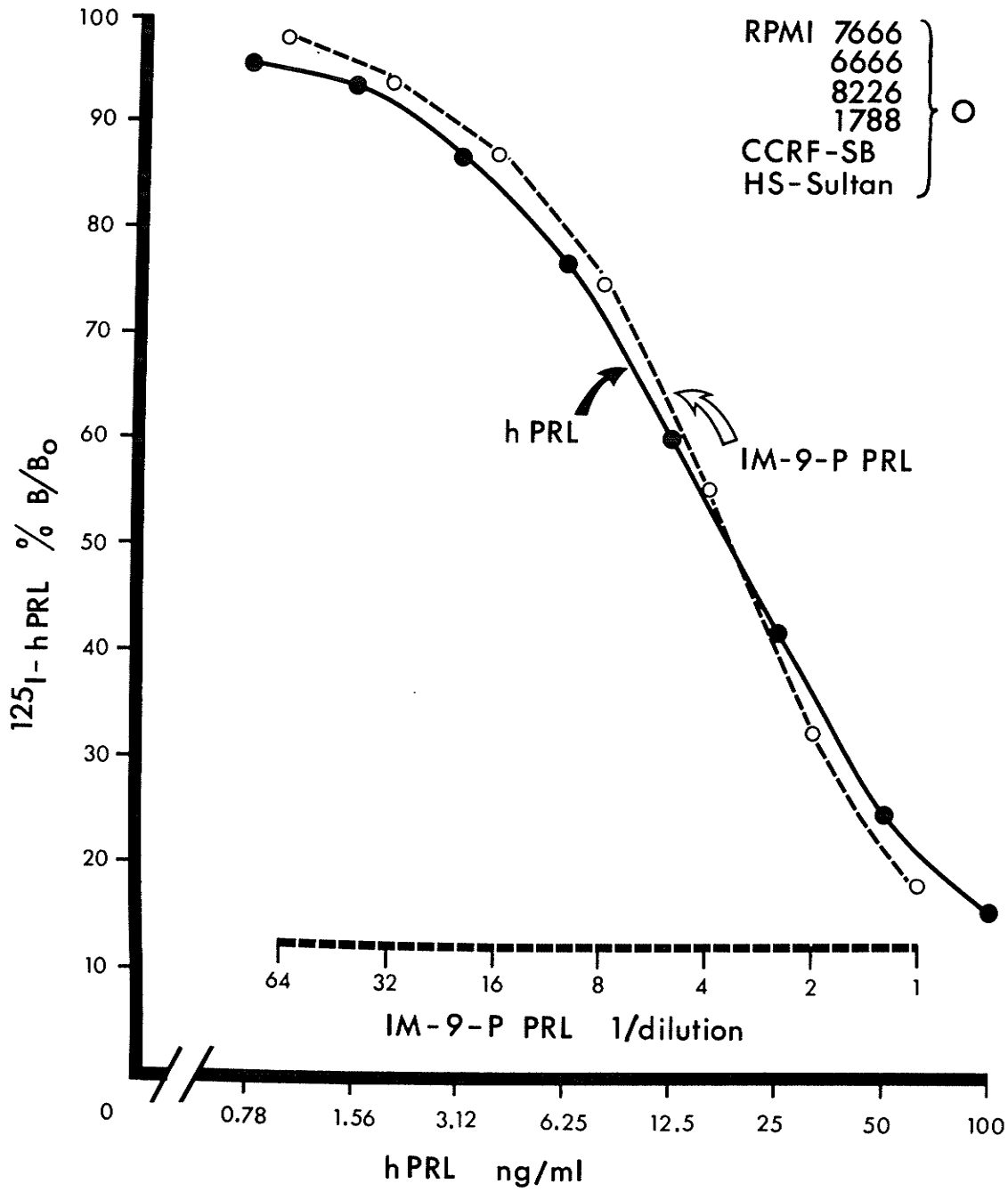
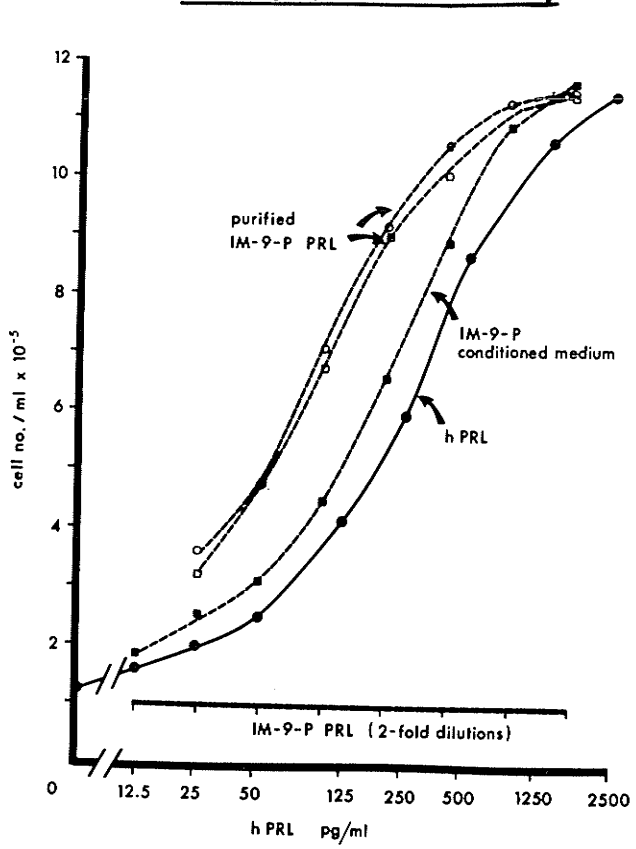

RPMI 7666
6666
8226
1788
CCRF-SB
HS-Sultan
O
h PRL
IM-9-P PRL
^{125}I-h PRL % B/Bo
100
90
80
70
60
50
40
30
20
10
0
64
32
16
8
4
2
1
IM-9-P PRL 1/dilution
0.78
1.56
3.12
6.25
12.5
25
50
100
h PRL ng/ml

Fig.10. Bioactivity of IM-9-P PRL.

a. The biological activity of crude conditioned medium from IM-9-P cells and that of IM-9-P PRL purified from this medium were analyzed and compared to that of pituitary hPRL in the Nb2 lymphoma bioassay. Conditioned media from the IM-9-P line was raised by culturing the cells in RPMI 1640 and 0.1% BSA for 4 days. Immunoaffinity purification of IM-9-P PRL was done as described in the "Materials and Methods". The two peak purified fractions, as well as crude serum-free IM-9-P conditioned medium and pituitary hPRL standard were serially diluted and added to growth-arrested Nb2 cells. After three days cell numbers were determined. The top point of the purified IM-9-P PRL curves and that of the IM-9-P conditioned medium curve represented the undiluted samples.

b. Serum-free conditioned medium (CM) from IM-9-P cells was assayed in the Nb2 lymphoma bioassay in the absence and presence of monoclonal hPRL antibody 9C3. The stippled bar represents the level of Nb2 cell growth in the absence of mitogens. The open bars denote the Nb2 cell response in the presence of added mitogen and the striped bars, the response upon addition of lactogen plus monoclonal hPRL antibody. oPRL, Ovine PRL; %, percent of Nb2 culture medium volume.

a.


Nb2 Lymphoma Bioassay
cell no./ml x 10^{-5}
purified IM-9-P PRL
IM-9-P conditioned medium
h PRL
IM-9-P PRL (2-fold dilutions)
h PRL pg/ml
0
12.5
25
50
125
250
500
1250
2500

b.

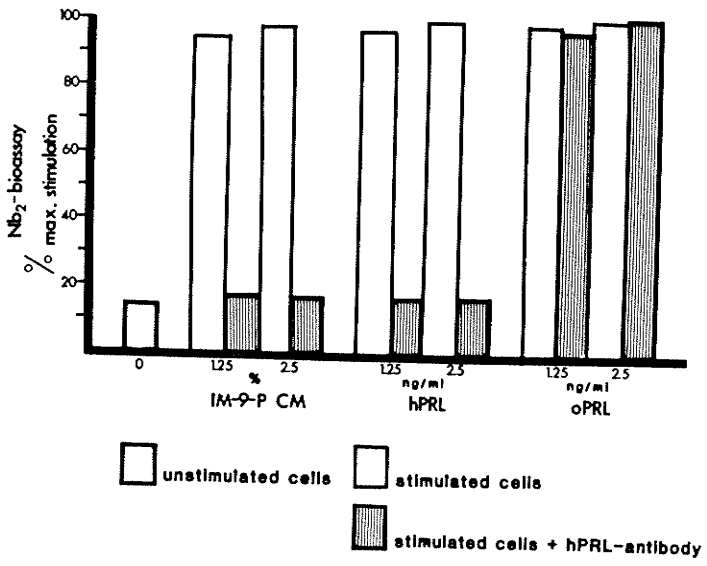

Neutralization of Bioactivity by Anti hPRL Ab
% max. stimulation
Nb_2-bioassay
IM-9-P CM
hPRL
oPRL
unstimulated cells
stimulated cells
stimulated cells + hPRL-antibody

Fig.11. PRL secretion and mRNA accumulation over the IM-9-P3 growth curve.

A. Cells in late log phase of growth were washed and plated at a density of 1 x 10^5 cells/ml. Daily 1ml aliquots (days 0-4), were removed for determination of cell number (■) and 0.5 ml of culture medium was taken for hPRL determination by RIA (●) after cells had been pelleted. The secretion rate of PRL averaged between 40-50ng/10^6cells/24hr. The coefficient of variation was less than 5% for cell counts and less than 2% for the RIA.

B. The relative abundance of PRL mRNA over the growth curve (□) of a separate experiment was analyzed by Northern blot hybridization. Every 24 hours, 1.5 x 10^7 cells were harvested for RNA extraction, of which 40μg were loaded in each lane and subsequently hybridized with hPRL cDNA at high stringency. Equal RNA loading was confirmed by examination of the EtBr-stained gels prior to transfer.

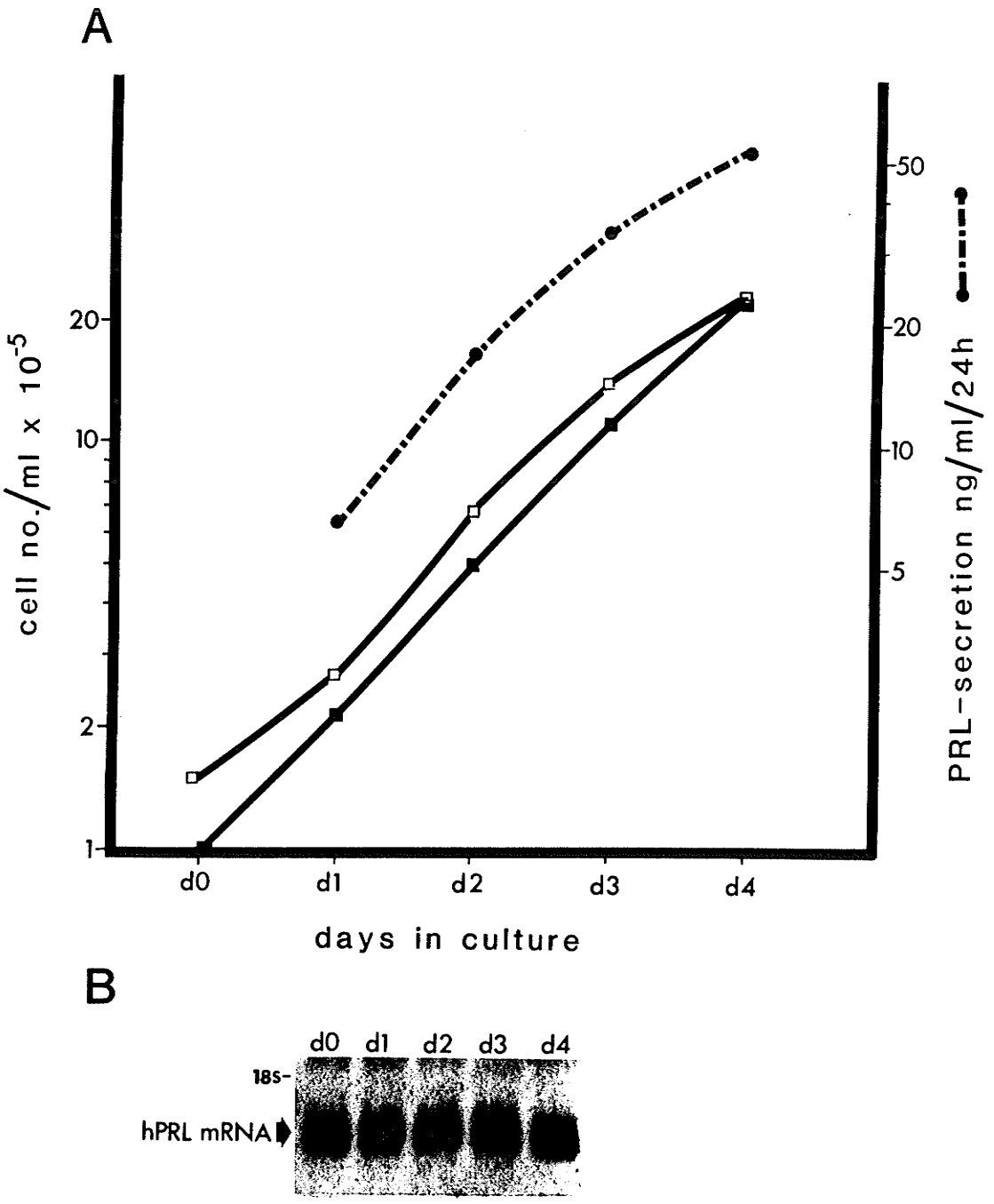

A
cell no./ml x 10^{-5}
20
10
2
1
d0
d1
d2
d3
d4
days in culture
50
20
10
5
PRL-secretion ng/ml/24h
B
d0 d1 d2 d3 d4
18s-
hPRL mRNA

Fig.12. Effect of neutralization of secreted PRL on IM-9-P cell growth.

IM-9-P cells were washed and placed in RPMI 1640/10% horse serum at a density of 1.25 x 10^5 cells/ml. To one set of cell cultures, monoclonal antibody (9C3) to hPRL was added on day 0 and day 2. On day 4, IM-9-P cell numbers were determined (left panel) and conditioned media harvested. In the right panel, the effective neutralization of secreted IM-9-P PRL was demonstrated. Conditioned media from IM-9-P cells cultured in the absence of antibody gave a significant stimulation of Nb2 cell growth (50% of a maximally stimulating does of hPRL), whereas conditioned media from cells grown in the presence of antibody exhibited no stimulatory effect. Data represent the mean ± SEM of triplicate determinations. d, day of experiment; Ab, antibody; O, represented the growth of Nb2 cells in the presence of RPMI 1640/10% horse serum.

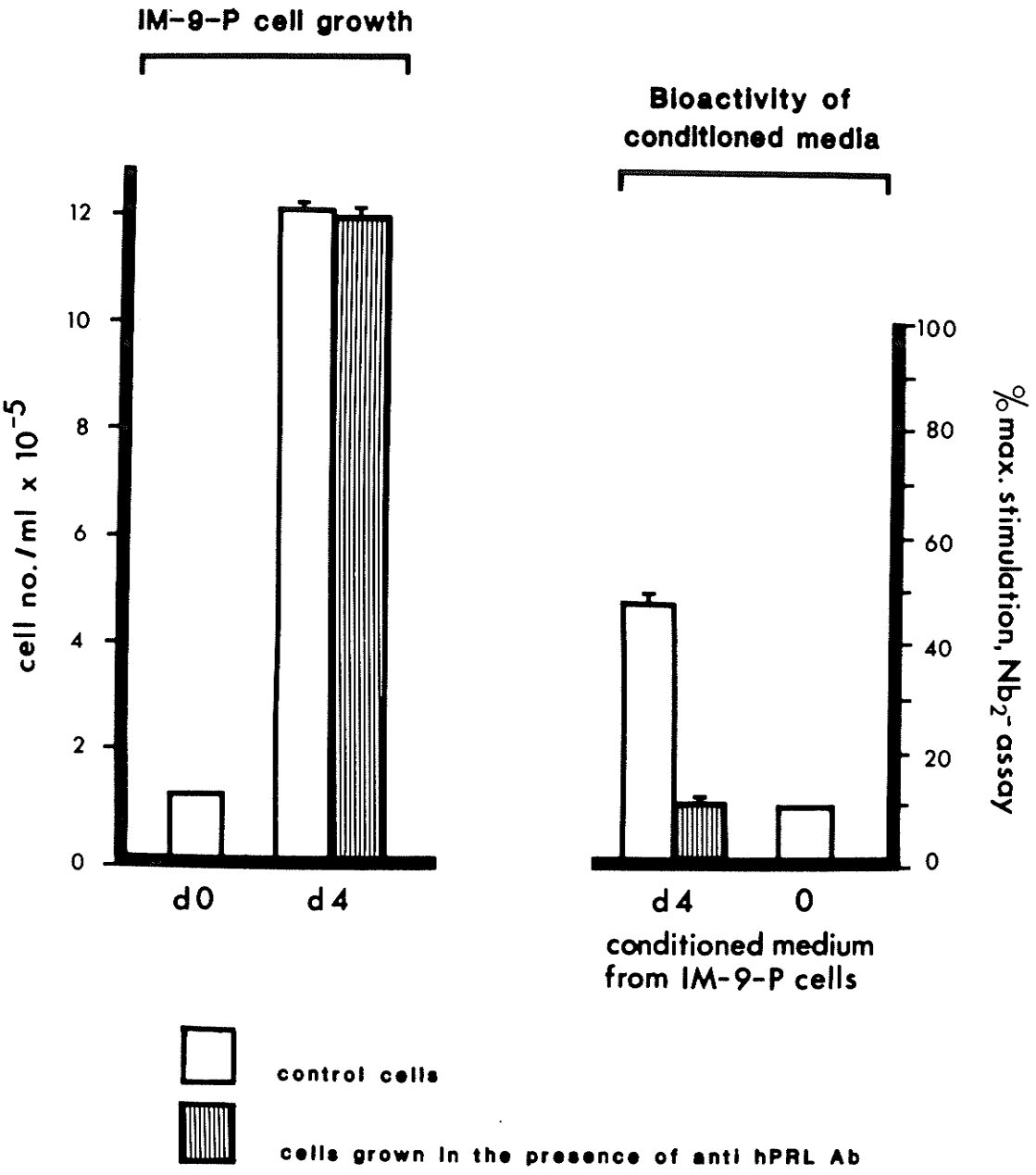

IM-9-P cell growth
Bioactivity of conditioned media
cell no./ml x 10^{-5}
12
10
8
6
4
2
0
d0
d4
100
80
60
40
20
0
% max. stimulation, Nb_2- assay
d4
0
conditioned medium from IM-9-P cells
control cells
cells grown in the presence of anti hPRL Ab

Fig.13. Molecular weight determination of IM-9-P PRL.

IM-9-P PRL was isolated from pooled conditioned medium by immunoaffinity chromatography, as described in "Materials and Methods". The two peak fractions were reconstituted in SDS-sample buffer and electrophoresed on a 15% SDS-polyacrylamide gel, which was subsequently silver stained. Lane A contained 2μg pituitary hPRL. In lanes B and C, 1.0 and 0.9μg were applied, respectively, of the two peak fractions of purified IM-9-P PRL. Kd, kilodaltons.

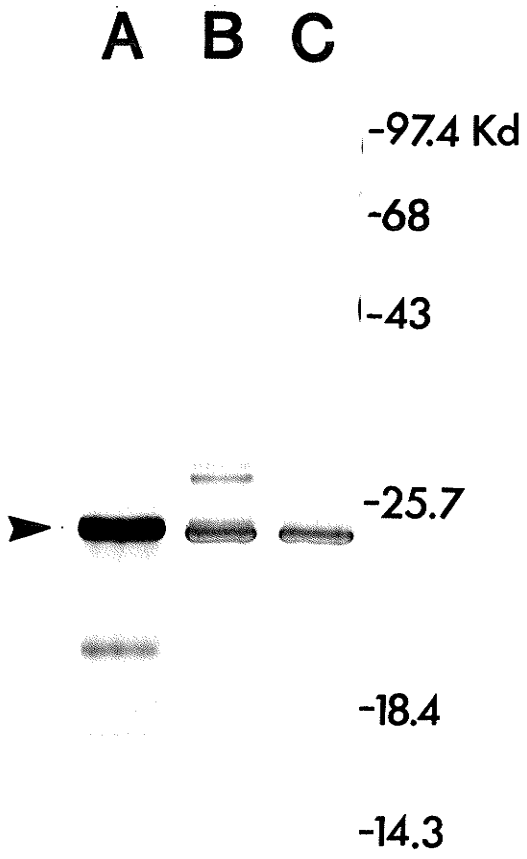

A B C
-97.4 Kd
-68
-43
-25.7
-18.4
-14.3

Fig.14. Northern blot hybridization analysis of IM-9-P and pituitary hPRL transcripts after removal of poly(A) tracts.

Oligo(dT) was hybridized to RNA samples of IM-9-P cells and human pituitary (Hum.Pit.) and the RNA was subsequently treated with RNase H (+) to remove poly(A) tails as described in the "Materials and Methods". Untreated RNA samples (-) showed the native size of the PRL transcript.

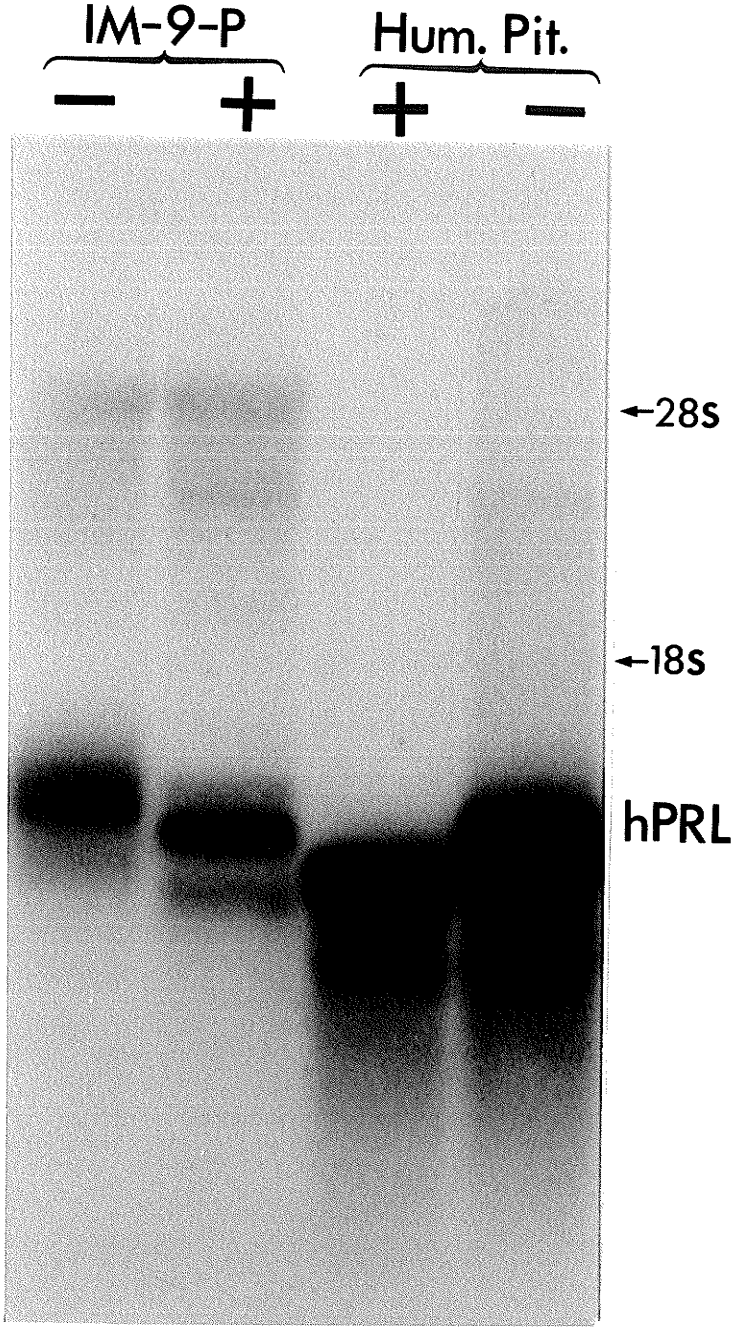

IM-9-P
Hum. Pit.
−
+
+
−
←28s
←18s
hPRL

Fig.15. (a & b) Genomic Southern blot analysis of the IM-9-P3 PRL gene.

DNA samples (20μg) from a human placenta (A), IM-9-P3 cells (B), and classical IM-9 cells (C) were digested by PstI (P), HindIII (H) or EcoRI (E) and transferred to nitrocellulose. The blot was hybridized at high stringency with pituitary hPRL cDNA and a 1132bp hPRL genomic fragment encompassing 978bp of 5' flanking DNA, exon 1, and 126bp of intron A. In "a" the blot was hybridized with the above probes simultaneously; after autoradiography, the blot was stripped and rehybridized with the 5' end-specific genomic probe only, "b". The low molecular weight band seen in the human placenta DNA lanes was unique to that sample of DNA and not reproducible.

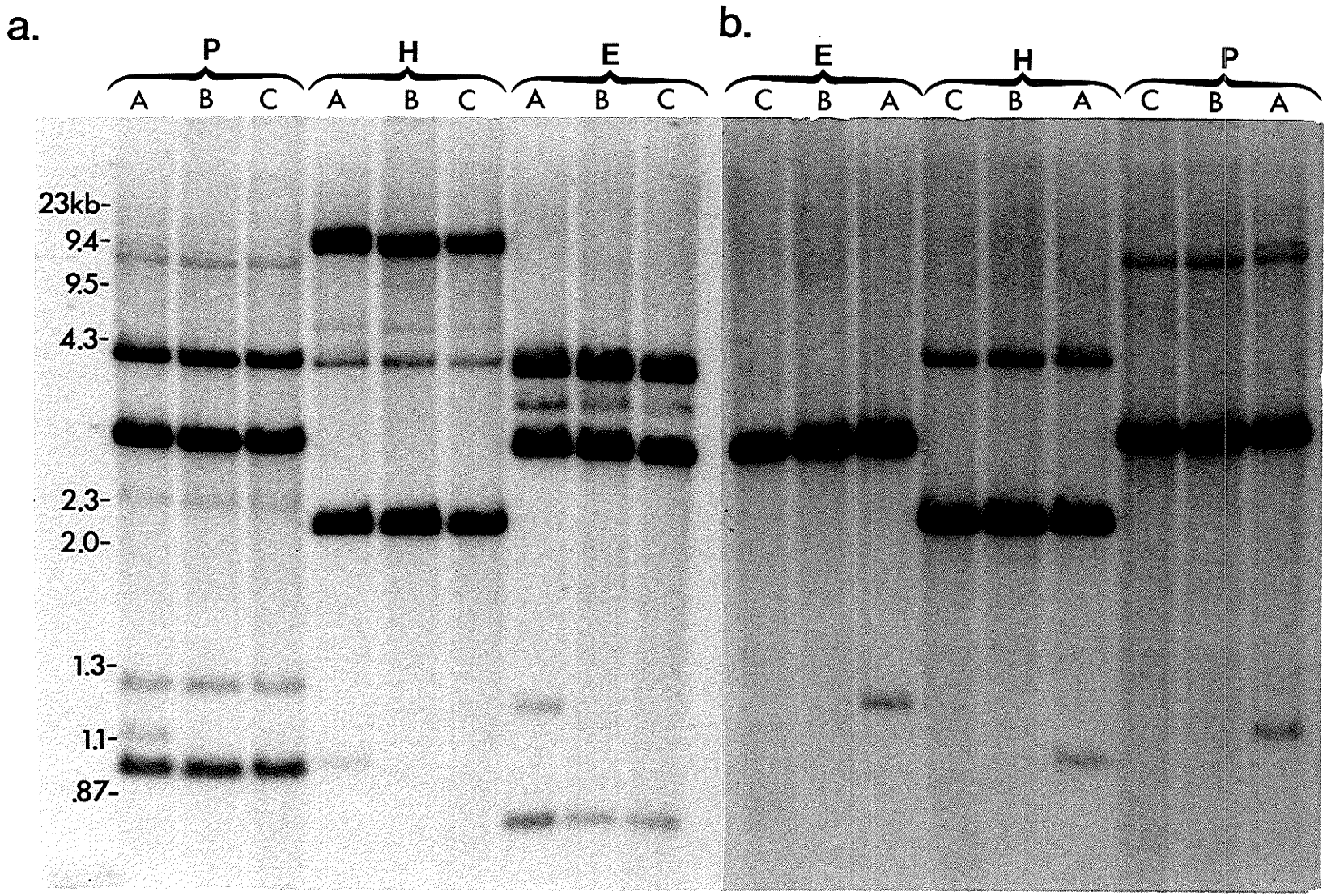

a.
P
H
E
A B C A B C A B C
23kb-
9.4-
9.5-
4.3-
2.3-
2.0-
1.3-
1.1-
.87-
b.
E
H
P
C B A C B A C B A

Fig.15. (c & d) Genomic Southern blot analysis of the IM-9-P3 PRL gene.

The genomic DNA blots in "c" and "d" were probed individually with three different DNAs. After each hybridization the radioactive signals were stripped from the nitrocellulose. The resultant autoradiograms have been dissected, aligned at the wells and rearranged for easier comparison of the fragments detected with each probe. In this way a direct comparison of the same three lanes of digested DNA with three different hPRL DNA probes was achieved. The identity of the DNAs in the lanes A, B and C are described above. The NcoI "c" and XbaI "d" digested DNAs were present on the same nitrocellulose filter and hybridized first with pituitary hPRL cDNA (hPRL cDNA), then with the 5' end-specific hPRL genomic probe (5' specific), and lastly with a 3' end-specific hPRL genomic probe covering a portion of exon 5 and 392bp of 3' flanking DNA. The 5' and 3' end-specific hPRL genomic fragments hybridized to comigrating DNA fragments in XbaI digests. In other experiments utilizing longer gels these bands could be differentiated with the 3' end genomic fragment migrating slightly faster than the 5' end genomic fragment.

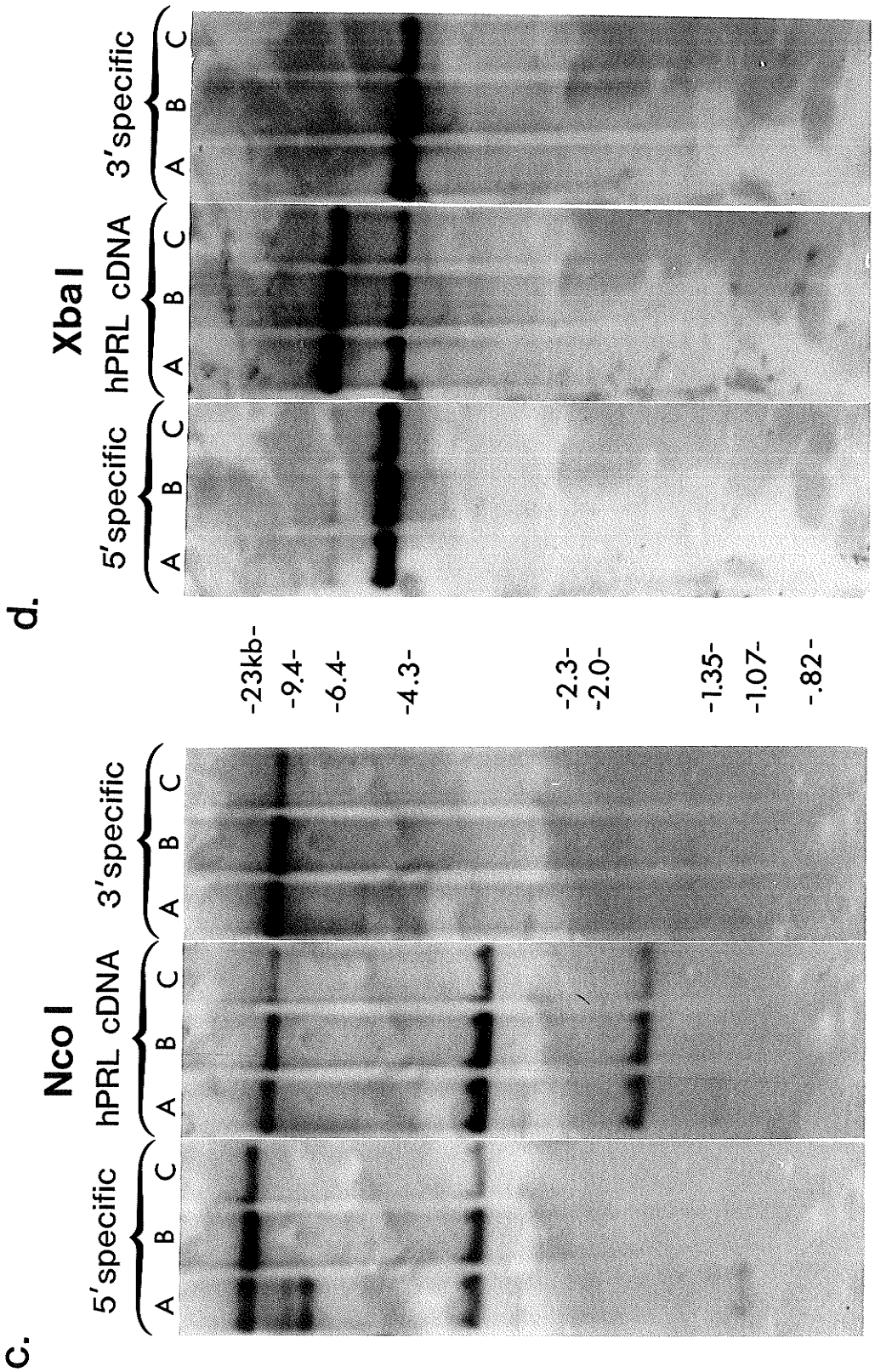

c.
Nco I
5'specific
A
B
C
hPRL cDNA
A
B
C
3'specific
A
B
C
d.
Xba I
5'specific
A
B
C
hPRL cDNA
A
B
C
3'specific
A
B
C
-23kb-
-9.4-
-6.4-
-4.3-
-2.3-
-2.0-
-1.35-
-1.07-
-.82-

Fig.16. Genomic Southern hybridization analysis of the IM-9-P3 PRL gene.

a. DNA samples (20μg) from a human placenta (A), IM-9-P3 cells (B), and classical IM-9 cells (C) obtained from M. Lesniak (NIH) were digested with BglII, fractionated and transferred to nitrocellulose. As in the previous figure, this blot was sequentially hybridized with pituitary hPRL cDNA, the 5' end-specific hPRL genomic fragment (5' specific) and lastly with the 3' end-specific hPRL genomic probe (3' specific). The blot was stripped between each hybridization. The autoradiograms were dissected and arranged to provide easy comparison of the restriction fragmentation pattern obtained with each probe.

b. Approximately 20μg samples of various human chromosomal DNAs were digested with BglII and used to construct a Southern blot that was hybridized with pituitary hPRL cDNA. DNA was isolated from various sources: P6, non-PRL-producing IM-9-P6 cell line; P3, PRL-producing IM-9-P3 cells; PL, human placenta; R.R., IM-9 cells from Dr. R. Rosenfeld (Stanford University); AT, IM-9 cells from the American Type Culture Collection (Rockville, MA); LES, IM-9 cells from M. Lesniak (NIH); PBL, peripheral blood lymphocytes of an individual; T47D, T47D breast cancer cell line; BR, a human brain. The restriction fragmentation pattern seen in the various lanes may be indicative of a BglII restriction fragment length polymorphism. No rearrangement of the hPRL gene was observed using the five different restriction enzymes described in Figure 15.

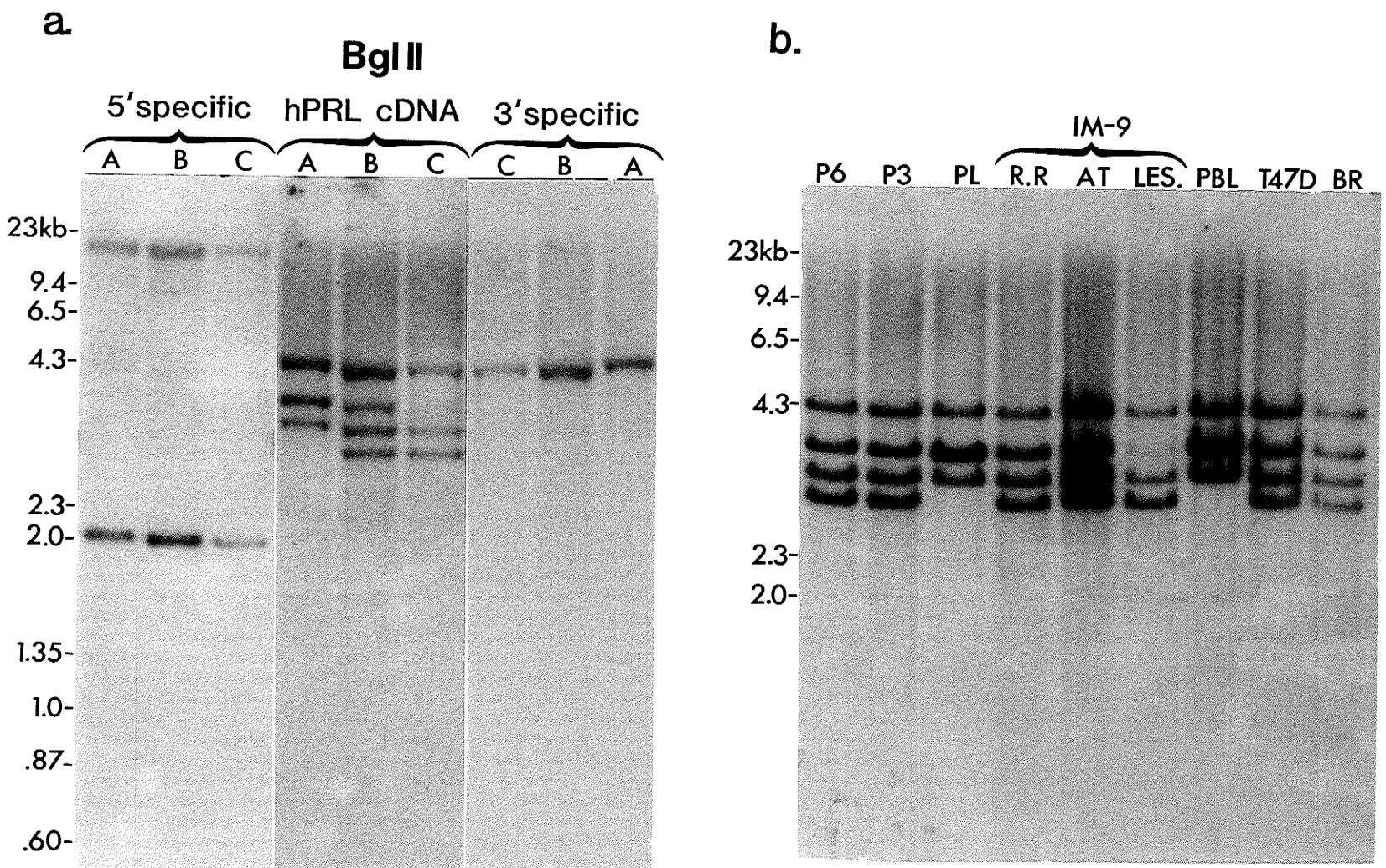
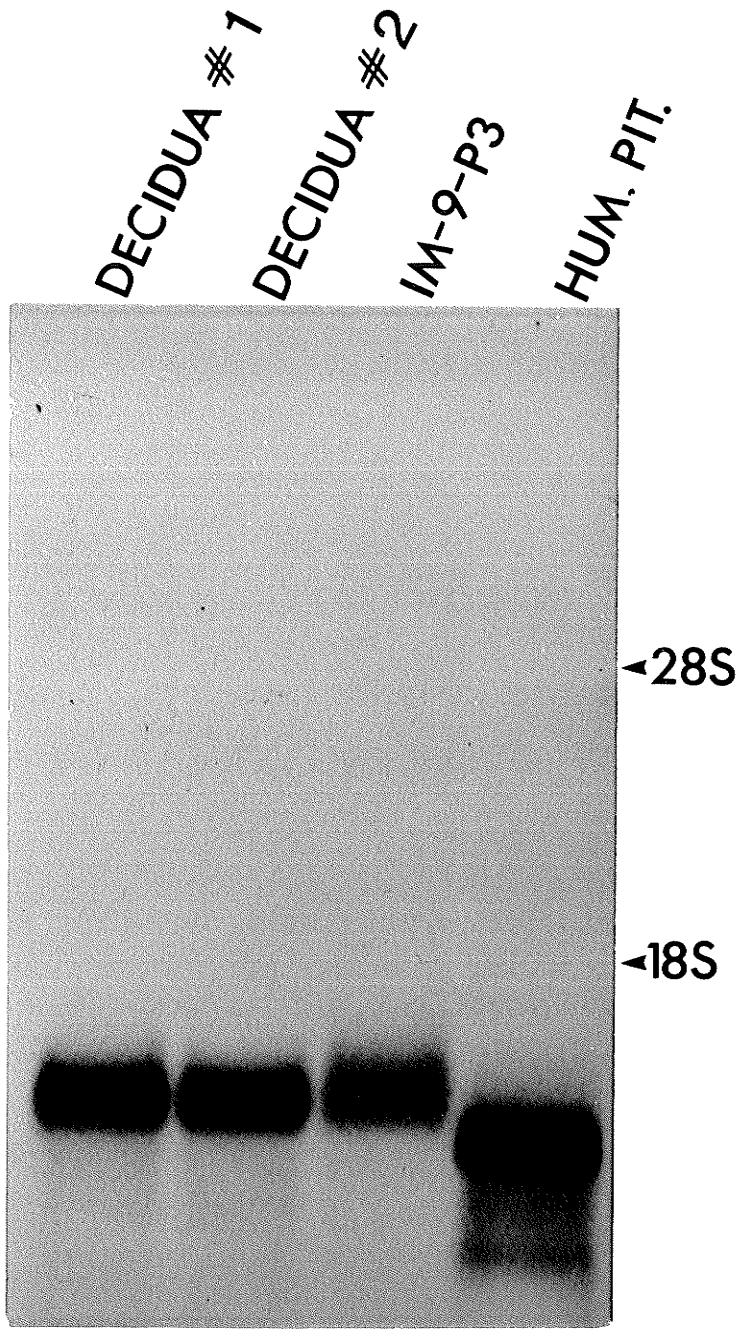

a.
BglII
5'specific
hPRL cDNA
3'specific
A B C A B C C B A
23kb-
9.4-
6.5-
4.3-
2.3-
2.0-
1.35-
1.0-
.87-
.60-
b.
IM-9
P6 P3 PL R.R AT LES. PBL T47D BR
23kb-
9.4-
6.5-
4.3-
2.3-
2.0-

Fig.17. Size comparison of IM-9-P3 PRL mRNA to the human decidual PRL transcript.

Total RNA (30μg) from two different human gestational decidua samples, human pituitaries (Hum.Pit.) and IM-9-P3 cells was fractionated and blotted to Hybond-N matrix as described in the "Materials and Methods". The blot was hybridized at high stringency with random-primer labelled pituitary hPRL cDNA. IM-9-P3 and decidual PRL transcripts comigrated in the denaturing formaldehyde-agarose gel at a larger size than pituitary hPRL mRNA. The location of 28S and 18S ribosomal RNAs is indicated by the arrows.



PROBE: hPRL cDNA

Fig.18. Comparison of the IM-9-P and decidual PRL cDNA structure to that of the human pituitary and sequencing strategy of the non-pituitary PRL cDNA clones.

A diagrammatic representation of a full-length pituitary hPRL cDNA excluding the poly(A) tail is shown at the top. Aligned below are the structures of IM-9-P and decidual PRL cDNAs isolated from four different libraries. Nineteen hPRL cDNAs were purified and eighteen are presented here; IM-9-P PRL cDNA #7 was found to be identical to IM-9-P PRL cDNA #6 by restriction enzyme analysis and not pursued further. The striped area on each schematic represents the 5' untranslated sequence (5'UT), the length of which in nucleotides is given as a negative number on the left end of each cDNA. The contiguous thick black area represents the protein coding region, 3' untranslated sequence and poly(A) tail. The designation of each cloned cDNA is given at the 3' end. Only IM-9-P PRL cDNA #9A was intact, almost all other IM-9-P PRL cDNAs were truncated at the unique internal EcoRI site because of inefficient methylation-protection of EcoRI recognition sequences during library construction. IM-9-P PRL cDNA 3, C and A were chimeric having a portion of PRL 3' UTR cDNA ligated to the 5' end as described in the "Results". The 3' UTR portion of chimeric clones C and 1A is depicted as separate cDNA aligned with the 3' end of pituitary hPRL cDNA and separated from the 5' region of the clone by a gap. IM-9-P PRL cDNA I-1 was also chimeric and contained 45bp of foreign cDNA ligated to the 5' end which is represented by the thin black line. Decidual PRL cDNAs were intact and not chimeric. The 3' EcoRI subfragment of dPRL cDNA 2A and 15A were present in the original lambda phage clone but not subcloned and analyzed. Decidual PRL cDNA F1-a was prematurely truncated during cDNA synthesis at cysteine $^{-6}$ of the signal

Fig.18 (cont'd)

signal sequence. The arrows beneath the schematic indicate the direction and distance of sequencing reactions performed such that the entire IM-9-P PRL cDNA sequence was obtained of which 88% of the protein coding region was determined from both strands. The 5' and 3' untranslated regions of individual cDNAs were totally sequenced. Whenever possible, sequences were obtained using the indicated mapped restriction sites. Sequences whose origin do not coincide with these sites were obtained from the 5' end of different cDNAs or the EcoRV site at -96 and the BglII site at -42 of the 5' non-coding region. The bar at the bottom represents the length of 100 basepairs (bp).

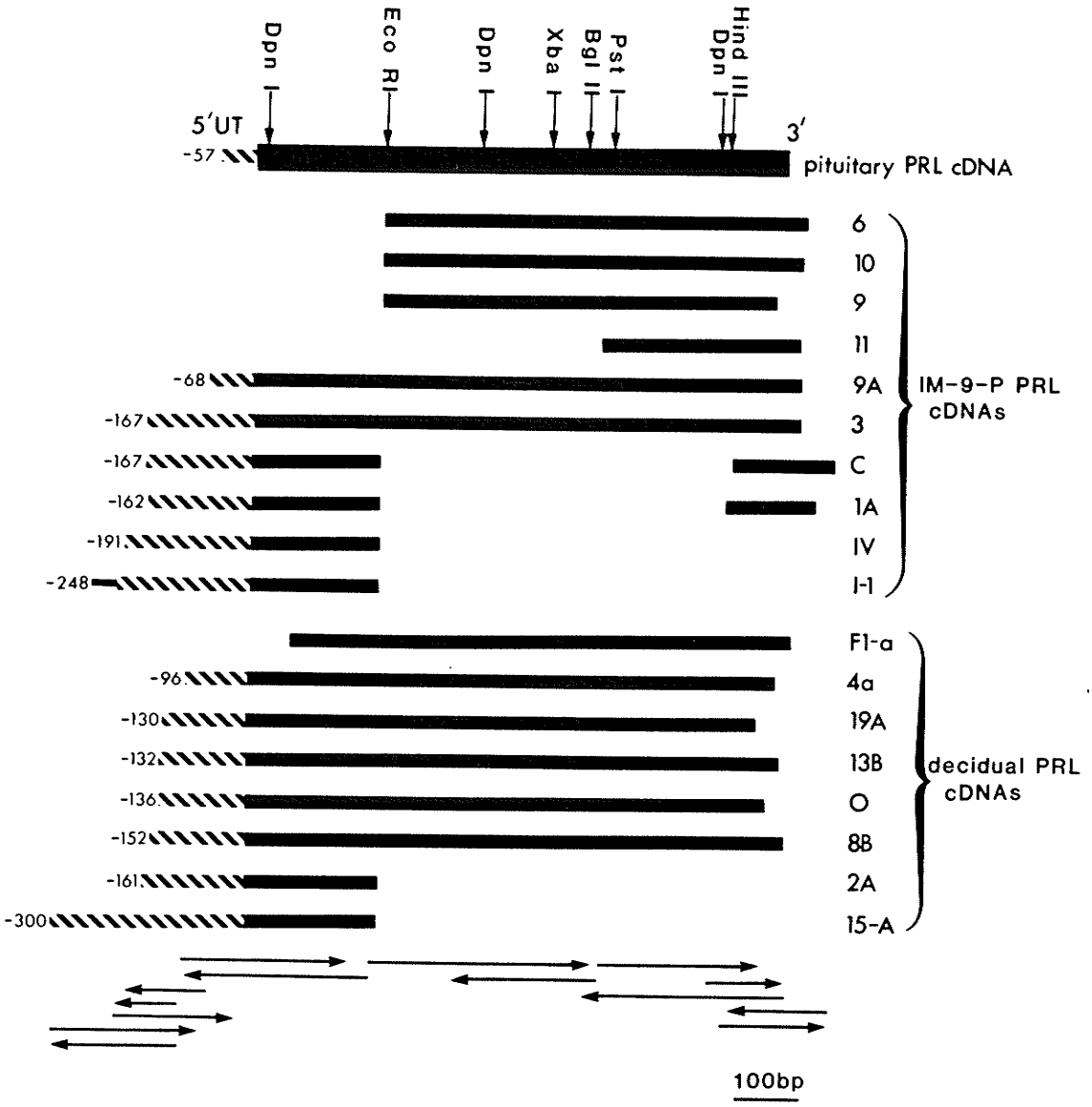

Dpn I
Eco RI
Dpn I
Xba I
Bgl II
Pst I
Hind III
Dpn I
5'UT
-57
3'
pituitary PRL cDNA
6
10
9
11
-68
9A
-167
3
-167
C
-162
1A
-191
IV
-248
I-1
IM-9-P PRL cDNAs
F1-a
-96
4a
-130
19A
-132
13B
-136
O
-152
8B
-161
2A
-300
15-A
decidual PRL cDNAs
100bp

Fig.19. Nucleotide and translated amino acid sequences of IM-9-P PRL cDNA #3.

IM-9-P PRL cDNA #3 was released from the lambda GT10 clone by EcoRI digestion and subcloned into pGEM-3. Various cDNA fragments were then subcloned and sequenced on both strands such that the entire sequence was determined. The 5' and 3' UTRs are shown as contiguous sequence and the protein coding region is broken up into codons with the corresponding amino acid three letter designation below each codon. The 28 amino acid signal sequence is double underlined and leucine 1 of the mature polypeptide is boxed as is the termination codon. The single base difference seen with pituitary hPRL cDNA in the 3' UTR is bolded. The length of the 5' UTR was 167bp and that of the 3' UTR, 148bp with a 3' poly(A) tail of approximately 45 bases denoted by A_{x45}. The putative polyadenylation signal is underlined and the sites of restriction endonuclease cleavage used for sequencing are indicated by the arrowheads: E, EcoRI; P, PstI; B, BglII; H, HindIII; D, DpnI; EV, EcoRV. The 3' UTR sequence which may have facilitated the chimeric construction of IM-9-P PRL cDNA 3, C and 1A is indicated by the broken underlining. IM-9-P PRL cDNA #3 was 996bp excluding the poly(A) tail.

```
                         TAAGAAC  CTTCATTCCA  GAAGTACCCT  CAAAGACAGA  GACACCAAGA   47
                                        EV
AGAATCGGAA  CATACAGGCT  TTGATATCAA  AGGTTTATAA  AGCCAATATC  TGGGAAAGAG   107
                   B
AAAACCGTGA  GACTTCCAGA  TCTTCTCTGG  TGAAGTGTGT  TTCCTGCAAC  GATCACGAAC   167

1                    D    7                               14
ATG AAC ATC AAA GGA TCG CCA TGG AAA GGG TCC CTC CTG CTG   209
met asn ile lys gly ser pro trp lys gly ser leu leu leu

                          21                              28
CTG CTG GTC TCA AAC CTG CTG CTG TGC CAG AGC GTG GCC CCC   251
leu leu val ser asn leu leu leu cys gln ser val ala pro

                          35                              42
[TTC] CCC ATC TGT CCC GGC GGG GCT GCC CGA TGC CAG GTG ACC   293
[leu] pro ile cys pro gly gly ala ala arg cys gln val thr

                          49                              56
CTT CGA GAC CTG TTT GAC CGC GCC GTC GTC CTG TCC CAC TAC   335
leu arg asp leu phe asp arg ala val val leu ser his tyr

                          63                  E           70
ATC CAT AAC CTC TCC TCA GAA ATG TTC AGC GAA TTC GAT AAA   377
ile his asn leu ser ser glu met phe ser glu phe asp lys

                          77                              84
CGG TAT ACC CAT GGC CGG GGG TTC ATT ACC AAG GCC ATC AAC   419
arg tyr thr his gly arg gly phe ile thr lys ala ile asn

                          91                              98
AGC TGC CAC ACT TCT TCC CTT GCC ACC CCC GAA GAC AAG GAG   461
ser cys his thr ser ser leu ala thr pro glu asp lys glu

                          105                             112
CAA GCC CAA CAG ATG AAT CAA AAA GAC TTT CTG AGC CTG ATA   503
gln ala gln gln met asn gln lys asp phe leu ser leu ile

                          119                             126
GTC AGC ATA TTG CGA TCC TGG AAT GAG CCT CTG TAT CAT CTG   545
val ser ile leu arg ser trp asn glu pro leu tyr his leu

                          133                             140
GTC ACG GAA GTA CGT GGT ATG CAA GAA GCC CCG GAG GCT ATC   587
val thr glu val arg gly met gln glu ala pro glu ala ile

                          147                             154
CTA TCC AAA GCT GTA GAG ATT GAG GAG CAA ACC AAA CGG CTT   629
leu ser lys ala val glu ile glu glu gln thr lys arg leu

                          161                             168
CTA GAG GGC ATG GAG CTG ATA GTC AGC CAG GTT CAT CCT GAA   671
leu glu gly met glu leu ile val ser gln val his pro glu

                    B   D 175                             182
ACC AAA GAA AAT GAG ATC TAC CCT GTC TGG TCG GGA CTT CCA   713
thr lys glu asn glu ile tyr pro val trp ser gly leu pro

         P                189                             196
TCC CTG CAG ATG GCT GAT GAA GAG TCT CGC CTT TCT GCT TAT   755
ser leu gln met ala asp glu glu ser arg leu ser ala tyr

                          203                             210
TAT AAC CTG GTC CAC TGC CTA CGC AGG GAT TCA CAT AAA ATC   797
tyr asn leu leu his cys leu arg arg asp ser his lys ile

                          217                             224
GAC AAT TAT CTC AAG CTC CTG AAG TGC CGA ATC ATC CAC AAC   839
asp asn tyr leu lys leu leu lys cys arg ile ile his asn

        227                                                       D
AAC AAC TGC [TAA]GCCCACA  TCCATTTCAT  CTATTTCTGA  GAAGGTCCTT  AATGATCCGT 898
asn asn cys
         H
TCCATTGCAA  GCTTCTTTTA  GTTGTATCTC  TTTTGAATCC  ATGCTTGGGT  GTAACAGGTC 958

TCCTCTTAAA  AAATAAAAAC  TGACTCGTTA  GAGACATCAx45                       996
```

Fig.20. Alignment of the 3' untranslated sequence of IM-9-P and decidual PRL cDNAs with the corresponding region of the hPRL gene.

Sequence alignment begins at the cytosine residue 110bp 3' of the terminal cysteine codon. Sequence upstream of this point amongst the decidua and IM-9-P PRL cDNAs was completely homologous with pituitary hPRL cDNA. The dotted nucleotide indicates the single base difference between IM-9-P/decidua PRL 3' UTR and that of the pituitary PRL mRNA. The polyadenylation signal is underlined in the hPRL gene sequence and the poly(A) tail on the 3' end of the cDNA sequences is denoted by A_n. IM-9-P PRL cDNA had a 3' end identical to that seen for human pituitary PRL cDNA. IM-9-P PRL cDNAs 10, 11, 9A, C and 1A, as well as dPRL clones 8B and F1-a, contained identical 7bp elongations relative to the 3' end of pituitary hPRL cDNA.

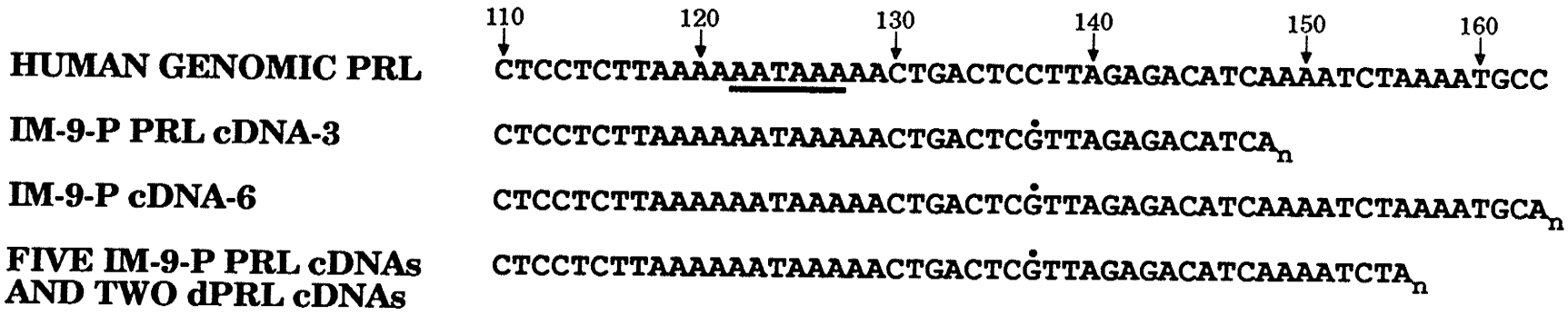

110
120
130
140
150
160
HUMAN GENOMIC PRL
CTCCTCTTAAAAAATAAAAACTGACTCCTTAGAGACATCAAAATCTAAAATGCC
IM-9-P PRL cDNA-3
CTCCTCTTAAAAAATAAAAACTGACTCGTTAGAGACATCAn
IM-9-P cDNA-6
CTCCTCTTAAAAAATAAAAACTGACTCGTTAGAGACATCAAAATCTAAAATGCAn
FIVE IM-9-P PRL cDNAs AND TWO dPRL cDNAs
CTCCTCTTAAAAAATAAAAACTGACTCGTTAGAGACATCAAAATCTAn

Fig.21. Comparison of the 5' untranslated sequence of IM-9-P PRL cDNA IV and decidual PRL cDNA 15A with the immediate 5' flanking DNA of the hPRL gene.

Alignment of these sequences begins at the initiator methionine codon of the preprolactin signal sequence and extends in a 5' direction to position -300 of the human genomic PRL sequence. The 9bp region over which pituitary-specific transcription begins is bracketed and the putative TATA box is underlined. The new 5' untranslated sequence in the IM-9-P and decidual PRL cDNAs which is not homologous to the corresponding hPRL gene sequence is indicated by white letters. The 5' UTR sequence of IM-9-P PRL cDNA IV was representative and identical to that found in nine other IM-9-P and dPRL cDNAs. The 5' UTR structure of decidual PRL cDNA 15A indicated that the unique sequence (in white letters) was spliced into the 5' flanking DNA of PRL exon 1 at -246. The diamonds give the location of the predominant 5' termini of IM-9-P and decidual PRL mRNAs based on primer extension studies. The dotted dinucleotides in the hPRL gene sequence denote the putative variant 3' splice junction between the unique 5' non-coding sequence of the cDNAs presented and the 5' DNA flanking the PRL exon 1. The negative numbers shown above each block of sequence are residue numbers for the 5' flanking DNA of PRL gene exon 1.

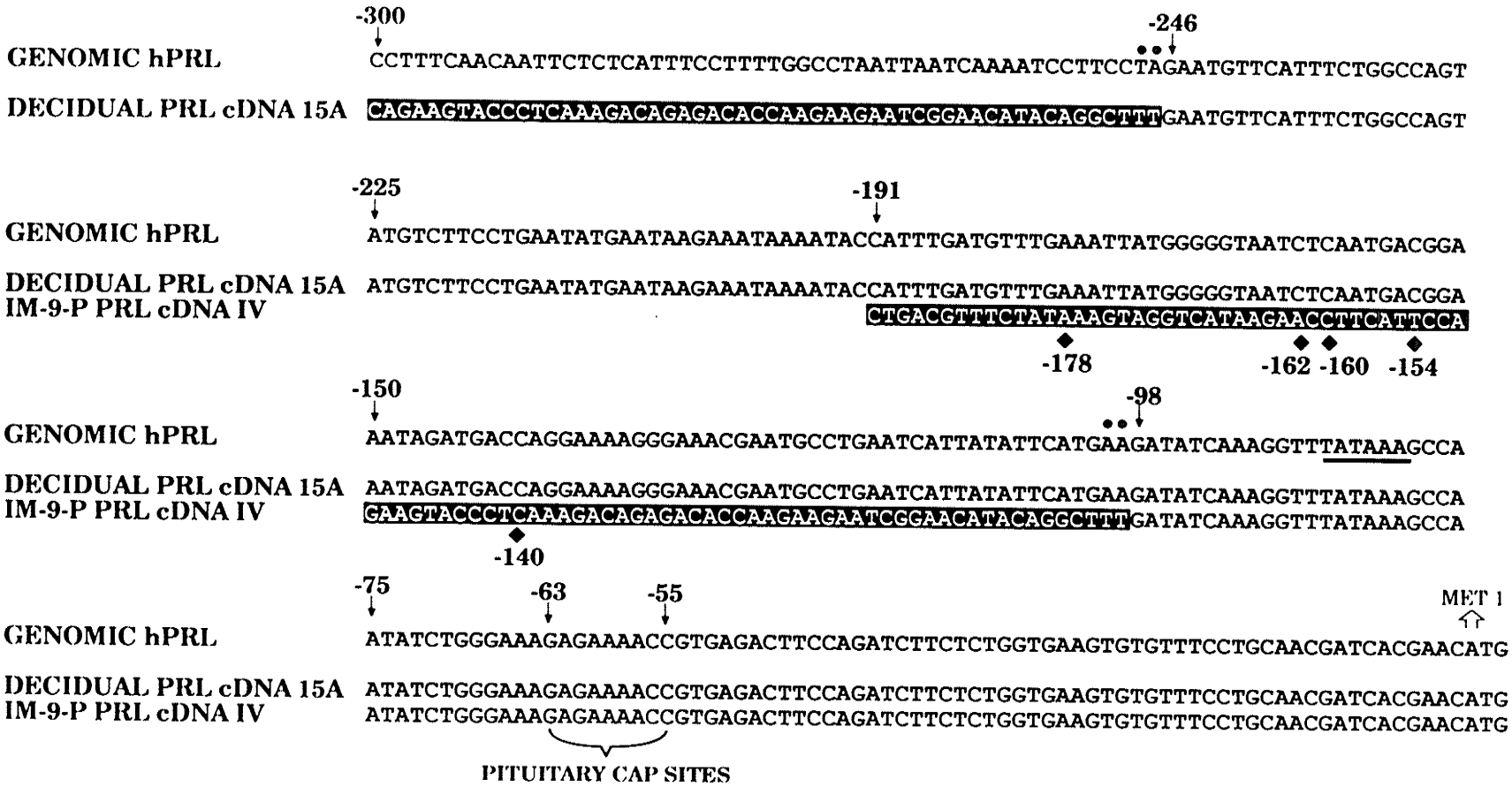

-300
-246
GENOMIC hPRL
CCTTTCAACAATTCTCTCATTTCCTTTTGGCCTAATTAATCAAAATCCTTCCTAGAATGTTCATTTCTGGCCAGT
DECIDUAL PRL cDNA 15A
CAGAAGTACCCTCAAAGACAGAGACACCAAGAAGAATCGGAACATACAGGCTTTGAATGTTCATTTCTGGCCAGT
-225
-191
GENOMIC hPRL
ATGTCTTCCTGAATATGAATAAGAAATAAAATACCATTTGATGTTTGAAATTATGGGGGTAATCTCAATGACGGA
DECIDUAL PRL cDNA 15A
ATGTCTTCCTGAATATGAATAAGAAATAAAATACCATTTGATGTTTGAAATTATGGGGGTAATCTCAATGACGGA
IM-9-P PRL cDNA IV
CTGACGTTTCTATAAAGTAGGTCATAAGAACCTTCATTCCA
-178
-162
-160
-154
-150
-98
GENOMIC hPRL
AATAGATGACCAGGAAAAGGGAAACGAATGCCTGAATCATTATATTCATGAAGATATCAAAGGTTTATAAAGCCA
DECIDUAL PRL cDNA 15A
AATAGATGACCAGGAAAAGGGAAACGAATGCCTGAATCATTATATTCATGAAGATATCAAAGGTTTATAAAGCCA
IM-9-P PRL cDNA IV
GAAGTACCCTCAAAGACAGAGACACCAAGAAGAATCGGAACATACAGGCTTTGATATCAAAGGTTTATAAAGCCA
-140
-75
-63
-55
MET 1
GENOMIC hPRL
ATATCTGGGAAAGAGAAAACCGTGAGACTTCCAGATCTTCTCTGGTGAAGTGTGTTTCCTGCAACGATCACGAACATG
DECIDUAL PRL cDNA 15A
ATATCTGGGAAAGAGAAAACCGTGAGACTTCCAGATCTTCTCTGGTGAAGTGTGTTTCCTGCAACGATCACGAACATG
IM-9-P PRL cDNA IV
ATATCTGGGAAAGAGAAAACCGTGAGACTTCCAGATCTTCTCTGGTGAAGTGTGTTTCCTGCAACGATCACGAACATG
PITUITARY CAP SITES

Fig.22. Comparison of the 5' UTRs of IM-9-P PRL cDNAs IV and I-1 with the hPRL gene sequence.

Alignment of these sequences is as in Figure 21. The new 5' untranslated sequence commonly found in the IM-9-P and decidual PRL cDNAs which is not homologous to the corresponding hPRL gene sequence is bordered by the broken line. The extra 12bp sequence unique to cDNA I-1 at the (-98) junction point with the known 5' flanking region of the hPRL gene is indicated by the dashes in the IM-9-P PRL cDNA IV sequence below. The extra 45 bases at the 5' end of cDNA I-1 is underlined and represents foreign cDNA sequence ligated to the IM-9-P PRL cDNA presumably at -203 in an inverted orientation during cDNA library construction, as described in the "Results". The negative numbers shown above each block of sequence are residue numbers for the 5' flanking DNA of hPRL exon 1. The bracketed -191 designates the 5' end of the IM-9-P PRL cDNA IV which did not contain the extra 12 nucleotides at the -98 splice junction.

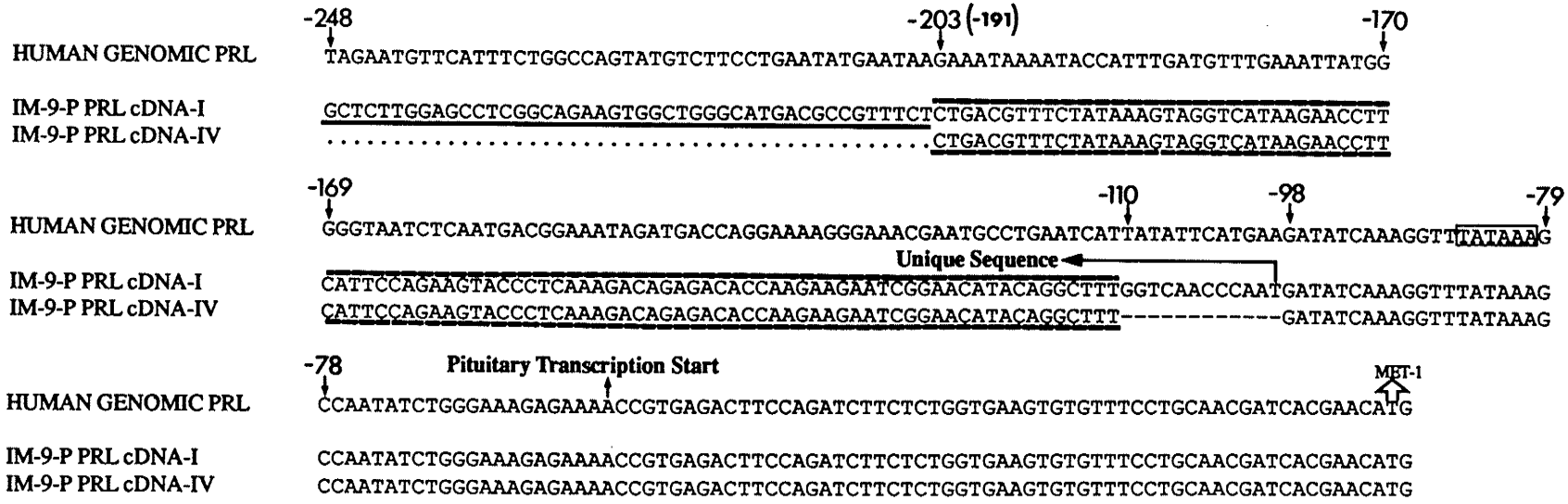

-248
-203 (-191)
-170
HUMAN GENOMIC PRL
TAGAATGTTCATTTCTGGCCAGTATGTCTTCCTGAATATGAATAAGAAATAAAATACCATTTGATGTTTGAAATTATGG
IM-9-P PRL cDNA-I
GCTCTTGGAGCCTCGGCAGAAGTGGCTGGGCATGACGCCGTTTCTCTGACGTTTCTATAAAGTAGGTCATAAGAACCTT
IM-9-P PRL cDNA-IV
.....CTGACGTTTCTATAAAGTAGGTCATAAGAACCTT
-169
-110
-98
-79
HUMAN GENOMIC PRL
GGGTAATCTCAATGACGGAAATAGATGACCAGGAAAAGGGAAACGAATGCCTGAATCATTATATTCATGAAGATATCAAAGGTTTATAAAG
Unique Sequence
IM-9-P PRL cDNA-I
CATTCCAGAAGTACCCTCAAAGACAGAGACACCAAGAAGAATCGGAACATACAGGCTTTGGTCAACCCAATGATATCAAAGGTTTATAAAG
IM-9-P PRL cDNA-IV
CATTCCAGAAGTACCCTCAAAGACAGAGACACCAAGAAGAATCGGAACATACAGGCTTT----------GATATCAAAGGTTTATAAAG
-78
Pituitary Transcription Start
MET-1
HUMAN GENOMIC PRL
CCAATATCTGGGAAAGAGAAAACCGTGAGACTTCCAGATCTTCTCTGGTGAAGTGTGTTTCCTGCAACGATCACGAACATG
IM-9-P PRL cDNA-I
CCAATATCTGGGAAAGAGAAAACCGTGAGACTTCCAGATCTTCTCTGGTGAAGTGTGTTTCCTGCAACGATCACGAACATG
IM-9-P PRL cDNA-IV
CCAATATCTGGGAAAGAGAAAACCGTGAGACTTCCAGATCTTCTCTGGTGAAGTGTGTTTCCTGCAACGATCACGAACATG

Fig.23. Tissue specificity of the new 5' untranslated sequence of IM-9-P/decidua PRL mRNA.

Total RNA was isolated from human decidual tissue, IM-9-P3 lymphoblast cells and human pituitaries obtained at autopsy. Total RNA (100μg) aliquots from 5 individual decidua and IM-9-P3 cells as well as 3μg of human pituitary total RNA was electrophoresed, photographed and blotted onto Hybond-N matrix. Equivalent loading of decidua and IM-9-P3 RNA was assessed by the intensity of ethidium bromide staining of the 18S rRNA band in each lane. Autoradiogram "a" is an approximately 2.5 day exposure after high stringency hybridization with a [^{32}P]-labelled antisense cRNA corresponding to the 93bp of unique 5' non-coding sequence in IM-9-P PRL cDNA IV. The faintly hybridizing low molecular weight bands are due to non-specific hybridization. This blot was then stripped and re-hybridized "b" under stringent conditions with random-primer labelled 555bp PstI fragment of pituitary hPRL cDNA as described previously (18). The locations of the 18S and 28S rRNA bands is indicated.

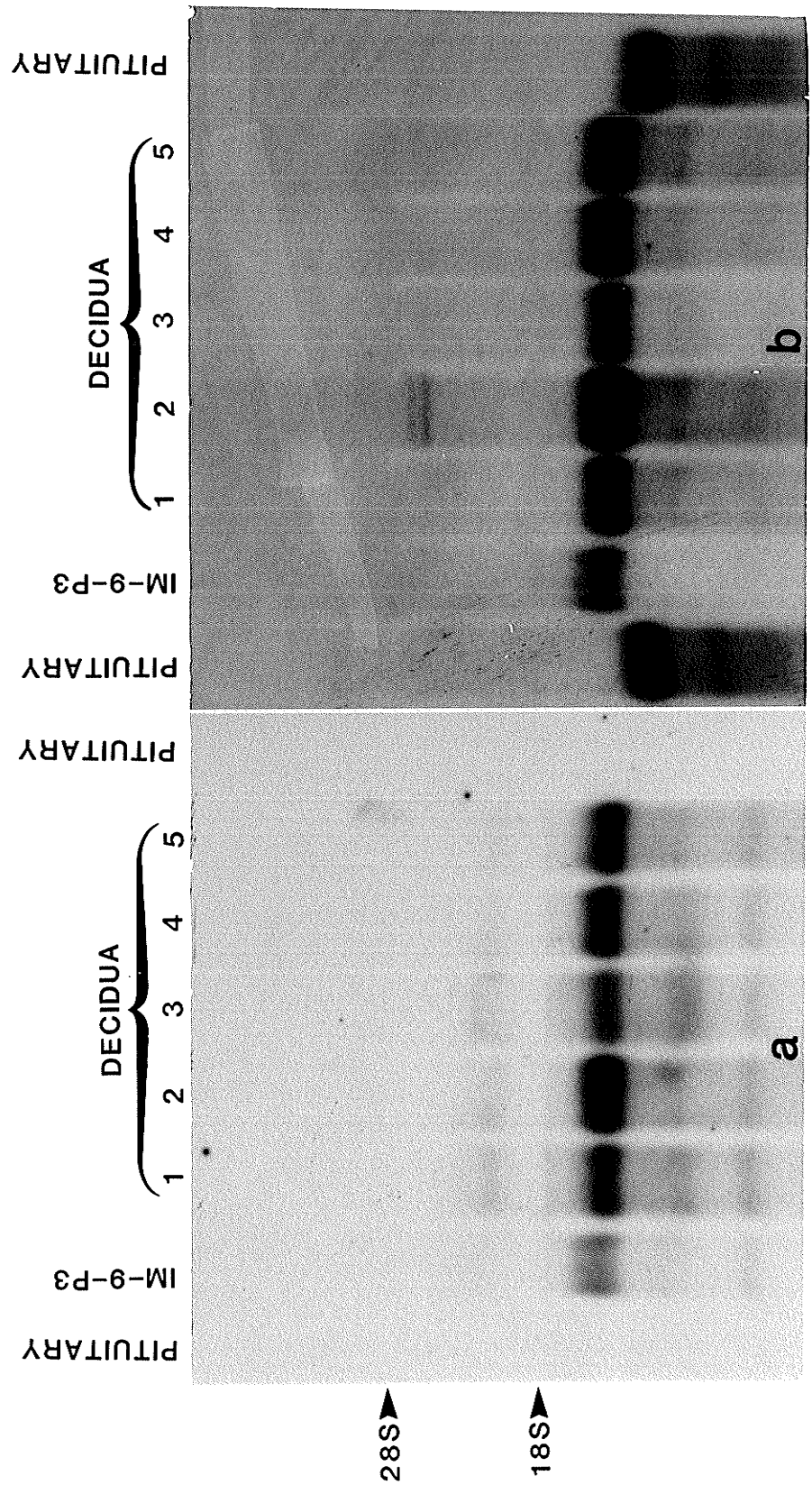

PITUITARY
IM-9-P3
DECIDUA
1
2
3
4
5
PITUITARY
PITUITARY
IM-9-P3
DECIDUA
1
2
3
4
5
PITUITARY
28S
18S
a
b

Fig.24. Primer extension analysis of hPRL mRNAs.

Primer extension was performed as described under "Materials and Methods" using a 21-base oligonucleotide whose complementary sequence in hPRL mRNA relative to the pituitary-specific 5' end (-57) is indicated in the line diagram below the autoradiogram. The primer was annealed to 40μg aliquots of batch-prepared poly(A)$^+$ enriched mRNA isolated from IM-9-P3 cells, non-PRL producing IM-9 cells and two different pools of decidual tissue as well as 15μg of total RNA and 2.5μg of poly(A)$^+$ enriched mRNA (Pituitary*) from human pituitaries. The lengths of the extension products are indicated at the left in nucleotides; only the sizes of the predominant and reproducible cDNAs are given. These sizes were determined by comparison of the band mobilities with the adjacent nucleotide sequence ladder and end-labelled Hpall digested pAT153 in lane M. Only the size, in nucleotides, of the relevant DNA marker bands are provided next to the corresponding fragment in lane M. A number of termination points between 140 and 149 nucleotides was obtained with total or poly(A)$^+$ enriched mRNA samples from human pituitaries. The primary exposure time was 24 hours at -70C with an intensifying screen, the last two lanes on the right are the result of an extended exposure of 4 days. The prolonged exposure was necessary to more clearly visualize the minor primer extended product at 410 nucleotides in decidua and IM-9-P3 RNA.

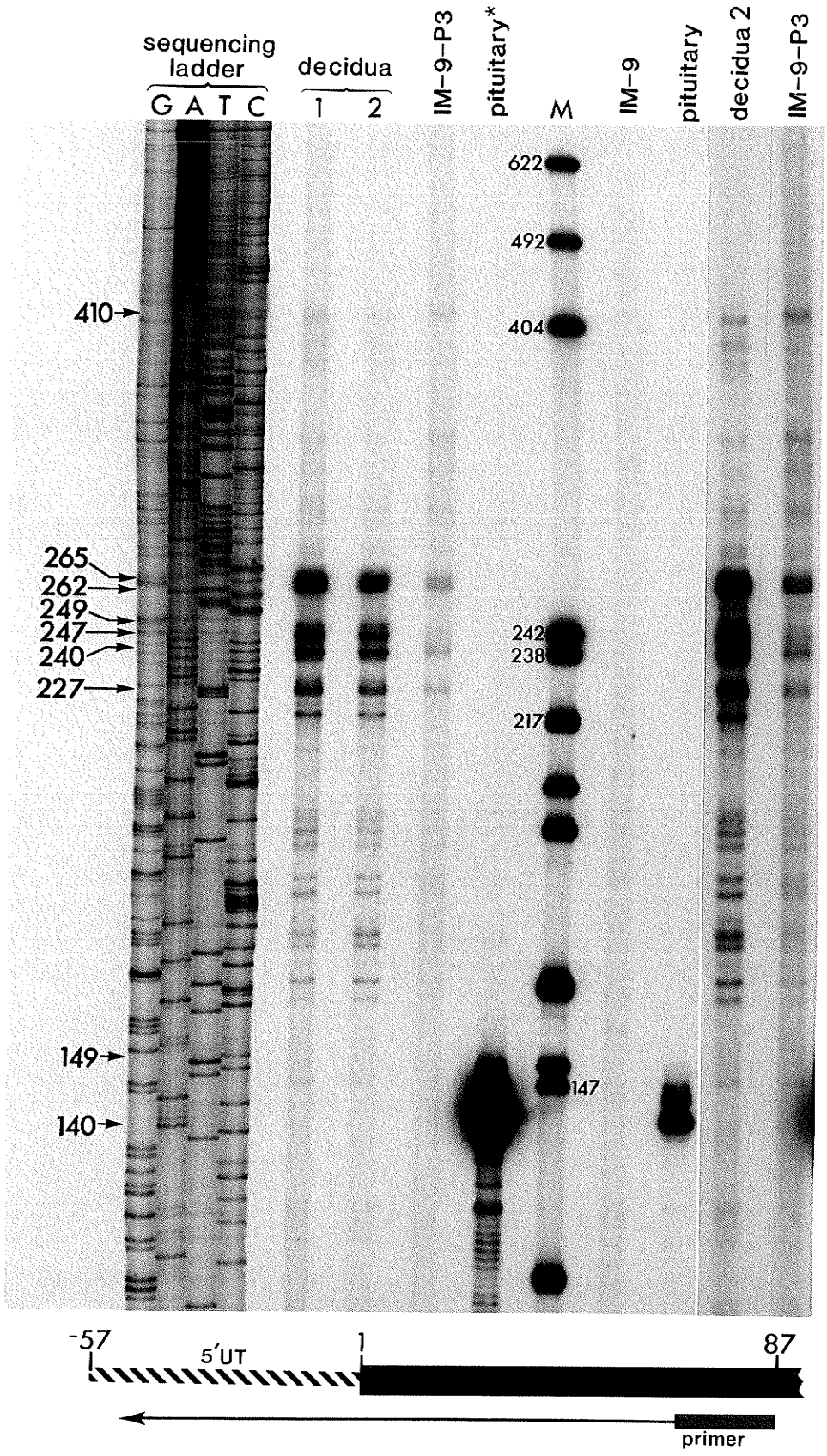

sequencing ladder
G A T C
decidua
1 2
IM-9-P3
pituitary*
M
IM-9
pituitary
decidua 2
IM-9-P3
622
492
404
410
265
262
249
247
240
227
242
238
217
149
147
140
-57
5'UT
1
87
primer

Fig.25. Localization of the unique 5' non-coding sequence relative to the human PRL gene exon 1 by genomic Southern hybridization analysis.

Human genomic DNA (20μg) from seven individuals (A-G) as well as DNA from the parental IM-9 line, the cloned IM-9-P6 (P6) non-PRL producing line and the PRL-producing clones IM-9-P3 (P3) and IM-9-P33 (P33) were digested with various restriction enzymes. These DNAs were cleaved with EcoRV and HindIII. An EcoRV site is located at the junction between the previously described 5' flanking DNA of the hPRL gene and the additional unique 5' non-coding sequence found in IM-9-P3 and decidual PRL transcripts. In "a", the resultant genomic Southern blot was hybridized, as described in the "Materials and Methods", with a 680bp EcoRV-HindIII fragment of the hPRL gene 5' flanking DNA shown above schematically. The blot was then stripped of the radioactive signal and hybridized with a 311bp probe containing three copies of a 72bp portion of the unique IM-9-P3 and decidual PRL 5' UTR ("b"), as described in "Materials and Methods". The DNA markers used were lambda digested with HindIII and ØX174 with HaeIII; sizes are given in kilobases.

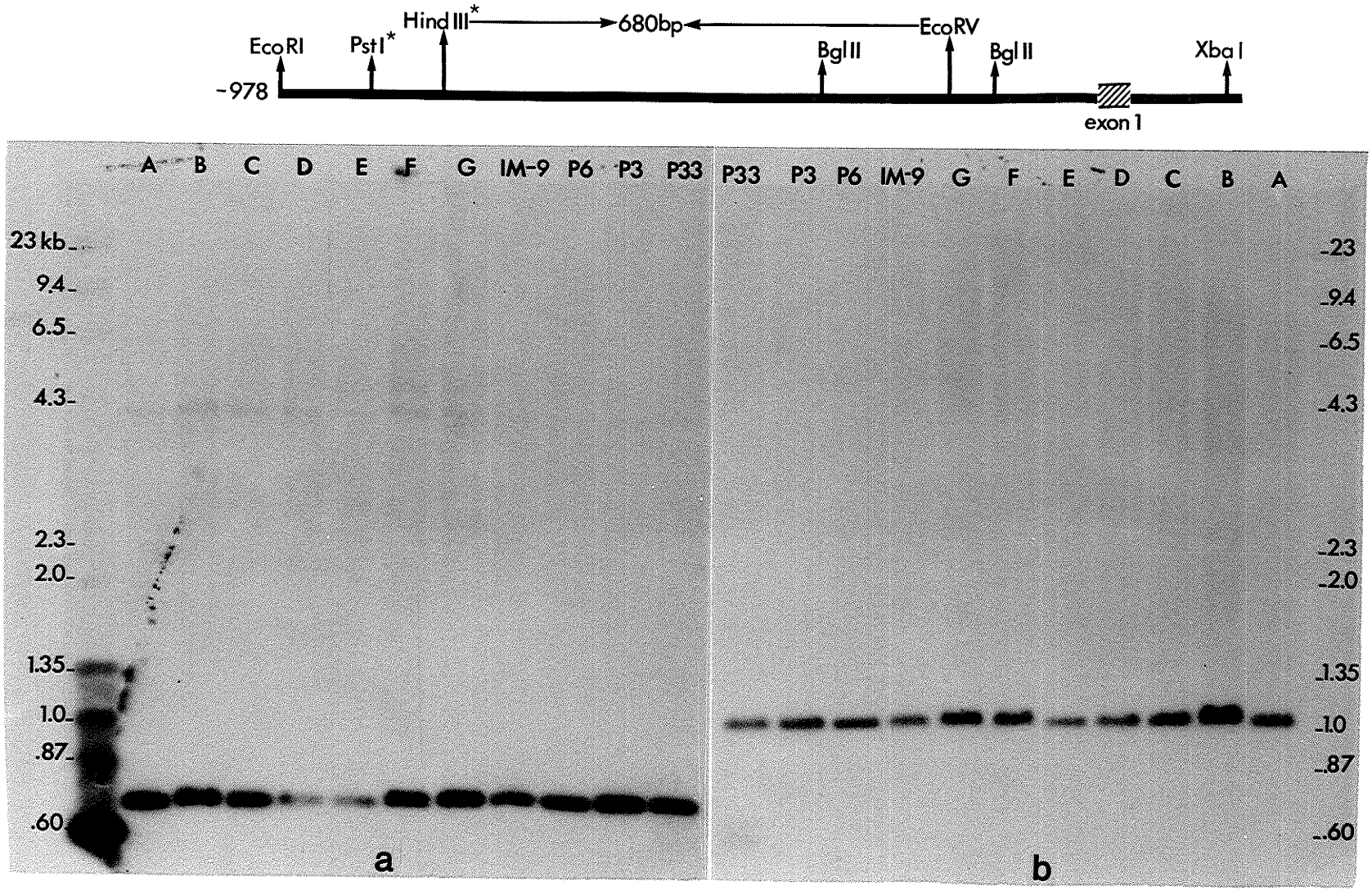

EcoRI
PstI*
HindIII*
680bp
EcoRV
BglII
BglII
XbaI
-978
exon 1
A B C D E F G IM-9 P6 P3 P33
23 kb
9.4
6.5
4.3
2.3
2.0
1.35
1.0
.87
.60
a
P33 P3 P6 IM-9 G F E D C B A
23
9.4
6.5
4.3
2.3
2.0
1.35
1.0
.87
.60
b

Fig.26. A partial restriction map of the hPRL gene redrawn from Truong *et al* (36).

a. This schematic covers approximately 11.1kb of the hPRL gene including 978bp of 5' flanking DNA and 392bp of 3' flanking DNA. The estimated sizes of the introns are as follows: intron A - 2176bp; intron B - 1788bp; intron C - 2660bp; and intron D - 2424bp. Exons 1 and 5, as depicted in this diagram, include the 5' and 3' UTRs, respectively. Only the restriction sites pertinent to the work described herein are indicated. The restriction enzyme designations are: E, EcoRI; P, PstI; H, HindIII; B, BglII; H_2, HincII; N, NcoI; X, XbaI. The bar below represents the length of 444bp.

b. This schematic is an enlargement of the 5' region of the hPRL gene extending from the 5' EcoRI site to the XbaI* site in intron A. This region encompasses 978bp of 5' flanking DNA, exon 1, and 126bp of intron A. The dotted area of exon 1 represents the 5' UTR of 57bp as determined by Truong *et al* (36). The adjacent darkened area of exon 1 denotes the first 28bp of hPRL coding sequence. This 1132bp EcoRI-XbaI fragment was used extensively in the studies described here and is referred to as the 5' end-specific hPRL genomic probe. The bar below represents the length of 50bp on the diagram.

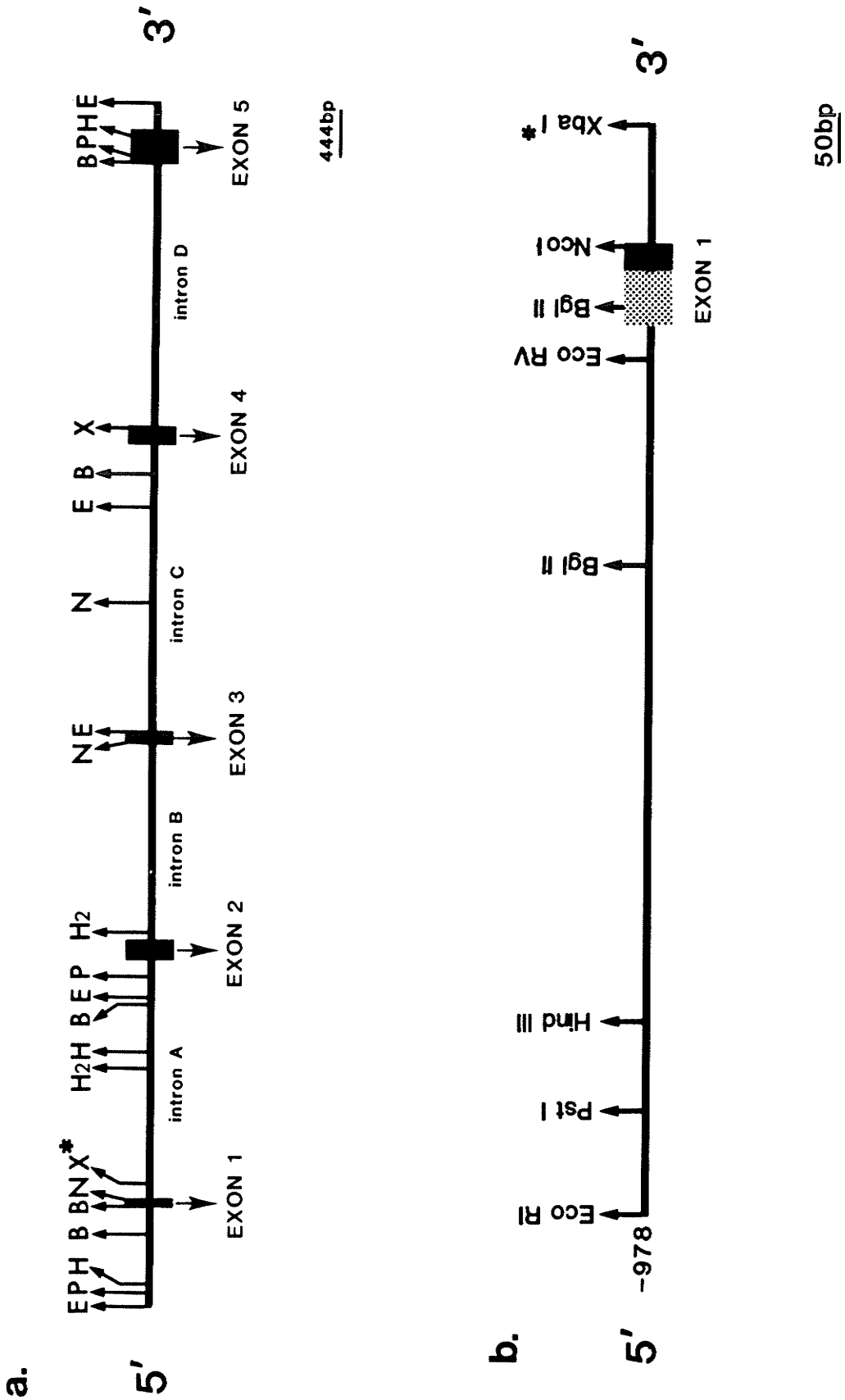
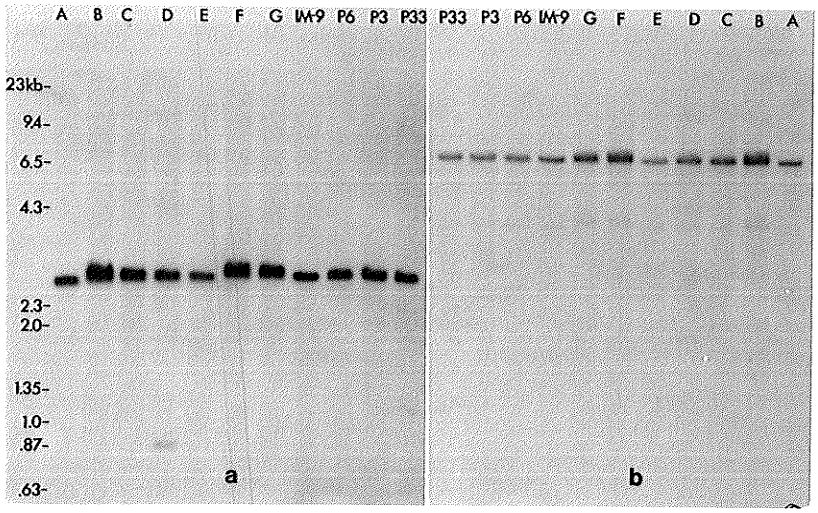
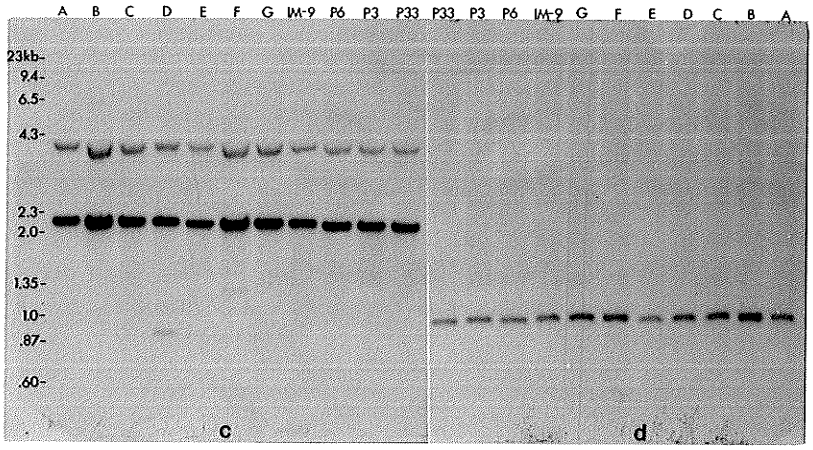

a.
5'
3'
EPH B BN X*
H2H B E P H2
N E
N
E B X
B P H E
EXON 1
EXON 2
EXON 3
EXON 4
EXON 5
intron A
intron B
intron C
intron D
444bp
b.
5'
3'
-978
Eco RI
Pst I
Hind III
Bgl II
Eco RV
Bgl II
Nco I
Xba I*
EXON 1
50bp

Fig.27. Localization of the unique 5' non-coding sequence of IM-9-P3/decidua PRL mRNA beyond 5.0kb of hPRL exon 1.

Human DNAs described in Fig.25 were digested with EcoRI ("a" and "b"), HindIII ("c" and "d") and HincII ("e" and "f"), blotted and probed first with the 5' end-specific genomic fragment of the hPRL gene containing 978bp of 5' flanking DNA, exon 1, and 126bp of intron A. The resultant autoradiorams are shown in "a", "c" and "e", respectively. The blots were stripped and reprobed with the 311bp tetramer of unique 5' non-coding sequence described in the "Materials and Methods". The results from this second hybridization are shown in "b", "d" and "f". The varying band intensities from lane to lane were indicative of unequal sample loading. DNA markers were as described for Figure 25.


A B C D E F G IM-9 P6 P3 P33
P33 P3 P6 IM-9 G F E D C B A
23kb-
9.4-
6.5-
4.3-
2.3-
2.0-
1.35-
1.0-
.87-
.63-
a
b


A B C D E F G IM-9 P6 P3 P33
P33 P3 P6 IM-9 G F E D C B A
23kb-
9.4-
6.5-
4.3-
2.3-
2.0-
1.35-
1.0-
.87-
.60-
c
d

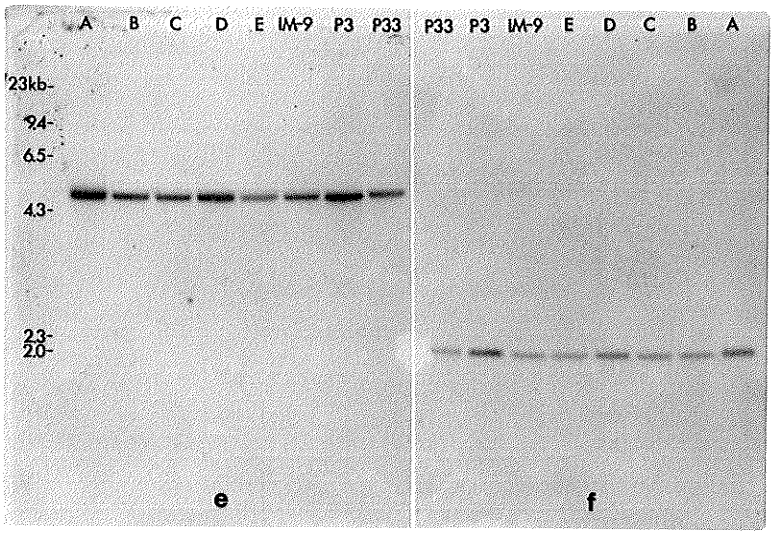
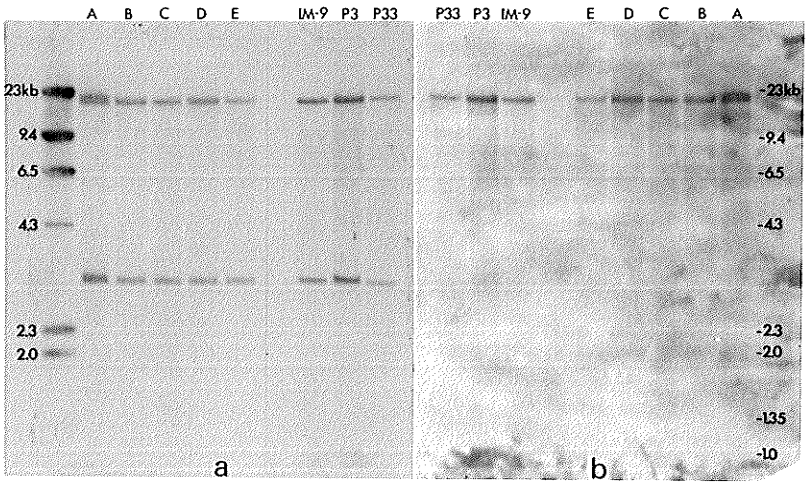
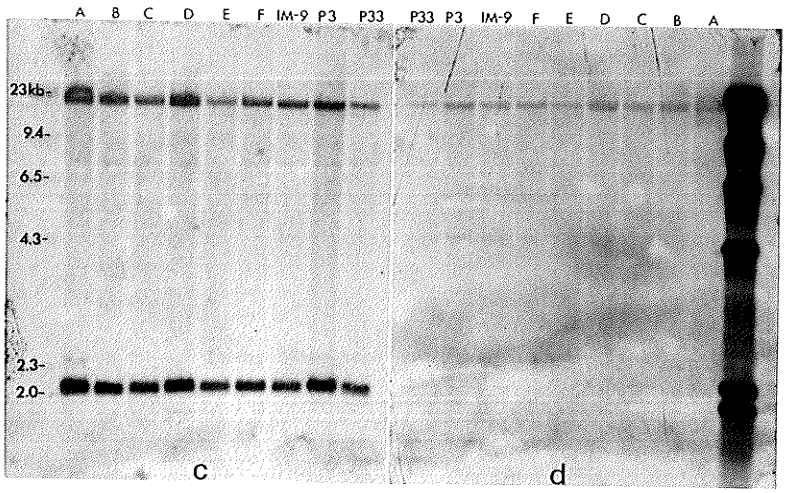

A B C D E IM-9 P3 P33
P33 P3 IM-9 E D C B A
23kb-
9.4-
6.5-
4.3-
2.3-
2.0-
e
f

Fig.28. Determining the outer limit size of the intron between hPRL gene exon 1 and the new 5' non-coding sequence.

Human DNAs described in Figure 25 were digested with NcoI ("a" and "b"), BglII ("c" and "d") and PstI ("e" and "f") and hybridized as described in Figure 27 with the 5' end-specific hPRL genomic probe and then with the 311bp tetramer of unique 5' non-coding region of IM-9-P3/decidua PRL mRNA. The autoradiograms in "a", "c" and "e" resulted from hybridization with the hPRL genomic probe and those in "b", "d" and "f" were generated by hybridization of the same blot with the IM-9-P3/decidua 5' UTR cDNA sequence. The very weak band which comigrated with the 1.35kb marker in "b" was due to hybridization of the 22bp of 3' UTR sequence found in the IM-9-P3/decidua unique 5' UTR probe due to a cDNA cloning artifact. This was confirmed by hybridization of this blot with a 3' end-specific hPRL genomic probe described previously.


A B C D E IM-9 P3 P33
23kb
9.4
6.5
4.3
2.3
2.0
a
P33 P3 IM-9 E D C B A
~23kb
~9.4
~6.5
~4.3
~2.3
~2.0
~1.35
~1.0
b


A B C D E F IM-9 P3 P33
23kb-
9.4-
6.5-
4.3-
2.3-
2.0-
c
P33 P3 IM-9 F E D C B A
d

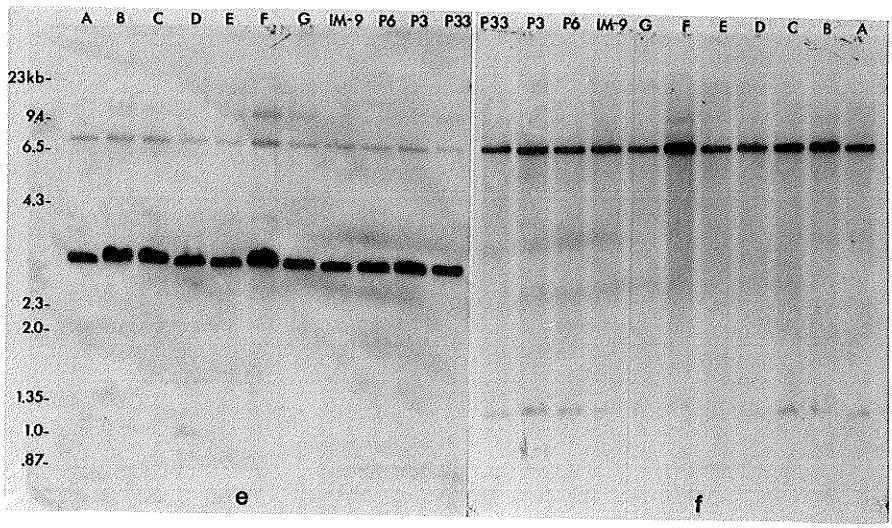

A B C D E F G IM-9 P6 P3 P33
23kb-
9.4-
6.5-
4.3-
2.3-
2.0-
1.35-
1.0-
.87-
e
P33 P3 P6 IM-9 G F E D C B A
f

Fig.29. Partial restriction map of additional 5' flanking DNA of the hPRL gene.

Human genomic clone phPRLg4800, provided by Dr. J. Martial (University of Liege, Belgium), contains an additional 4800bp of DNA 5' to the EcoRI site at -978 of phPRLg2750 described in the "Materials and Methods". The 2620bp SacI-EcoRI fragment was sequenced by members of Dr. J. Martial's laboratory and a partial restriction map for the 5' portion of the clone was also provided. The locations of the HindIII, HincII, XbaI and MspI sites were more precisely localized by additional restriction digests and are indicated on the map. The thin black line denotes the previously reported 5' portion of the hPRL gene (36) (see Fig.26), the thicker black line extending 5' from -978 represents the additional 4800bp of 5' flanking DNA to -5780bp. The broken black line upstream of -5780 corresponds to DNA which has not yet been cloned. The location of the 830bp SalI-HindIII fragment containing DNA from the 5' end of phPRLg4800 and used in experiments described herein, is also indicated. The bar at the bottom represents the length of 290bp.

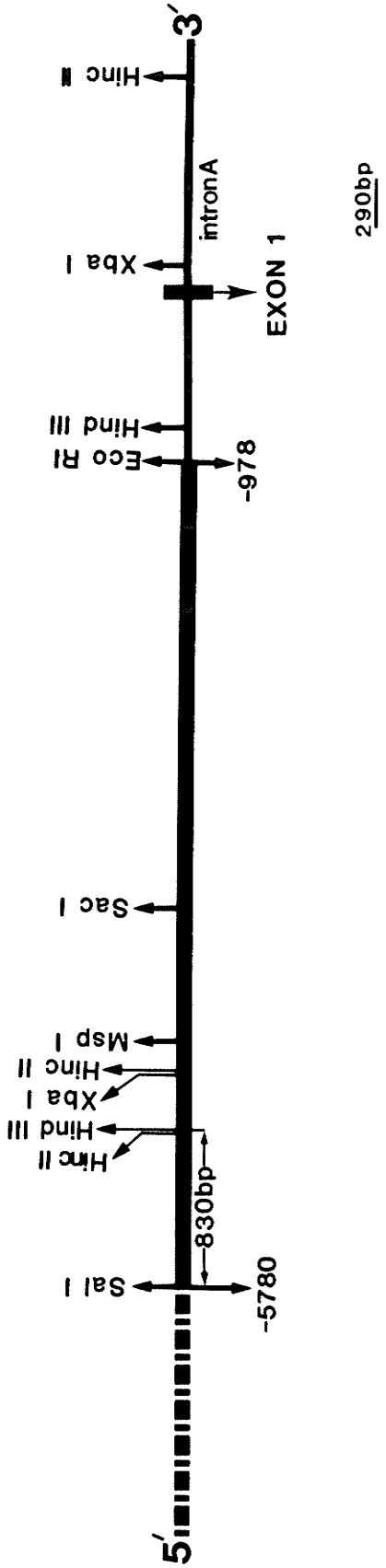

3′
Hinc II
intronA
290bp
Xba I
EXON 1
Hind III
Eco RI
-978
Sac I
Msp I
Hinc II
Xba I
Hind III
Hinc II
830bp
Sal I
-5780
5′

Fig.30. Identification of common genomic fragments with the unique 5' UTR of IM-9-P3/decidua PRL mRNA and a genomic probe extending 5.8kb upstream of the pituitary PRL cap site.

Approximately 15μg of IM-9-P33 cell DNA was digested with HincII (lane 1), HincII/XbaI (lane 2), HindIII (lane 3), HindIII/XbaI (lane 4) and fractionated, blotted to nitrocellulose and probed first with the 311bp tetramer of IM-9-P3/decidua PRL unique 5' UTR ("b") described in the "Materials and Methods". The blot was then stripped and rehybridized with the 1132bp EcoRI/XbaI genomic fragment (see Fig.26) containing 978bp of 5'DNA flanking exon 1 of the hPRL gene ("a"). Lastly, this blot was stripped and probed with an 830bp SalI-HindIII fragment encompassing the 5' end of a genomic clone (phPRLg4800) extending 5.8 kb upstream of the pituitary PRL RNA start site ("c"). The hybridizing bands in autoradiogram "b" comigrate and were directly superimposable with those present on autoradiogram "c".

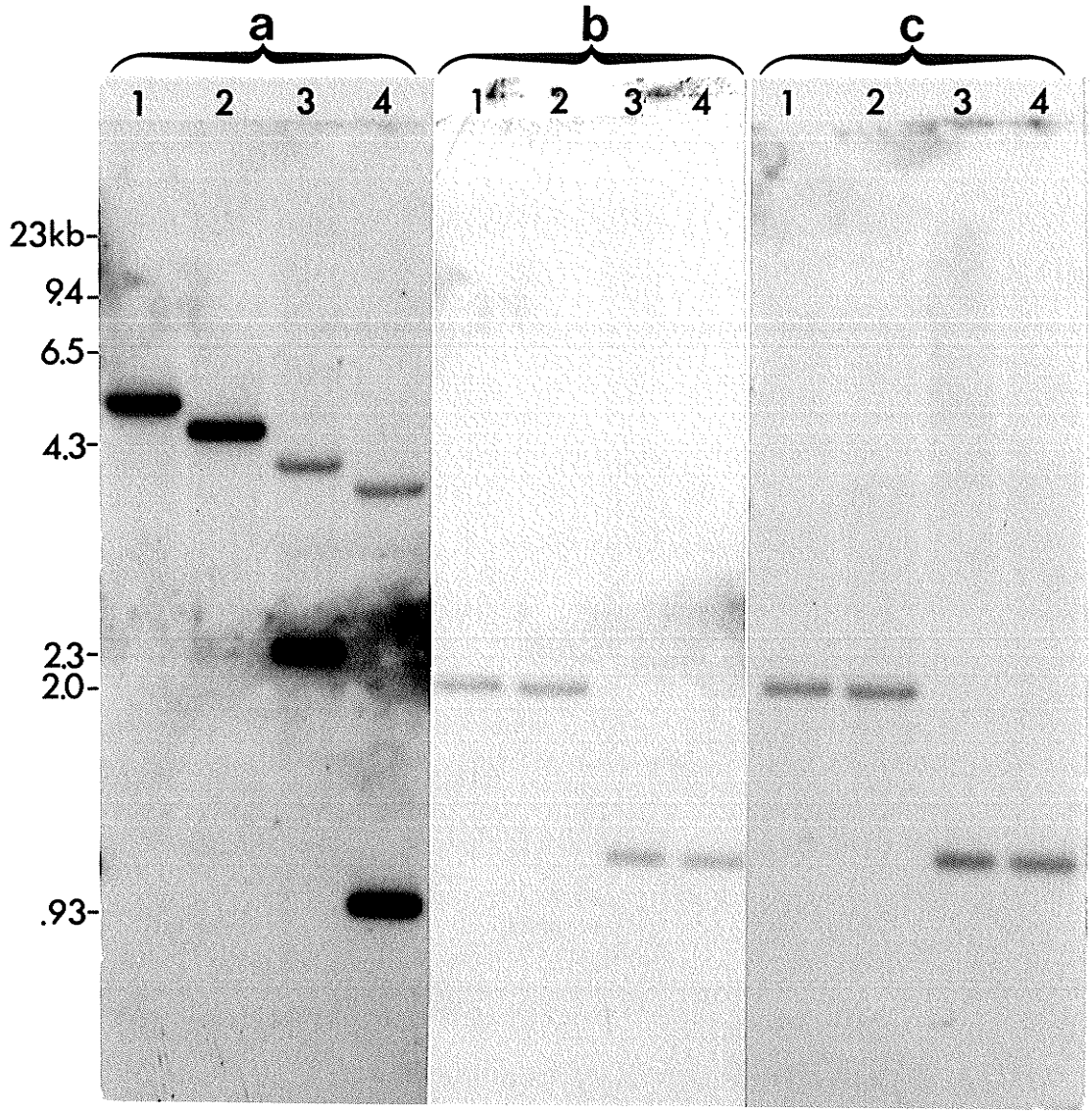

a
b
c
1
2
3
4
23kb
9.4
6.5
4.3
2.3
2.0
.93

Fig.31. Direct linkage between the unique 5' non-coding sequence of IM-9-P3/decidua PRL mRNA and the 5' flanking DNA of the hPRL gene.

These autoradiograms resulted from hybridization of the same genomic DNA blot described in Figure 15 with an 830bp SalI-HindIII genomic fragment ("a") which represented the 5' end of DNA extending 5.8kb upstream of hPRL exon 1 (see Fig.29). The autoradiogram in "b" resulted from a previous experiment in which this DNA blot had been hybridized with IM-9-P PRL cDNA #3 which contained 72bp of unique 5' UTR. The genomic fragments detected in common by these probes are indicated by the arrowheads on the autoradiogram in "b". DNA fractionated in lane A was isolated from a human placenta, in lane B from IM-9-P3 cells and in lane C from parental IM-9 cells. These DNAs were digested with the indicated restriction enzymes.

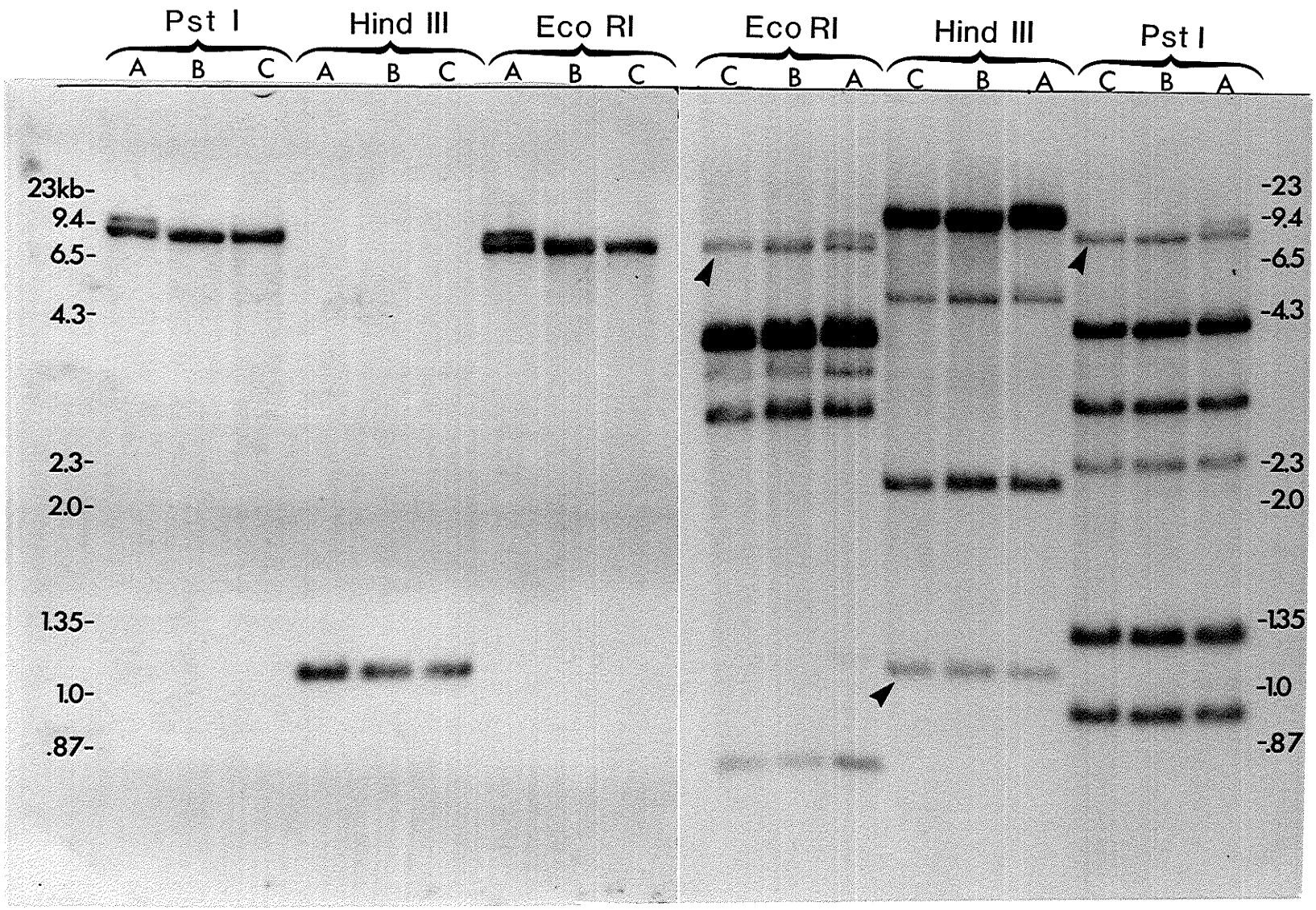

Pst I
Hind III
Eco RI
Eco RI
Hind III
Pst I
A B C A B C A B C C B A C B A C B A
23kb-
9.4-
6.5-
4.3-
2.3-
2.0-
1.35-
1.0-
.87-
-23
-9.4
-6.5
-4.3
-2.3
-2.0
-1.35
-1.0
-.87

Fig.32. A schematic representation of the revised structure of the hPRL gene 5' end.

The 5' non-coding sequence is indicated by the hatched lines and the thick black line represents the coding region of the hPRL gene. The top diagram depicts the operational structure of the pituitary PRL gene with the RNA start sites around -57 (perpendicular arrow) relative to the initiator codon (ATG) of exon 1b. The schematic below shows the multiple, clustered start sites of the IM-9-P3/decidua PRL gene in the new 5' non-coding exon 1a located approximately 5.9kb upstream of the pituitary specific start site. The size of exon 1a is shown as 93bp which corresponds to the longest unique 5' UTR found in IM-9-P PRL cDNA IV. Primer extension studies indicated that the size of exon 1b may be larger. In IM-9-P3 and decidual cells, intron A-1 is processed such that exon 1a is spliced to -98 of exon 1b and rarely at -246, the latter location of which is represented by the thin broken line. The bar at the bottom represents the length of 32bp which pertains to the exons; intron A-1 is not drawn to scale.

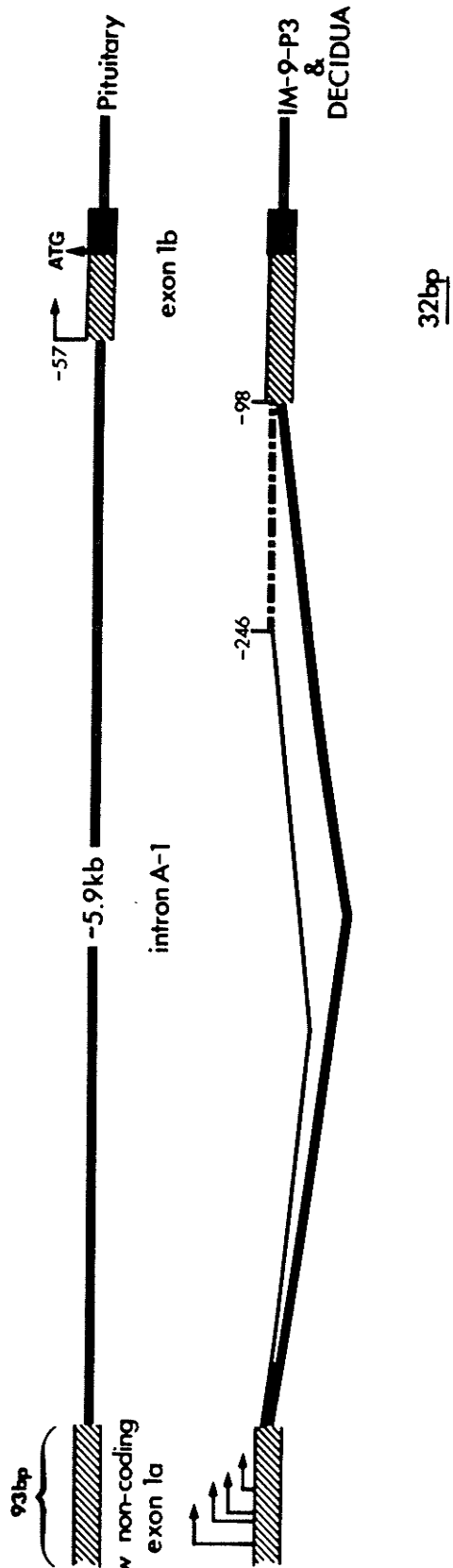

Pituitary
IM-9-P3
&
DECIDUA
ATG
-57
exon 1b
-98
-246
32bp
-5.9kb
intron A-1
93bp
new non-coding
exon 1a

TABLE 1: EVALUATION OF HUMAN LEUKOCYTE CELL SURFACE ANTIGENS BY FLUORESCENCE-ACTIVATED CELL SORTING.

	MONOCLONAL ANTIBODY	ATCC IM-9 CELLS	IM-9-P3 CELLS
	T-CELL MARKERS: T3,T4,T8	---	---
	MYELOID MARKERS: MY9,Mo1,Mo2	---	---
	NKH-1	---	---
	IL-2R1	---	---
	J5 (CALLA)	---	---
	I2	100%	99.7%
B-CELL MARKERS	B1	74.7%	---
	KAPPA	97.5%	---
	LAMBDA	---	---
	IgM	---	---
	IgG	88.7%	---
	HLA TYPING	A2,28 B21,22,13 BW4,6	A2,28 B21,22,13 BW4,6

The monoclonal antibody designations are defined in the "Materials and Methods". Percentages refer to the proportion of cells reactive with a particular monoclonal antibody and a dashed line indicates that the reactivity was lower than background.

REFERENCES

1. Stricker P, Grueter F (1928) Action du lobe anterieur de l'hypophyse sur la montee laiteuse. Compt Rend Soc de Biol 99:1978-1980.

2. Riddle O, Bates RW, Dykshorn SW (1932) A new hormone of the anterior pituitary. Proc Soc Exper Biol Med 29:1211-1212.

3. Riddle O, Bates RW, Dykshorn SW (1932) Prolactin, a new and third hormone of the anterior pituitary. Anat Rec (Suppl) 54:25.

4. Riddle O, Bates RW, Dykshorn SW (1933) The preparation, identification and assay of prolactin - a hormone of the anterior pituitary. Amer J Physiol 105:191-216.

5. Schulster D, Gaines Das RE, Jeffcoate SL (1989) International standards for human prolactin: calibration by international collaborative study. J Endocrinol 121:157-166.

6. Lewis UJ, Singh NP, Seavey BK (1971) Human prolactin: isolation and some properties. Biochem Biophys Res Commun 44:1169-1176.

7. Hwang P, Guyda H, Friesen HG (1972) Purification of human prolactin. J Biol Chem 247:1955-1958.

8. Hwang P, Guyda H, Friesen HG (1971) A radioimmunoassay for human prolactin. Proc Nat Acad Sci USA 68:1902-1906.

9. Shome B, Parlow AR (1977) Human pituitary prolactin (hPRL): the entire linear amino acid sequence. J Clin Endocrinol Metab 45:112-115.

10. Cooke NE, Coit D, Shine J, Baxter JD, Martial JA (1981) Human prolactin. J Biol Chem 256:4007-4016.

11. Shuh HK, Frantz AG (1974) Size heterogeneity of human prolactin in plasma and pituitary extracts. J Clin Endocrinol Metab 39:928-935.

12. Meuris S, Svoboda M, Christophe J, Robyn C (1985) Evidence for a glycosylated prolactin variant in human pituitary and amniotic fluid. In MacLeod RM, Thorner MO, Scapagnin U (eds), Prolactin. Basic and Clinical Correlates. Fidia Research Series 1:487-493, Liviana Press, Padova.

13. Larrea F, Escorza A, Granados J, Valencia X, Valero A, Cravioto M, Perez-Palacious G (1987) Familial occurrence of big-big prolactin as the predominant immunoreactive human prolactin species in blood. Fertil Steril 47:956-963.

14. Jackson R, Wortsman J, Malarkey W (1985) Characterization of a large molecular weight prolactin in women with idiopathic hyperprolactinemia and normal menses. J Clin Endocrinol Metab 61:258-264.

15. Asawaroengchai H, Russell SM, Nicoll CS (1978) Electrophoretically separable forms of rat prolactin with different bioassay and radioimmunoassay activities. Endocrinology 102:407-413.

16. Sinha YN, Jacobsen BP (1988) Structural and immunological evidence for a small molecular weight ("21K") variant of prolactin. Endocrinology 123:1364-1370.

17. Mittra I (1980) A novel "cleaved prolactin" in the rat pituitary. Part I. Biosynthesis, characterization and regulatory control. Biochem Biophys Res Commun 95:1750-1759.

18. Casabiell X, Robertson MC, Friesen HG, Casanueva F (1989) Cleaved prolactin and its 16K fragment are generated by an acid protease. Endocrinology 125:1967-1972.

19. Oetting W, Tuazon P, Traugh J, Walker A (1986) Phosphorylation of prolactin. J Biol Chem 261:1649-1652.

20. RobynC, Meuris S (1988) The state of the art in human prolactin. In Hoshino K (ed) Prolactin Gene Family and its Receptors. Excerpta Medica International Congress Series 819:29-41. Elsevier Science Publishers B.U., Amsterdam.

21. Lewis U, Singh R, Lewis L, Seavey B, Sinha Y (1984) Glycosylated ovine prolactin. Proc Natl Acad Sci USA 81:385-389.

22. Markoff E, Lee DW (1987) Glycosylated PRL is a major circulating variant in human serum. Endocrinology 65:1102-1106.

23. Markoff E, Sigel M, Lacour N, Seavey B, Friesen HG, Lewis UJ (1988) Glycosylation selectively alters the biological activity of prolactin. Endocrinology 123:1303-1306.

24. Markoff E, Lee D, Hollingsworth D (1988) Glycosylated and nonglycosylated prolactin in serum during pregnancy. Endocrinology 67:519-523.

25. Nicoll CS, Mayer GL, Russel SM (1986) Structural features of prolactins and growth hormones that can be related to their biological properties. Endo Rev 7:169-203.

26. Duckworth ML, Kirk KL, Friesen HG (1986) Isolation and identification of a cDNA clone of rat placental lactogen II. J Biol Chem 261:10871-10878.

27. Schuler LA, Shimomura K, Kessler MA, Zieler CG, Bremel RD (1988) Bovine placental lactogen: Molecular cloning and protein structure. Biochemistry 27:8443-8448.

28. Duckworth ML, Peden LM, Friesen HG (1986) Isolation of a novel prolactin-like cDNA clone from developing rat placenta. J Biol Chem 261:10879-10884.

29. Duckworth ML, Peden LM, Friesen HG (1988) A third prolactin-like protein expressed by the developing rat placenta: Complementary deoxyribonucleic acid sequence and partial structure of the gene. Mol Endocrinol 2:912-920.

30. Linzer DI, Lee S-J, Ogren L, Talamantis F, Nathani D (1985) Identification of proliferin mRNA and protein in mouse placenta. Proc Natl Acad Sci USA 82:4356-4359.

31. Linzer DI, Nathans D (1985) A new member of the prolactin-growth hormone gene family expressed in mouse placenta. EMBO J 4:1419-1423.

32. Schuler LA, Hurley WL (1987) Molecular cloning of a prolactin related mRNA expressed in bovine placenta. Proc Natl Acad Sci USA 84:5650-5654.

33. Kessler MA, Milosavljevic M, Zieler CG, Schuler LA (1989) A subfamily of bovine prolactin-related transcripts distinct from placental lactogen in the fetal placenta. Biochemistry 28:5154-5161.

34. Jackson-Grusby L, Pravtcheva D, Ruddle FH, Linzer DI (1988) Chromosomal mapping of the prolactin/growth hormone gene family in the mouse. Endocrinology 122:2462-2466.

35. Owerbach D, Rutter WJ, Cooke NE, Martial JA, Shows TB (1981) The prolactin gene is located on chromosome 6 in humans. Science 212:815-816.

36. Truong AT, Duez C, Belayew A, Renard A, Pictet R, Bell GI, Martial JA (1984) Isolation and characterization of the human prolactin gene. EMBO J 3:429-437.

37. Nicoll C (1974) Physiological actions of prolactin. In Greep R, Astwood E (eds): Handbook of Physiology. Vol. IV. Waverly Press Inc., Baltimore, pp 253-292.

38. Nicoll C (1980) Ontogeny and evolution of prolactin's functions. Fed Proc 39:2563-2566.

39. Lee K-F, Atiee S, Henning S, Rosen J (1989) Relative contribution of promoter and intragenic sequences in the hormonal regulation of rat ß-casein transgenes. Mol Endocrinol 3:447-453.

40. Pittius C, Sankaran L, Topper Y, Hennighausen L (1988) Comparison of the regulation of the whey acid protein gene with that of a hybrid gene containing the whey acid protein gene promoter in transgenic mice. Mol Endocrinol 2:1027-1032.

41. Alila HW, Rogo KO, Gombe S (1987) Effects of prolactin on steroidogenesis by human luteal cells in culture. Fertil Steril 47:947-955.

42. Poindexter AN, Buttram VC, Besch P, Smith RG (1979) Prolactin receptors in the ovary. Fertil Steril 31:273-277.

43. Barkey RJ, Shoni J, Amik J, Barzilai D (1979) Characterization of the specific binding of prolactin to binding sites in the seminal vesicle of the rat. J Endocrinol 80:181-189.

44. Aragona C, Friesen HG (1975) Specific prolactin binding sites in the prostate and testis of rats. Endocrinology 97:677-684.

45. Barthe A, Hafiez AA, Bex FJ, Dalterio S (1978) Hormonal interaction in relation of androgen secretion. Biol Reprod 18:44-54.

46. Slaunwhite WR, Sharma M (1977) Effects of hypophysectomy and prolactin replacement therapy on prostatic response to androgen in orchiectomized rats. Biol Reprod 17:489-492.

47. Vance ML, Thorner MO (1987) Prolactinomas. Endocrinol Metab Clin 16:731-753.

48. Yen SSC (1986) Prolactin in human reproduction. In Yen SSC, Jaffe RB (eds), Reproductive Endocrinology, 2nd Edition, W.B. Saunders Co., Philadelphia, PA. pp 237-261.

49. Nagasawa H, Sakai S, Banerjee MR (1979) Prolactin receptor. Life Sci 24:193-208.

50. Boutin J-M, Jolicoeur C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-Fourt I, Djiane J, Kelly PA (1988) Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. Cell 53:69-72.

51. Bolander FF (1984) Possible roles of calcium and calmodulin in mammary gland differentiation *in vitro* . J Endocrinol 104:29-34.

52. Rillema JA, Etindi RN, Ofenstein JP, Waters SB (1988) In Knobil E, Neill J (eds), Mechanisms of Prolactin Action in the Physiology of Reproduction, Vol. 2. Raven Press Ltd, NY, pp 2217-2235.

53. Walters CA, Daly DC, Chapits J, Kuslis ST, Prior JC, Kusmik WF, Riddick DH (1983) Human myometrium: a new potential source of prolactin. Am J Obstet Gynecol 147:639-644.

54. Chapitis J, Riddick DH, Betz LM, Brumsted JR, Gibson M, Prior JC, Gout PW (1988) Physicochemical characterization and functional activity of fibroid prolactin produced in cell culture. Am J Obstet Gynecol 158:846-853.

55. Daly DC, Walters CA, Prior JC, Kuslis ST, Chapitis J, Andreoli J, Riddick DH (1984) Prolactin production from proliferative phase leiomyoma. Am J Obstet Gynecol 148:1059-1063.

56. Boyd JD, Hamilton WJ (1970) The Human Placenta. W Heffer and Sons Ltd, Cambridge, pp 24-44.

57. Brenner RM, Maslar IA (1988) The primate oviduct and endometrium. In Knobil E, Neill JD (eds), The Physiology of Reproduction, Vol 1. Raven Press, NY, pp 303-331.

58. Berne RM, Levy MN (1988) Physiology, 2nd Edition, C.V. Mosby Co, St Louise, MI., pp 101.

59. Healy DL, Cameron IT (1987) Clinical physiology of the endometrium. In Riddick DH (ed), Reproductive Physiology and Clinical Practice, pp 51-70.

60. Weitlauf HM (1988) Biology of implantation. In Knobil E, Neill JD (eds), The Physiology of Reproduction, Vol 1. Raven Press, NY, pp 231-265.

61. Benirschke K (1986) The endometrium. In Yen SSC, Jaffe RB (eds), Endocrinology, 2nd Edition. WB Saunders Co, Philadelphia, pp 385-406.

62. Bell SC (1985) Comparative aspects of decidualization in rodents and human: cell types, secreted products, and associated function. In Edwards RG, Purdy JM, Steptoe PC (eds), Implantation of the Human Embryo, Academic Press Inc, London, pp 71-122.

63. England MA (1983) Color Atlas of Life Before Birth. Year Book Medical Publishers Inc, Chicago, pp 40-46.

64. Golander A, Hurley T, Barrett F, Hizi A, Handwerger S (1978) Prolactin synthesis by human chorion-decidual tissue: a possible source of prolactin in the amniotic fluid. Science 202:311-312.

65. Riddick DH, Luciano AA, Kusmik WF, Maslar IA (1978) De novo synthesis of prolactin by human decidua. Life Sciences 23:1913-1922.

66. Daly DC, Maslar IA, Riddick DH (1983) Prolactin production during *in vitro* decidualization of proliferative endometrium. Am J Obstet Gynecol 145:672-678.

67. Maslar IA, Riddick DH (1979) Prolactin production by human endometrium during the normal menstrual cycle. Am J Obstet Gynecol 135:751-754.

68. Maslar IA, Kaplan BM, Luciano AA, Riddick DH (1980) Prolactin production by the endometrium of early human pregnancy. J Clin Endocrinol Metab 51:78-83.

69. Maslar IA, Ansbacher R (1986) Effects of progesterone on decidual prolactin production by organ cultures of human endometrium. Endocrinology 118:2102-2108.

70. Huang JR, Tseng L, Bischof P, Janne OA (1987) Regulation of prolactin production by progestin, estrogen, and relaxin in human endometrial stromal cells. Endocrinology 121:2011-2017.

71. Irwin JC, Kirk D, King RJ, Quigley M, Gwatkin RB (1989) Hormonal regulation of human endometrial stromal cells in culture: an *in vitro* model for decidualization. Fertil Steril 52:761-768.

72. McRae MA, Newman GR, Walker SM, Jasani B (1986) Immunohistochemical identification of prolactin and 24K protein in secretory endometrium. Fertil Steril 45:643-648.

73. Bryant-Greenwood GD, Rees MC, Turnbull AC (1987) Immunohistochemical localization of relaxin, prolactin, and prostaglandin synthase in human amnion, chorion and decidua. J Endocrinol 114:491-496.

74. Clements J, Whitfeld P, Cooke N, Healy D, Matheson B, Shine T, Funder J (1983) Expression of the prolactin gene in human decidua-chorion. Endocrinology 112:1133-1134.

75. Takahashi H, Nabeshima Y, Nabeshima Y-I, Ogata K, Takeuchi S (1984) Molecular cloning and nucleotide sequence of DNA complementary to human decidual prolactin mRNA. J Biochem 95:1491-1499.

76. Golander A, Barrett J, Hurley T, Barry S, Handwerger S (1979) Failure of bromocriptine, dopamine, and thyrotropin-releasing hormone to affect prolactin secretion by human decidual tissue *in vitro* . J Clin Endocrinol Metab 49:787-789.

77. Maslar IA, Laur JJ, Norman RL, Spies HG (1988) Amniotic fluid and decidual prolactin during pregnancy in Rhesus Macaques. Biol Reprod 38:1067-1076.

78. Thrailkill KM, Golander A, Underwood LE, Handwerger S (1988) Insulin-like growth factor-I stimulates the synthesis and release of prolactin from human decidual cells. Endocrinology 123:2930-2934.

79. Handwerger S, Capel D, Korner G, Richards R (1987) Purification of decidual prolactin-releasing factor, a placental protein that stimulates prolactin release from human decidual tissue. Biochem Biophys Res Commun 147:452-459.

80. Markoff E, Howell S, Handwerger S (1983) Inhibition of decidual prolactin release by a decidual peptide. J Clin Endocrinol Metab 57:1282-1286.

81. Josimovich JB, Merisko K, Boccella L (1977) Amniotic prolactin control over amniotic and fetal extracellular fluid water and electrolytes in Rhesus monkey. Endocrinology 100:564-570.

82. Bigazzi M, Rouga R, Lancranjan I, Ferraro S, Branconi F, Buzzoni P, Martorana G, Scarselli G, Del Pozo E (1979) A pregnancy in an acromegalic woman during bromocryptine treatment: effects on growth hormone and prolactin in the maternal, fetal, and amniotic compartments. J Clin Endocrinol Metab 48:9-12.

83. Riddick DH, Luciano AA, Kusmik WF, Maslar IA (1979) Evidence for a non-pituitary source of amniotic fluid prolactin. Fertil Steril 31:35-39.

84. Walsh SW, Meyer RK, Wolf RC, Friesen HG (1977) Corpus luteum and fetoplacental functions in monkeys hypophysectomized during late pregnancy. Endocrinology 100:845-850.

85. Fukamatsu Y, Tomita K, Fukata T (1984) Further evidence of prolactin production from human decidua and its transport across fetal membranes. Gynecol Obstet Invest 17:309-316.

86. Rosenberg SM, Maslar IA, Riddick DH (1980) Decidual production of prolactin in late gestation: further evidence for a decidual source of amniotic fluid prolactin. Am J Obstet Gynecol 138:681-685.

87. Ussing HH, Zerahn K (1951) Active transport of sodium as the source of electric current in the short circuited isolated frog skin. Acta Physiol Scand 23:110-127.

88. Riddick DH, Maslar IA (1981) The transport of prolactin by human fetal membranes. J Clin Endocrinol Metab 52:220-224.

89. McCoshen JA, Tagger OY, Wodzicki A, Tyson JE (1982) Choriodecidual adhesion promotes decidual prolactin transport by human fetal membranes. Am J Physiol 243:R552-557.

90. McCoshen JA, Barc J (1985) Prolactin bioactivity following decidual synthesis and transport by amniochorion. Am J Obstet Gynecol 153:217-223.

91. Riddick DH (1985) Regulation and physiological relevance of non-pituitary prolactin. In MacLeod RM, Thorner MO, Scapagnini U (eds), Prolactin. Basic and Clinical Correlates, Fidia Research Series, Vol 1. Liviani Press, Padova, pp 463-473.

92. Herrington AC, Graham J, Healy DL (1980) The presence of lactogen receptors in human chorion laeve. J Clin Endocrinol Metab 51:1466-1468.

93. McWey LA, Singhas CA, Rogol AD (1982) Prolactin binding sites on human chorion-decidua tissue. Am J Obstet Gynecol 144:283-288.

94. Healy DL, Herrington AC, O'Herlithy C (1983) Chronic idiopathic polyhydramnious: evidence for a defect in the chorion laeve receptor for lactogenic hormones. J Clin Endocrinol Metab 56:520-523.

95. Boutin J-M, Edery M, Shirota M, Jolicoeur C, Lesueur L, Ali S, Gould D, Djiane J, Kelly PA (1989) Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. Mol Endocrinol 3:1455-1461.

96. Leontic EA, Tysone JE (1977) Prolactin and fetal osmoregulation: water transport across isolated human amnion. Am J Physiol 232:R124-127.

97. Leontic EA, Schruefer JJ, Andreassen B, Pinto H, Tyson JE (1979) Further evidence for the role of prolactin on human fetoplacental osmoregulation. Am J Obstet Gynecol 133:435-438.

98. Tyson JE, Mowat GS, McCoshen JA (1984) Stimulation of a probable biological action of decidual prolactin on fetal membranes. Am J Obstet Gynecol 148:296-302.

99. Tyson TE, McCoshen JA (1985) Inhibition of fetal membrane prostaglandin production by prolactin. Am J Obstet Gynecol 151:1032-1038.

100. Chapitis J, Betz LM, Brumsted JR, Gibson M, Kuslis ST, McDaniel H, Riddick DH (1989) Observation of production of immunoreactive prolactin by normal human connective tissue in cell culture. In Vitro Cell Develop Biol 25:564-570.

101. Einspanier R, Pitzel L, Wuttke W, Hagendorff G, Preu K-D, Kardalinou E, Scheit K (1986) Demonstration of mRNAs for oxytocin and prolactin in porcine granulosa and luteal cells. FEBS Letters 204:37-40.

102. Khan-Dawood FS (1988) Human corpus luteum: immunocytochemical evidence for presence of prolactin. Cell Tissue Res 251:233-236.

103. Roux M, Martinat N, Richoux J-P, Grignon G (1985) Histochemical identification of a prolactin-like substance in rodent testis. Cell Tissue Res 240:663-667.

104. Fuxe K, Hokfelt T, Eneroth P, Gustafsson J-A, Skett P (1977) Prolactin-like immunoreactivity: localization in nerve terminals of rat hypothalamus. Science 196:899-900.

105. Toubeau G, Desclin J, Parmentier M, Pasteels JL (1979) Compared localization of prolactin-like and adrenocorticotropin immunoreactivities within the brain of the rat. Neuroendocrinology 29:374-384.

106. Toubeau G, Desclin J, Parmentier M, Pasteels JL (1979) Cellular localization of a prolactin-like antigen in the rat brain. J Endocrinol 83:261-266.

107. Hansen BL, Hansen GN, Hagen C (1982) Immunoreactive material resembling ovine prolactin in perikaya and nerve terminals of the rat hypothalamus. Cell Tissue Res 226:121-131.

108. Harlan RE, Shivers BD, Fox SR, Kaplove KA, Schachter BS, Pfaff DW (1989) Distribution and partial characterization of immunoreactive prolactin in the rat brain. Neuroendocrinology 49:7-22.

109. Schachter BS, Durgerian S, Harlan RE, Pfaff DW, Shivers BD (1984) Prolactin mRNA exists in rat hypothalamus. Endocrinology 114:1947-1949.

110. DeVito WJ (1989) Comparison of brain and pituitary immunoreactive prolactin by peptide mapping and lectin affinity chromatography. Endocrinology 125:2439-2444.

111. Liddle GW, Givens JR, Nicholson WE, Island DP (1965) The ectopic ACTH syndrome. Cancer Res 25:1057-1061.

112. DeBold CR, Menefee JK, Nicholson WE, Orth DN (1988) Proopiomelanocortin gene is expressed in many normal human tissues and in tumours not associated with ectopic adrenocorticotropin syndrome. Mol Endocrinol 2:862-870.

113. Oates EL, Allaway GP, Armstrong GR, Boyajian RA, Kehil JH, Prabhakar BS (1988) Human lymphocytes produce pro-opiomelanocortin gene-related transcript. J Biol Chem 263:10041-10044.

114. Elsui T, Nakai Y, Toshihiko T, Jingami H, Takahashi H, Fukata J, Imura H (1988) Expression of adrenocorticotropin-releasing hormone precursor gene in placenta and other non-hypothalamic tissues in man. Mol Endocrinol 2:871-875.

115. Howlett TA, Rees LH (1985) Ectopic hormones. Special Topics in Endocrinol Metab 7:1-44.

116. Baylin SB, Mendelsohn G (1980) Ectopic (inappropriate) hormone production by tumors: mechanisms involved and the biological and clinical implications. Endo Rev 1:45-77.

117. DeBustros A, Baylin SB (1985) Hormone production by tumours: biological and clinical aspects. Clin Endocrinol Metab 14:221-256.

118. Molitch ME, Schwartz S, Mukherji B (1981) Is prolactin secreted ectopically? Am J Med 70:803-807.

119. Ozarda AT (1983) Prolactin secreting tumours. J Surg Oncol 22:9-10.

120. Stanisic TH, Donovan J (1986) Prolactin secreting renal cell carcinoma J Urol 136:85-86.

121. Hoffman WH, Gala RR, Kovacs K, Subramanian MG (1987) Ectopic prolactin secretion from a gonadoblastoma. Cancer 60:2690-2695.

122. Rosen SW, Weintraub BD, Aaronson SA (1980) Non-random ectopic protein production by malignant cells: direct evidence *in vitro* . J Clin Endocrinol Metab 50:834-841.

123. Beck C, Burger HG (1972) Evidence for the presence of immuno-reactive growth hormone in cancers of the lung and stomach. Cancer 30:75-79.

124. Kaganowicz A, Farkouh N, Frantz AG, Blaustein AU (1979) Ectopic human growth hormone in ovaries and breast cancer. J Clin Endocrinol Metab 48:5-8.

125. Greenberg PB, Beck C, Martin TJ, Burger HG (1972) Synthesis and release of human growth hormone from lung carcinoma in cell culture. Lancet 1:350-352.

126. Fusco FD, Rosen SW (1966) Gonadotropin-producing anaplastic large-cell carcinomas of the lung. N Engl J Med 178:507-515.

127. Weintraub BD, Rosen SW (1971) Ectopic production of human chorionic sommatomammotropin by non-trophoblastic cancer. J Clin Endocrinol Metab 32:94-107.

128. Sheth NA, Suraiya JN, Sheth AR, Ranadive KJ, Jussawalla DJ (1977) Ectopic production of human placental lactogen by human breast tumours. Cancer 39:1693-1699.

129. Smith EM, Morrill AC, Meyer WJ, Blalock JE (1986) Corticotropin releasing factor induction of leukocyte-derived immunoreactive ACTH and endorphins. Nature 321:881-881.

130. Smith EM, Phan M, Kruger TE, Coppenhaver DH, Blalock JE (1983) Human lymphocyte production of immuno-reactive thyrotropin. Proc Natl Acad Sci USA 80:6010-6013.

131. Harbour-McMenamin D, Smith EM, Blalock JE (1986) Production of immunoreactive chorionic gonadotropin during mixed lymphocyte reactions: a possible selective mechanism for genetic diversity. Proc Natl Acad Sci USA 83:6834-6838.

132. Weigent DA, Baxter JB, Wear WE, Smith LR, Bost KL, Blalock JE (1988) Production of immunoreactive growth hormone by mononuclear leukocytes. FASEB J 2:2812-2818.

133. Buzzetti R, McLoughlin L, Lavender PM, Clark AJ, Rees LH (1989) Expression of pro-opiomelanocortin gene and quantification of adrenocorticotropin hormone-like immunoreactivity in human normal peripheral mononuclear cells and lymphoid and myeloid malignancies. J Clin Invest 83:733-737.

134. Lacaze-Masmonteil T, DeKeyzer Y, Laton J-P, Kahn A, Bertagna X (1987) Characterization of POMC transcripts in human non-pituitary tissues. Proc Natl Acad Sci USA 84:7261-7265.

135. Morley SD, Ivell R (1987) Vasopressin gene transcripts in the bovine corpus luteum are defective. Mol Cell Endocrinol 53:255-258.

136. Zurawski G, Benedik M, Kamb BJ, Abrams JS, Zurawski SM, Lee FD (1986) Activation of mouse T-helper cells induces abundant preproenkephalin mRNA synthesis. Science 232:772-775.

137. Goetzl EJ, Sreedharan SP, Truck CW (1988) Structurally distinctive vasoactive intestinal peptides from rat basophilic leukemia cells. J Biol Chem 263:9083-9087.

138. Blalock JE (1988) Production of neuroendocrine peptide hormones by the immune system. Prog Allergy 43:1-13.

139. Spangelo BL, MacLeod RM, Isakson PC (1990) Production of interleukin-6 by anterior pituitary cells *in vitro* . Endocrinology 126:582-586.

140. Vankelecom H, Carmeliet P, Van Damme J, Billiau A, Denef C (1989) Production of interleukin-6 by folliculo-stellate cells of the anterior pituitary gland in a histiotypic cell aggregate culture system. Neuroendocrinology 49:102-106.

141. Breder CD, Dinarello CA, Saper CB (1988) Interleukin-1 immunoreactive innervation of the human hypothalamus. Science 240:321-323.

142. Elias JA, Trinchieri G, Beck JM, Simon PL, Sehgal PB, May LT, Kern JA (1989) A synergistic interaction of IL-6 and IL-1 mediates the thymocyte-stimulating activity produced by recombinant IL-1 stimulated fibroblast. J Immunol 142:509-514.

143. Sironi M, Breviario F, Proserpio P, Biondi A, Vecchi A, Van Damme J, Dejana E, Mantovani A (1989) IL-1 stimulates IL-6 production in endothelial cells. J Immunol 142:549-553.

144. Woloski B, Smith EM, Meyer WJ, Fuller GM, Blalock JE (1985) Corticotropin-releasing activity of monokines. Science 230:1035-1037.

145. Besedovsky H, Del Rey A, Sorkin E, Dinarello CA (1986) Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. Science 233:652-654.

146. Sapolsky R, Rivier C, Yamamoto G, Plotsky P, Vale W (1987) Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. Science 238:522-524.

147. Berkenbosch F, Van Oers J, Del Rey A, Tilders F, Besedovsky H (1987) Corticotropin releasing factor-producing neurons in the rat activated by interleukin-1. Science 238:524-526.

148. Tsagarakis S, Gillies G, Rees LH, Besser M, Grossman A (1989) Interleukin-1 directly stimulates the release of corticotropin releasing factor from rat hypothalamus. Neuroendocrinology 49:98-101.

149. Bernton EW, Beach JE, Holaday JW, Smallridge RC, Fein HG (1987) Release of multiple hormones by a direct action of interleukin-1 on pituitary cells. Science 238:519-521.

150. Fukata J, Usui T, Naitoh Y, Nakai Y, Imura H (1989) Effects of recombinant human interleukin-1alpha, -1ß, 2 and 6 on ACTH synthesis and release in the mouse pituitary tumour cell line AtT-20. J Endocrinol 122:33-39.

151. Gottschall PE, Uehara A, Hoffman S, Arimura A (1987) Interleukin-1 inhibits follicle stimulating hormone-induced differentiation in rat gramulosa cells *in vitro*. Biochem Biophys Res Commun 149:502-509.

152. Fukuoka M, Mori T, Taii S, Yasuda K (1988) Interleukin-1 inhibits luteinization of porcine granulosa cells in culture. Endocrinology 122:367-369.

153. Gottschall PE, Katsuura G, Hoffman S, Arimura A (1988) Interleukin-1: an inhibitor of luteinizing hormone receptor formation in cultured rat granulosa cells. FASEB J 2:2492-2496.

154. Rivier C, Vale W (1989) In the rat, interleukin-1 acts at the level of the brain and the gonads to interfere with gonadotropin and sex steroid secretion. Endocrinology 124:2105-2109.

155. Calkins JH, Sigel MM, Nankin HR, Lin T (1988) Interleukin-1 inhibits Leydig cell steroidogenesis in primary culture. Endocrinology 123:1605-1610.

156. Verhoeven G, Cailleau J, Van Damme J, Billiau A (1988) Interleukin-1 stimulates steroidogenesis in cultured rat Leydig cells. Mol Cell Endocrinol 57:51-60.

157. Harris RD, Kay NE, Seljeskog EL, Murray KJ, Douglas SD (1979) Prolactin suppression of leukocyte chemotaxis *in vitro*. J Neurosurg 50:462.

158. Karmali RA, Lauder I, Horrobin DF (1974) Prolactin and the immune response. Lancet 2:106-107.

159. Berczi I, Nagy E, Kovacs K, Horvath E (1981) Regulation of humoral immunity in rats by pituitary hormones. Acta Endocrinol 98:506-513.

160. Nagy E, Berczi I, Friesen, HG (1983) Regulation of immunity in rats by lactogenic and growth hormones. Acta Endocrinol 102:351-357.

161. Berczi I, Nagy E, Asa SL, Kovacs K (1983) Pituitary hormones and contact sensitivity in rats. Allergy 38:325-330.

162. Kelley KW, Brief S, Westly HJ, Novakofski J, Bechtel PJ, Simon J, Walker EB (1986) GH_3 pituitary adenoma cells can reverse thymic aging in rats. Proc Natl Acad Sci USA 83:5663-5667.

163. Russell DH, Matrisian L, Kibler R, Larson DF, Poulos B, Magun BE (1984) Prolactin receptors on human lympohocytes and their modulation by cyclosporine. Biochem Biophys Res Commun 145:692-698.

164. Russell DH, Kibler R, Matrisian L, Larson DF, Poulos B, Magun BE (1985) Prolactin receptors on human T and B lymphocytes: antagonism of prolactin binding by cyclosporine. J Immunol 134:3027-3031.

165. Hiestand PC, Mekler P, Nordmann R, Grieder A, Permmargkol C (1986) Prolactin as a modulator of lymphocyte responsiveness provides a possible mechanism of action for cyclosporine. Proc Natl Acad Sci USA 83:2599-2603.

166. Montgomery DW, Zukoski CF, Shah GN, Buckley AR, Pacholczyk T, Russell DH (1987) Concanavalin A stimulated murine spleenocytes produce a factor with prolactin-like bioactivity and immunoreactivity. Biochem Biophys Res Commun 145:692-698.

167. Too CKL, Cragoe EJ, Friesen HG (1987) Amiloride-sensitive Na^+/H^+ exchange in rat Nb2 node lympohoma cells. Stimulation by prolactin and other mitogens. Endocrinology 121:1512-1520.

168. Tanaka T, Shui RPC, Gout PW, Beer CT, Noble RL, Friesen HG (1980) A new sensitive and specific bioassay for lactogenic hormones: measurement of prolactin and growth hormone in human serum. J Clin Endocrinol Metab 51:1058-1063.

169. Shiu RPC, Elsholtz HP, Tanaka T, Friesen HG, Gout PN, Beer CT, Noble RL (1983) Receptor-mediated mitogenic action of prolactin in a rat lymphoma cell line. Endocrinology 113:159-164.

170. Varma S, Ebner KE (1988) The effect of cyclosporin A on the growth and prolactin binding to Nb-2 rat lymphoma cells. Biochem Biophys Res Commun 156:233-239.

171. Mahajan PB, Ebner KE (1985) Cyclosporin A and rabbit mammary prolactin receptors. Biochem Biophys Res Commun 133:753-758.

172. Harding MW, Handschumacher RE, Speicher DW (1986) Isolation and amino acid sequence of cyclophilin. J Biol Chem 261:8547-8555.

173. Takahashi N, Hayano T, Suzuki M (1989) Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. Nature 337:473-476.

174. Tropschug M, Barthelmess IB, Neupert W (1989) Sensitivity to cyclosporin A is mediated by cyclophilin in Neurospora crassa and Saccharomyces cerevisiae. Nature 342:953-955.

175. Bellusi G, Muccioli G, Ghe C, DiCarlo R (1987) Prolactin binding sites in human erythrocytes and lymphocytes. Life Sciences 41:951-959.

176. Spangelo BL, Hall NR, Goldstein AL (1985) Evidence that prolactin is an immunomodulatory hormone. In MacLeod RM, Thorner MO, Scapagnini U (eds), Prolactin. Basic and Clinical Correlates. Fidia Research Series, Vol 1, Liviana Press, Padova, pp 343-349.

177. Bernton EW, Meltzer MS, Holaday JW (1988) Suppression of macrophage activation and T-lymphocyte function in hypoprolactinemic mice. Science 239:401-404.

178. Spangelo BL, Hall NR, Ross PC, Goldtein AL (1987) Stimulation of *in vivo* antibody production and concanavalin-A induced mouse spleen cell mitogenesis by prolactin. Immunopharmacology 14:11-20.

179. Russell DH, Mills KT, Talamantes FJ, Bern HA (1988) Neonatal administration of prolactin antiserum alters the developmental pattern of T- and B-lymphocytes in the thymus and spleen of BALB/c female mice. Proc Natl Acad Sci USA 85:7404-7407.

180. Spangelo BL, Judd AM, Isakson PC, MacLeod RM (1989) Interleukin-6 stimulates anterior pituitary hormone release *in vitro*. Endocrinology 125:575-577.

181. Powell DJ, Friedman J, Oulette A, Krauter K, Darnell J (1984) Transcriptional and post-transcriptional control of specific messenger RNAs in adult and embryonic liver. J Mol Biol 179:21-35.

182. Maniatis T, Goodbourn S, Fischer JA (1987) Regulation of inducible and tissue-specific gene expression. Science 236:1237-1244.

183. Beato M, Chalepakis G, Schauer M, Slater EP (1989) DNA regulatory elements for steroid hormones. J Ster Biochem 32:737-748.

184. Evans R (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889-895.

185. Beato M (1989) Gene regulation by steroid hormones. Cell 56:335-344.

186. Guarente L (1987) Regulatory proteins in yeast. Ann Rev Genet 21:425-452.

187. Chen W, Struhl K (1988) Saturation mutagenesis of a yeast Lis 3 "TATA element": genetic evidence for a specific TATA binding protein. Proc Natl Acad Sci USA 85:2691-2695.

188. Grosscheld R, Birnstiel ML (1980) Identification of regulatory sequences in the prelude sequences of an H2A histone gene by the study of specific deletion mutants *in vivo* . Proc Natl Acad Sci USA 77:1432-1436.

189. Ghosh PK, Lebowitz P, Frisque RJ, Gluzman Y (1981) Identification of a promoter component involved in positioning the 5' termini of simian virus 40 early mRNAs. Proc Natl Acad Sci USA 78:100-104.

190. Hirokoshi M, Wang KC, Fuji H, Cromlish JA, Weil PA, Roeder RG (1989) Cloning and structure of a yeast gene encoding a general transcription initiation factor TFIID that binds to the TATA box. Nature 341:299-303.

191. Van Dyke MW, Roeder RG, Sawadogo M (1988) Physical analysis of transcription pre-initiation complex assembly on a class II gene promoter. Science 241:1335-1338.

192. Buratowski S, Hahn S, Guarente L, Sharp PA (1989) Five intermediate complexes in transcription initiation by RNA polymerase II. Cell 56:549-561.

193. Klein-Hitpass L, Tsai S, Weigel N, Allan G, Riley D, Rodriguez R, Schader W, Tsai M-J, O'Malley B (1990) The progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable pre-initiation complex. Cell 60:247-257.

194. Fire A, Samuels M, Sharp PA (1984) Interactions between RNA polymerase II, factors, and template leading to acccurate transcription. J Biol Chem 259:2509-2516.

195. Davison BL, Egly J, Mulvihill ER, Chambon P (1983) Formation of stable pre-initiation complexes between eukaryotic class B transcription factors and promoter sequences. Nature 301:680-686.

196. Workman J, Roeder G (1987) Binding of transcription factor TFIID to the major late promoter during *in vitro* nucleosome assembly potentiates subsequent initiation by RNA polymerase II. Cell 51:613-622.

197. Santoro C, Mermod N, Andrews PC, Tjian R (1988) A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature 334:218-224.

198. Raymondjean M, Cereghini S, Yaniv M (1988) Several distinct "CCAAT" box binding proteins coexist in eukaryotic cells. Proc Natl Acad Sci USA 85:757-761.

199. Chodosh LA, Baldwin AS, Carthew RW, Sharp PA (1988) Human CCAAT-binding proteins have heterologous subunits. Cell 53:25-35.

200. Birkenmeier EH, Gwynn B, Howard S, Jerry J, Gordon JI, Landschulz WH, McKnight SL (1989) Tissue-specific expression, developmental regulation and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev 3:1146-1156.

201. Kim S-J, Glick A, Sporn MB, Roberts AB (1989) Characterization of the promoter region of the human transforming growth factor-beta 1 gene. J Biol Chem 264:402-408.

202. Lion GI, Ma D-P, Yang YW, Geng L, Zhu C, Baehr W (1989) Human interstitial retinoid-binding protein. J Biol Chem 264:8200-8206.

203. Visvader J, Verma I (1989) Differential transcription of exon 1 of the human c-fms gene in placental trophoblasts and monocytes. Mol Cell Biology 9:1336-1341.

204. Adler HT, Reynolds PJ, Kelley CM, Sefton B (1988) Transcriptional activation of LCK by retrovirus promoter insertion between two lymphoid-specific promoters. J Virol 62:4113-4122.

205. Hammond GL, Underhill DA, Rykse HM, Smith CL (1989) The human sex hormone-binding globulin gene contains exons for androgen-binding protein and two other testicular messenger RNAs. Mol Endocrinol 3:1869-1876.

206. Sehgal A, Patil N, Chao M (1988) A constitutive promoter directs expression of the nerve growth factor receptor gene. Mol Cell Biol 8:3160-3167.

207. Ingraham HA, Evan GA (1986) Characterization of two atypical promoters and alternate mRNA processing in the mouse Thy-1.2 glycoprotein gene. Mol Cell Biol 6:2923-2931.

208. Otani T, Otani F, Krych M, Chaplin D, Boime I (1988) Identification of a promoter region in the CG-beta gene cluster. J Biol Chem 263:7322-7329.

209. Osumi T, Ishii N, Miyazawa S, Hashimoto T (1987) Isolation and structural characterization of the rat acyl-CoA oxidase gene. J Biol Chem 262:8138-8143.

210. Saito H, Koyama T, Georgopoulous K, Clevers H, Haser W, LeBien T., Tonegawa S, Terhorst C (1987) Close linkage of the mouse and human CD3 gamma-and sigma-chain genes suggests that their transcription is controlled by common regulatory elements. Proc Natl Acad Sci USA 84:9131-9134.

211. Dynan WS, Tjian R (1983) The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell 35:79-87.

212. Kadonaga JT, Carner KR, Masiarz FR, Tjian R (1987) Isolation of a cDNA encoding transcription factor Sp1 and functional analysis of other DNA binding domain. Cell 51:1079-1090.

213. Feng P, Ohlsson M, Ny T (1990) The structure of the TATA-less rat tissue-type plasminogen activator gene. J Biol Chem 265:2022-2027.

214. Smale ST, Baltimore D (1989) The initiator as a transcription control element. Cell 57:103-113.

215. Johnson PF (1990) Transcriptional activators in hepatocytes. Cell Growth Diff 1:47-52.

216. Davis RL, Weintraub H, Lassar AB (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987-1000.

217. Hoeffler JP, Meyer TE, Yun Y, Jameson JL, Habener JB (1988) Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. Science 242:1430-1433.

218. Johnson PF, McKnight SL (1989) Eukaryotic transcriptional regulatory proteins. Ann Rev Biochem 58:799-839.

219. Winoto A, Baltimore D (1989) A novel, inducible and T-cell specific enhancer located at the 3' end of the T cell receptor alpha locus. EMBO J 8:729-733.

220. McDouglass S, Peterson CL, Calame K (1988) A transcriptional enhancer 3' of C-beta 2 in the T cell receptor beta locus. Science 241:205-241.

221. Scotto L, Vaduva PI, Wger RE, Assoiau RK (1990) Type beta-1 transforming growth factor gene expression. J Biol Chem 265:2203-2208.

222. Pettersson S, Cook GP, Bruggemann M, Williams GT, Neuberger MS (1990) A second B cell-specific enhancer 3' of the immunoglobulin heavy-chain locus. Nature 344:165-168.

223. Queen C, Baltimore D (1983) Immunoglobulin gene transcription is activated by downstream sequence elements. Cell 33:741-748.

224. Slater EP, Rabenau O, Karin M, Baxter JD, Beato M (1985) Glucocorticoid receptor binding and activation of a heterologous promoter by dexamethasone in the first intron of the hGH gene. Mol Cell Biol 5:2984.

225. Vu TH, Martin GR, Lee P, Mark D, Wang A, Williams LT (1989) Developmentally regulated use of alternative promoters creates a novel platelet-derived growth factor receptor transcript in mouse teratocarcinoma and embryonic stem cells. Mol Cell Biol 9:4563-4567.

226. Kudrycki KE, Shull GE (1989) Primary structure of the rat kidney band 3 anion exchange protein deduced from a cDNA. J Biol Chem 264:8185-8192.

227. Sakai DD, Helms S, Carlstedt-Duke J, Gustafsson J-A, Rottaran FR, Yamamoto KR (1988) Hormone mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev 2:1144-1154.

228. Levine M, Manley JL (1989) Transcriptional repression of eukaryotic promoters. Cell 59:405-408.

229. Kageyama R, Pastan I (1989) Molecular cloning and characterization of a human DNA binding factor that represses transcription. Cell 59:815-825.

230. Schibler U, Hagenbuchle O, Wellauer P, Pittet A (1983) Two promoters of different strengths control the transcription of the mouse alpha-amylase gene Amy-la in the parotid gland and the liver. Cell 33:501-508.

231. Benyajati C, Spoerel N, Haymerle H, Ashburner M (1983) The messenger RNA for alcohol dehydrogenase in Drosophila melanogaster differs in its 5' end in different developmental stages. Cell 33:125-133.

232. Lopez-Casillas F, Kim K-H (1989) Heterogeneity at the 5' end of rat acetyl-coenzyme A carboxylase mRNA. J Biol Chem 264:7176-7184.

233. Lebacq-Verheyden A-M, Krystal G, Sartor O, Way J, Batley JF (1988) The rat preprogastrin releasing peptide gene is transcribed from two initiation sites in the brain. Mol Endocrinol 2:556-563.

234. Chobert MN, Lahuna O, Lebargy F, Kurauchi O, Darbouy M, Bernardin J, Guellaen G, Barouki R, Laperche Y (1990) Tissue-specific expression of two gamma-glutamyl transpeptidase mRNAs with alternative 5' ends encoded by a single copy gene in the rat. J Biol Chem 265:2352-2357.

235. Sartor O, Gregory FS, Smyth-Templeton N, Power S, Perlmutter RM, Rosen N (1989) Selective expression of alternative lck mRNAs in human malignant cell lines. Mol Cell Biol 9:2983-2988.

236. Bennett UD, Adams SL (1990) Identification of a cartilage-specific promoter within intron 2 of the chick alpha 2 (I) collagen gene. J Biol Chem 265:2223-2230.

237. Streuli M, Saito H (1989) Regulation of tissue-specific alternative splicing: exon specific *cis*-elements govern the splicing of leukocyte common antigen pre-mRNA. EMBO J 8:787-796.

238. Barone MV, Henchcliffe C, Baralle FE, Paolella G (1989) Cell type specific *trans*-acting factors are involved in alternative splicing of human fibronectin pre-mRNA. EMBO J 8:1079-1085.

239. Padgett RA, Graboski PJ, Konarska MM, Seiler S, Sharp PA (1986) Splicing of messenger RNA precursors. Ann Rev Biochem 55:1119-1150.

240. Leff SE, Rosenfeld MG, Evans RM (1986) Complex transcriptional units: diversity in gene expression by alternative RNA processing. Ann Rev Biochem 55:1091-1117.

241. Cooke NE, Ray J, Watson MA, Estes PA, Kuo BA, Liebhaber SA (1988) Human growth hormone gene and the highly homologous growth hormone variant gene display different splicing patterns. J Clin Invest 82:270-275.

242. Masuda N, Watahiki M, Tanaka M, Yamakawa M, Shimizu K, Nagai J, Nakashima K (1988) Molecular cloning of cDNA encoding 20kD variant human growth hormone and the alternative splicing mechanism. Biochem Biophys Acta 949:125-131.

243. Cooke NE, Ray J, Emery JG, Liebhaber SA (1988) Two distinct species of human growth hormone-variant mRNA in the human placenta predict the expression of novel growth hormone proteins. J Biol Chem 263:9001-9006.

244. Hampson RK, Rottman FM (1987) Alternative processing of bovine growth hormone mRNA: non-splicing of the final intron predicts a high molecular weight variant of bovine growth hormone. Proc Natl Acad Sci USA 84:2673-2677.

245. Chen EY, Liao Y-C, Smith DH, Barrera-Saldana H, Gelinas RE, Seeburg PH (1989) The human growth hormone locus: nucleotide sequence, biology, and evolution. Genomics 4:479-497.

246. Levy-Wilson B, Fortier C (1989) Tissue-specific undermethylation of DNA sequences at the 5' end of the human apolipoprotein B gene. J Biol Chem 264:9891-9896.

247. Grainger R, Hazard-Leonards R, Samaha F, Hougan L, Lesk M, Thomsen G (1983) Is hypomethylation linked to activation of delta-crystallin genes during lens development. Nature 306:88-90.

248. Dynan WS (1989) Understanding the molecular mechanism by which methylation influences gene expression. Trends Genet 5:35-36.

249. Holler M, Westin G, Jiricny J, Schaffner W (1988) Spl transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev 2:1127-1135.

250. Watt F, Molloy PL (1988) Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes Dev 2:1136-1143.

251. Elgin SCR (1988) The formation and function of DNase I hypersensitive sites in the process of gene activation. J Biol Chem 263:19259-19262.

252. Usala SJ, Young WS, Morioka H, Nikodem UM (1988) The effect of thyroid hormone on the chromatin structure and expression of the malic enzyme gene in hepatocytes. Mol Endocrinol 2:619-626.

253. Nelson C, Albert VR, Elsholtz HP, Lu L, Rosenfeld MG (1988) Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. Science 239:1400-1405.

254. Gutierrez-Hartmann A, Siddiqui S, Loukin S (1987) Selective transcription and DNase I protection of the rat prolactin gene by GH_3 pituitary cell-free extracts. Proc Natl Acad Sci USA 84:5211-5215.

255. Schuster WA, Treacy MN, Martin F (1988) Tissue-specific *trans*-acting factor interaction with proximal rat prolactin gene promoter sequences. EMBO J 7:1721-1733.

256. Sharp ZD, Helsel S, Cao Z, Barron EA, Sanchez Y (1989) DNA recognition element required for PUF-1 mediated cell-type-specific transcription of the rat prolactin gene. Nucleic Acid Res 17:2705-2722.

257. Kim KE, Day RN, Maurer RA (1988) Functional analysis of the interaction of a tissue-specific factor with an upstream enhancer element of the rat prolactin gene. Mol Endocrinol 2:1374-1381.

258. Ingraham HA, Chen R, Mangalam HJ, Elsholtz HP, Flynn SE, Lin CR, Simmons DM, Swanson L, Rosenfeld MG (1988) A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell 55:519-529.

259. Bodner M, Castrillo J-L, Theill LE, Deerinck T, Ellisman M, Karin M (1988) The pituitary-specific transcription factor GHF-I is a homeobox-containing protein. Cell 55:505-518.

260. Herr W, Sturm R, Clerc R, Corcoran L, Baltimore D, Sharp P, Ingraham H, Rosenfeld MG, Finney M, Ruvkun G, Horvitz H (1988) The POU domain: a large conserved region in the mammalian pit-1, oct-1, oct-2 and Caenorhabditis elegans unc-86 gene products. Genes Dev 2:1513-1516.

261. Sturm RA, Herr W (1988) The POU domain is a bipartite DNA-binding structure. Nature 336:601-604.

262. Theill LE, Castrillo J-L, Wu D, Karin M (1989) Dissection of functional domains of the pituitary-specific transcription factor GHF-1. Nature 342:945-948.

263. Mangalam HJ, Albert VR, Ingraham HA, Kapiloff M, Wilson L, Nelson C, Elsholtz H, Rosenfeld MG (1989) A pituitary POU domain protein, Pit-I, activates both growth hormone and prolactin promoters transcriptionally. Genes Dev 3:946-958.

264. Crenshaw EB, Kalla K, Simmons DM, Swanson LW, Rosenfeld MG (1989) Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergistic interactions between promoter and enhancer elements. Genes Dev 3:959-972.

265. Elsholtz HP, Albert VR, Treacy MN, Rosenfeld MG (1990) A two-base change in a POU factor-binding site switches pituitary-specific to lympohoid-specific gene expression. Genes Dev 4:43-51.

266. Siler-Khodr TM, Morgenstern LL, Greenwood FC (1974) Hormone synthesis and release from human fetal adenohypophyses *in vitro* . J Clin Endocrinol Metab 39:891-905.

267. Lipson LG, Beitins IR, Korablith PL, McArthur JW, Friesen HG, Kliman B, Kjellberg RN (1979) Tissue culture studies on human pituitary tumours: long term release of anterior pituitary hormones into the culture medium. Acta Endocrinol (Copenh) 90:421-433.

268. Kletzy OA, Marrs RP, Rundall TT, Weiss MH, Beierle JW (1980) Monolayer and suspension culture of human prolactin-secreting pituitary adenoma. Am J Obstet Gynecol 138:660-666.

269. Rodgers-Neame NT, Bradley EL, Blackwell RE (1984) Long-term monolayer culture of human anterior pituitary cells: functional validation of a method. Fertil Steril 41:75-80.

270. Kikuchi Y, Seki K, Momose E, Kizawa I, Oomori K, Shima K, Mukai K, Kato K (1985) Establishment and characterization of a new human cultured cell line from a prolactin-secreting pituitary adenoma. Cancer Res 45:5722-5727.

271. Prysor-Jones RA, Silverlight JJ, Jenkins JS (1987) Vasoactive intestinal peptide increases intracellular free calcium in rat and human pituitary tumour cells *in vitro*. J Endocrinol 114:119-123.

272. Hay R, Macy M, Chen TR, McClintock P, Reid Y (1988) American Type Culture Collection Catalogue of Cell Lines and Hybridomas, 6th edition. (Rockville, MA).

273. Lemaigre FP, Peers B, LaFontaine DA, Mathy-Hartert M, Rousseau GG, Belayew A, Martial JA (1989) Pituitary-specific factor binding to the human prolactin, growth hormone and placental lactogen genes. DNA 8:149-159.

274. Cooke NE, Coit D, Weiner RI, Baxter JD, Martial JA (1980) Structure of cloned DNA complementary to rat prolactin mRNA. J Biol Chem 255:6502-6510.

275. Seeburg PH, Shine J, Martial JA, Baxter JD, Goodman HM (1977) Nucleotide sequence and amplification in bacteria of the structural gene for rat growth hormone. Nature 270:486-490.

276. Shine J, Seeburg PH, Martial JA, Baxter JD, Goodman HM (1977) Construction and analysis of recombinant DNA for human chorionic sommatomammotropin. Nature 270:494-499.

277. Rubin CM, Houck CM, Deininger PL, Friedman T, Schmid CW (1980) Partial nucleotide sequence of the 300-nucleotide interspersed repeated human DNA sequence. Nature 284:72-74.

278. Lau, Y-F, Dozy AM, Huang JC, Kan YW (1984) A rapid screening test for antenatal sex determination. Lancet i:14-16.

279. Spence AM, Sheppard PC, Davie JR, Matuo Y, Nishi N, McKeehan WL, Dodd JG, Matusik RJ (1989) Regulation of a bifunctional mRNA results in synthesis of secreted and nuclear probasin. Proc Natl Acad Sci USA 86:7843-7847.

280. Berwaer M, Mathy-Hartert M, Bellefroid E, Morin A, Belayew A, Martial JA (1989) Cloning and sequencing of upstream, potential regulatory regions of the human prolactin gene. Arch Internat Physiol Biochim 97(2) B7.

281. Julkunen M, Seppala M, Janne OA (1988) Complete amino acid sequence of human placental protein 14: a progesterone-regulated uterine protein homologous to beta-lactoglobulins. Proc Natl Acad Sci USA 85:8845-8849.

282. Gout PW, Beer CT, Nobel RL (1980) Prolactin stimulated growth of cell cultures established from malignant Nb rat lymphomas. Cancer Research 40:2433-2436.

283. Stinchcombe U, Jones T, Bradley BA (1985) HLA typing of Epstein-Barr virus transformed lymphoblastoid cell lines (LCL). Tiss Ant 26:161-167.

284. Voller A, Bidwell D, Bartlet A (1976) Microplate enzyme immunoassay for the immunodiagnosis of virus infections. In Rose NR, Friedman H (eds), Manual of Clinical Immunology, American Society for Microbiology, Washington, DC, Chapter 69, p 506.

285. Tolis G, Goldstein M, Friesen HG (1973) Functional evaluation of prolactin secretion in patients with hypothalamic pituitary disorders. J Clin Invest 52:783-788.

286. Haggi E, Aoki A (1981) Prolactin content in rat pituitary gland. RIA of prolactin after different extraction procedures. Acta Endocrinol (Copenh) 97:338-342.

287. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680.

288. Wray W, Boulikas T, Wray VP, Hancock R (1981) Silver staining of proteins in polyacrylamide gels. Anal Biochem 118:197-203.

289. Mandel M, Higa, A (1970) Calcium dependent bacteriophage DNA infection. J Mol Biol 53:154-162.

290. Maniatis T, Fritch EF, Sanbrook J (1982) In Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

291. DiMattia GE (1985) Albumin gene expression by the liver and yolk sac of the developing chicken embryo. Master of Science Thesis, Dalhousie University, Halifax, Nova Scotia.

292. Rigby PWJ, Dreckmann M, Rhodes C, Berg PJ (1977) Labelling deoxyribonucleic acid to high specific activity *in vitro* by translation with DNA polymerase I. J Mol Biol 113:237-251.

293. Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13.

294. Davis LG, Dibner MD, Battey JF (1986) Basic Methods in Molecular Biology, Elsevier, New York.

295. Protter AA, Wang S-Y, Shelness G, Ostapchuk P, Williams DL (1982) Isolation and characterization of a cDNA clone specific for avian vitellogenin II. Nucleic Acid Res 10:4935-4949.

296. Chirgwin J, Pryzbyla A, MacDonald R, Rutter W (1979) Isolation of biologically active ribonucleic acid from sources enriched for ribonuclease. Biochemistry 18:5294-5299.

297. Chromczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid-guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159.

298. Aviv H, Leder P (1972) Purification of biologically active globin mRNA by chromatography on oligo-thymidylic acid cellulose. Proc Natl Acad Sci USA 69:1408-1412.

299. Thomas PS (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201-5205.

300. Melton DA, Krieg P, Rebagliati M, Maniatis T, Zinn K, Green MR (1984) Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage Sp6 promoter. Nucleic Acid Res 12:7035-7070.

301. Sippel AE, Stavrianopoulos J, Schutz G, Feigelson P (1974) Translational properties of rabbit globin mRNA after specific removal of poly(A) with ribonuclease H. Proc Natl Acad Sci USA 71:4635-4639.

302. Huynh TV, Young RA, Davis RW (1985) DNA Cloning: A Practical Approach, 1st Edition. IRL Press, Oxford.

303. Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.

304. Benton WD, Davis RW (1977) Screening lambda GTII recombinant clones by hybridization to single plaques *in situ*. Science 196:180-182.

305. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467.

306. Maxam A, Gilbert W (1980) Sequencing end-labelled DNA with base-specific chemical cleavages. Methods in Enzymology 65:499-560.

307. Fahey JL, Buell DN, Sox HC (1971) Proliferation and differentiation of lymphoid cells: studies with human lymphoid cell lines and immunoglobulin synthesis. Annals NY Acad Sci 189-190:221-234.

308. Lesniak MA, Gorden P, Roth J, Gavin JR (1974) Binding of ^{125}I-human growth hormone to specific receptors in human cultured lymphocytes. J Biol Chem 249:1661-1667.

309. Gavin JR, Roth J, Neville DM, DeMeyts P, Buell DN (1974) Insulin-dependent regulation of insulin receptor concentrations: a direct demonstration in cell culture. Proc Natl Acad Sci USA 71:84-88.

310. Dalla Favera R, Gelman EP, Martinotti S, Franchini G, Paps T, Gallo RC, Wong Stael F (1982) Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). Proc Natl Acad Sci USA 76:6497-6501.

311. Shevach EM, Herberman R, Frank MM, Green I (1972) Receptors for complement and immunoglobulin on human leukemic cells and human lymphoblastoid cell lines. J Clin Invest 51:1933-1938.

312. Paul WE (1984) Fundamental Immunology. Raven Press, New York, NY.

313. Swartwout SG, Preisler H, Guan W, Kinniburgh AJ (1987) Relatively stable population of c-myc RNA that lacks long poly(A). Mol Cell Biol 7:2052-2058.

314. Brilliant MH, Sueoka N, Chiraraishi DM (1984) Cloning of DNA corresponding to rare transcripts of rat brain: evidence of transcriptional and post-transcriptional control and of the existence of nonpolyadenylated transcripts. Mol Cell Biol 4:2187-2197.

315. Dworkin MB, Dworkin-Rash E (1985) Changes in RNA titers and polyadenylation during oogenesis and oocyte maturation in Xenopus laevis. Dev Biol 112:451-457.

316. Shah GN, Montgomery DW, Laird HE, Russell DH (1988) Characterization of a prolactin-like polypeptide (Ly-PLP) required for mitogenesis of Con-A stimulated murine spleenocytes. J Cell Biol 107(6)pt 3, p 505a, abstract 2840, ASCB and ASBMB 1989 Joint Meeting.

317. Woldeyesus J, Cair F, Bernton E (1989) Cultured mouse lymphocytes express RNA message with complementarity to prolactin DNA sequence. J Leukocyte Biol 46:294. Abstract 14, Annual Meeting of the Reticuloendothelial Society.

318. Clevenger C, Russell D, Shipman P, Prystowsky M (1989) IL-2 driven T-cells secrete and require a prolactin-like lymphokine for proliferation. FASEB J 3:A316. Abstract 523, Fed Amer Soc Exp Biol 73rd Annual Meeting.

319. Linzer DIH, Talamantes F (1985) Nucleotide sequence of mouse prolactin and growth hormone mRNAs and expression of these mRNAs during pregnancy. J Biol Chem 260:9574-9579.

320. Lewin B (1987) Genes III, John Wiley & Sons, NY, p 148.

321. Demczuk S, Baumberger C, Mach B, Dayer J-M (1988) Differential effects of *in vitro* mycoplasma infection on interleukin-1 alpha and ß mRNA expression in U937 and A431 cells. J Biol Chem 263:13039-13045.

322. Robinson M-A, Kindt TJ (1989) Major histocompatability complex antigens and genes, Paul WE (ed), chapter 17, Raven Press Ltd, NY.

323. Ferrero D, Rovera G (1984) Human leukemic cell lines. Clin Haematol 13:461-487.

324. Gellersen B, DiMattia GE, Kempf R, Bohnet HG (1989) Ectopically produced prolactin: an autocrine growth factor? Acta Endocrinologica 120(suppl 1):222.

325. Bonhoff A, Gellersen B, Held KR, Bohnet HG (1990) Clonal derivatives of a human B-lymphoblastoid cell line producing prolactin: a cytogenetic characterization. Cytotechnol. 3:43-50.

326. Nilsson K, Klein G (1982) Phenotypic and cytogenetic characteristics of human B-lymphoid cell lines and their relevance for the etiology of Burkitt's lymphoma. Adv Cancer Res 37:319-380.

327. Russell DH (1989) New aspects of prolactin and immunity: a lymphocyte-derived prolactin-like product and nuclear protein kinase C activation. Trends Pharm Sciences 10:40-44.

328. DiMattia GE, Gellersen B, Bohnet HG, Friesen HG (1987) A human B-lymphoblastoid cell line produces prolactin. Endocrinology 122:2508-2517.

329. Gellersen B, DiMattia GE, Friesen HG, Bohnet HG (1989) Phorbol ester stimulates prolactin release but reduces prolactin mRNA in the human B-lymphoblastoid cell line IM-9-P3. Mol Cell Endocrinol 66:153-161.

330. Gellersen B, DiMattia GE, Friesen HG, Bohnet HG (1989) Regulation of prolactin secretion in the human B-lymphoblastoid cell line IM-9-P3 by dexamethasone but not other regulators of pituitary PRL secretion. Endocinology 125:2853-2861.

331. Prysor-Jones RA, Silverlight JJ, Jenkins JS (1989) Estradiol, vasoactive intestinal peptide, and fibroblast growth factor in the growth of human pituitary tumour cells *in vitro* . J Endocrinol 120:171-177.

332. Markoff E, Zeitler P, Peleg S, Handwerger S (1983) Characterization of the synthesis and release of prolactin by an enriched fraction of human decidual cells. J Clin Endocrinol Metab 56:962-968.

333. Goodyer C, St.George Hall C, Guyda H, Robert F, Giroud C (1976) Human fetal pituitary in culture: hormone secretion and response to somatostatin, luteinizing hormone releasing factor, thyrotropin releasing factor and dibutyryl cyclic AMP. J Clin Endocrinol Metab 45:73-85.

334. Snyder J, Hymer WC, Wilfinger WW (1978) Culture of human pituitary prolactin and growth hormone cells. Cell Tissue Res 191:379-388.

335. Bethea CL, Weiner RI (1981) Human prolactin secreting adenoma cells maintained on extracellular matrix. Endocrinol 108:357-360.

336. Adams E, Brajkovich I, Mashiter K (1979) Hormone secretion by dispersed cell cultures of human pituitary adenomas: effects of theophylline, thyrotropin-releasing hormone, somatostatin, and 2-bromo-alpha-ergocryptine. J Clin Endocrinol Metab 49:120-126.

337. Hwang P, Robertson M, Guyda H, Friesen H (1973) The purification of human prolactin from frozen pituitary glands. J Clin Endocrinol Metab 36:1110-1118.

338. Handwerger S, Wilson S, Conn PM (1984) Different subcellular storage sites for decidual- and pituitary-derived prolactin: possible explanation for differences in regulation. Mol Cell Endocrinol 37:83-87.

339. Harman I, Costello A, Ganong J, Bell RM, Handwerger S (1986) Activation of protein kinase C inhibits synthesis and release of decidual prolactin. Am J Physiol 251:E172-E177.

340. Klein B, Jourdan M, Vazquez A, Dugas B, Bataille R (1987) Production of growth factors by human myeloma cells. Can Res 47:4856-4860.

341. DeMeyts P (1976) Insulin and growth hormone receptors in human cultured lymphocytes and peripheral blood monocytes. In Blecher M (ed) Methods in Receptor Research, part 1. Marcel Dekker, NY, pp 301-383.

342. Hintz RL, Clemmons DR, Underwood LE, Van Wyk JJ (1972) Competitive binding of somatomedin to insulin receptors of adipocytes, chondrocytes and liver membranes. Proc Natl Acad Sci USA 69:2351-2353.

343. Lesniak MA, Gorden P, Roth J (1977) Reactivity of non-primate growth hormones and prolactins with human growth hormone receptors on cultured human lymphocytes. J Clin Endocrinol Metab 44:838-849.

344. Brawerman G (1987) Determinants of messenger RNA stability. Cell 48:5-6.

345. Shaw P, Sordat B, Schibler U (1986) Developmental coordination of alpha-amylase and Psp gene expression during mouse parotid gland differentiation is controlled post-transcriptionally. Cell 47:102-112.

346. Robinson BG, Firm DM, Schwartz WJ, Majzoulo JA (1988) Vasopressin mRNA in the suprachiasmatic nuclei: daily regulation of polyadenylate tail length. Science 241:342-344.

347. Muschel R, Khoury G, Reid LM (1986) Regulation of insulin mRNA abundance and adenylation: dependence on hormones and matrix substrata. Mol Cell Biol 6:337-341.

348. Edwards D, Waterhouse P, Holman M, Denhardt DT (1986) A growth-responsive gene (16C8) in normal mouse fibroblasts homologous to a human collagenase inhibitor with erythyroid-potentiating activity: evidence for inducible and constitutive transcripts. Nucleic Acid Res 14:8863-8876.

349. Guggenheim NZ, Permutt MA (1986) Identical transcription initiation sites for proinsulin messenger ribonucleic acid in three insulin-expressing tissues. Endocrinology 118:1710-1715.

350. Gellersen B, DiMattia GE, Freisen HG, Bohnet HG (1989) Prolactin (PRL) mRNA from human decidua differs from pituitary PRL mRNA but resembles the IM-9-P3 lymphoblast PRL transcript. Mol Cell Endocrinol 64:127-130.

351. Ziemiecki A, Muller RG, Xiao-Chang F,. Hynes NE, Kozma S (1990) Oncogenic activation of the human trk proto-oncogene by recombination with the ribosomal large subunit protein L7a. EMBO J 9:191-196.

352. Adams JM, Harris A, Pinkert C, Corcoran L, Alexander W, Cory S, Palmiter R, Brinster R (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318:533-538.

353. Stravenhagen JB, Robins DM (1988) An ancient provirus has imposed androgen regulation on the adjacent mouse sex-limited protein gene. Cell 55:247-254.

354. Martin D-Z, Hughes S, Barbacid M (1986) A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature 319:743-748.

355. Park M, Dean M, Cooper C, Schmidt M, O'Brian S, Blair D, Vande Woude GF (1986) Mechanism of met oncogene activation. Cell 45:895-904.

356. Klein G, Klein E (1985) Myc/Ig juxtaposition by chromosomal translocations: some new insights, puzzles and paradoxes. Immunology Today 6:208-215.

357. Ben-Neriah Y, Daley G, Mes-Masson A, Witte O, Baltimore D (1986) The chronic myelogeneous leukemia specific P210-protein is the product of the BCR/ABL hybrid gene. Science 233:212-214.

358. Tsujimoto Y, Yunis J, Showe L, Erikson J, Nowell P, Croce CM (1984) Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t (11;14) chromosome translocation. Science 224:1403-1406.

359. Tsujimoto Y, Jaffe E, Cossman J, Gorham J, Nowell J, Croce CM (1985) Clustering of breakpoints on chromosome 11 in human B-cell neoplasia with the t (11;14) chromosome translocation. Nature 315:340-343.

360. Liang W, Hopper J, Rowley JD (1979) Karyotypic abnormalities and clinical aspects of patients with multiple myeloma and related paraproteinemic disorders. Cancer 44:630-644.

361. Shadle KM, Meltzer PS, Durie BGM (1988) Somatic mutation alters a critical region of the c-myc gene in multiple myeloma. Clin Res 36:499A.

362. Vorona AF, Adler HT, Sefton BM (1987) Two lck transcripts containing different 5' untranslated regions are present in T cells. Mol Cell Biol 7:4407-4413.

363. Adler HT, Reynold P, Kelley C, Sefton BM (1988) Transcriptional activation of lck by retrovirus promoter insertion between two lymphoid-specific promoters. Mol Cell Biol 62:4113-4122.

364. Teo CG, Griffin BE (1987) Epstein-Barr virus genomes in lymphoid cells: activation in mitosis and chromosomal location. Proc Natl Acad Sci USA USA 84:8473-8477.

365. Rickstein A, Olsson A, Andersson T, Rymo L (1988) The 5' flanking region of the gene for the Epstein-Barr virus-encoded nuclear antigen 2 contains a cell-type specific *cis*-acting regulatory element that activates transcription in transfected B-cells. Nucl Acid Res 16:8391-8342.

366. Shapiro MB, Senapathy P (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nuc Acid Res 17:7155-7175.

367. Gao F, Endo H, Yamamoto M (1989) Length heterogeneity in rat salivary gland alpha-2 microglobulin mRNAs: multiple splice acceptor and polyadenylation sites. Nuc Acid Res 17:4629-4636.

368. Kiss I, Deak F, Holloway R, Delius H, Mebust K, Frimberger E, Argraves W, Tsonis P, Winterbottom N, Goetinck PF (1989) Structure of the gene for cartilage matrix protein, a modular protein of the extracellular matrix. J Biol Chem 264:8126-8134.

369. Danoff A, Shields D (1988) Differential translation of two distinct preprosomatostatin messenger RNAs. J Biol Chem 263:16461-16466.

370. Pelletier J, Sonenberg N (1985) Insertion mutagenesis to increase secondary structure within the 5' noncoding region of a eukaryotic mRNA reduces translational efficiency. Cell 40:515-526.

371. Chitpatima ST, Brawerman G (1988) Shifts in configuration of the 5' noncoding region of a mouse messenger RNA under translational control. J Biol Chem 263:7164-7169.

372. Casey JL, Hentze M, Koeller D, Caughman S, Rouault T, Klausner R, Harford J (1988) Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. Science 240:924-928.

373. Rouault TA, Hentze M, Caughman S, Harford J, Klausner R (1988) Binding of a cytosolic protein to the iron-responsive element of human ferritin messenger RNA. Science 241:1207-1210.

374. Mueller PP, Jackson BM, Miller PF, Hinnebusch AG (1988) The first and fourth upstream open reading frames in GCN4 mRNA have similar initiation efficiencies but respond differently in translational control to change in length and sequence. Mol Cell Biol 108:229-241.

375. Ozawa K, Ayub J, Young N (1988) Translational regulation of B19 parvovirus capsid protein production by multiple upstream AUG triplets. J Biol Chem 263:10922-10926.

376. Kozak M (1989) The scanning model for translation: an update. J Cell Biol 108:229-241.

377. He X, Treacy MN, Simmons D, Ingraham H, Swanson L, Rosenfeld MG (1989) Expression of a large family of POU-domain regulatory genes in mammalian brain development. Nature 340:35-42.

378. Sturm RA, Das G, Herr W (1988) The ubiquitous octamer-binding protein Oct-1 contains a POU-domain with a homeo box subdomain. Genes Dev 2:1582-1599.

379. Frain M, Swart G, Monair P, Nicosia A, Stampfli S, Frank R, Cortese R (1989) The liver-specific transcription factor LF-B1 contains a higly diverged homeobox DNA binding domain. Cell 59:1455-157.

380. Elsholtz HP, Mangalam H, Potter E, Albert UR, Supowit S, Evans RM, Rosenfeld MG (1986) Two different *cis*-active elements transfer the transcriptional effects of both EGF and phorbol ester. Science 234:1552-1557.

381. Day RN, Maurer RA (1989) The distal enhancer region of the rat prolactin gene contains elements conferring response to multiple hormones. Mol Endocrinol 3:3-9.

382. Jackson AE, Bancroft C (1988) Proximal upstream flanking sequence direct calcium regulation of the rat prolactin gene. Mol Endocrinol 2:1139-1144.

383. Huang D-H, Horikoshi M, Roeder RG (1988) Activation of the adenovirus EIIa late promoter by a single point mutation which enhances binding of transcription factor IID. J Biol Chem 263:12596-12601.

384. Zajac-Kaye M, Gelmann E, Levens D (1988) A point mutation in the c-myc locus of a Burkitt lymphoma abolishes binding of a nuclear protein. Science 240:1776-1780.

385. McCormick A, Wu D, Castrillo J-L, Dana S, Strobl J, Thompson E, Karin M (1988) Extinction of growth hormone expression in somatic cell hybrids involves expression of the specific *trans*-activator GHF-1. Cell 55:379-389.

386. Tripputi P, Guerin S, Moore DD (1988) Two mechanisms for the extinction of gene expression in hybrid cells. Science 241:1205-1207.

387. Brasier AR, Philippe J, Campbell D, Habener JF (1986) Novel expression of the angiotensionogen gene in a rat pancreatic islet cell line. J Biol Chem 261:16148-16154.

388. Ozturk M, Bellet D, Isselbacher K, Wands J (1987) Ectopic beta-human chorionic gonadotropin production by a human hepatoma cell line (FOCUS): isolation and immunochemical characterization. Endocrinology 120:559-566.

389. Sausville E, Carney D, Battey J (1985) The human vasopressin gene is linked to the oxytocin gene and is selectively expressed in a cultured lung cancer cell line. J Biol Chem 260:10236-10241.

390. Cosgrove DE, Campain JA, Cox GS (1989) Chorionic gonadotropin synthesis by human tumour cell lines: examination of subunit accumulation, steady state levels of mRNA, and gene structure. Biochim Biophys Acta 1007:44-54.

391. Arceci RJ, Shanahan F, Stanley ER, Pollard JW (1989) Temporal expression and location of colony stimulating factor 1 (CSF-1) and its receptor in the female reproductive tract are consistent with CSF-1 regulated placental development. Proc Natl Acad Sci USA 86:8818-8822.

392. Uzumaki H, Okanabe T, Saskai N, Hagiwara K, Takaku F, Tobita M, Yasukawa K, Ito S, Umezawa Y (1989) Identification and characterization of receptors for granulocyte colony-stimulating factor on human placenta and trophoblastic cells. Proc Natl Acad Sci USA 86:9323-9326.

393. Casey ML, Cox S, Beutler B, Mitewich L, MacDonald PC (1989) Cachectin/tumour necrosis factor-alpha formation in human decidua. J Clin Invest 83:430-436.

394. Boehm KD, Kelley M, Ilan J, Ilan J (1989) The interleukin 2 gene is expressed in the syncytiotrophoblast of the human placenta. 86:656-660.

395. Shorter SC, Vince G, Starkey PM (1989) The identification of mRNA for the IL-3-related cytokines in placental and decidual tisssue. Placenta 10:486.

396. Vince GS, Shorter S, Starkey P, Sargent I, Redman SWG (1989) Cytokine production by human cell decidual macrophages and cytotrophoblast cells. Placenta 10:487.

UNIVERSITY OF MANITOBA
FACULTY COMMITTEE ON THE USE OF HUMAN SUBJECTS IN RESEARCH

NAME: Mr. Gabriel DiMattia OUR REFERENCE: E88:199

DATE: January 24, 1989

YOUR PROJECT ENTITLED:

Maternal Decidual Tissue/Prolactin Study.

HAS BEEN APPROVED BY THE COMMITTEE AT THEIR MEETING OF:

January 23, 1989.

COMMITTEE PROVISOS OR LIMITATIONS:

Approved as per correspondence dated December 16th from Dr. F. Manning and January 11th from Dr. Alguacil-Garcia.

You will be asked at intervals for a status report. Any significant changes of the protocol should be reported to the Chairman for the Committee's consideration, in advance of implementation of such changes.

** This is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

J. P. Maclean, M.D.,
Chairman,
Faculty Committee on the Use of Human Subjects in Research

JPM/11

TELEPHONE ENQUIRIES:
788-6376 - Lorraine Lester