THE UNIVERSITY OF MANITOBA

STRUCTURAL CHARACTERIZATION OF COMPLEMENTARY DNA CLONES TO RAT PLACENTAL LACTOGEN II MESSENGER RNA

by

KATHRYN LEE KIRK

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"... we consist of the whole existence of the world, each one of us, and just as our body bears in it the various stages of our evolution back to the fish and further back still, we have in our soul everything that has ever existed in the human mind ..."

Pistorius in Herman Hesse's Demian

To

My friends and family

for their support and encouragement

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LIST OF ABBREVIATIONS

PEPTIDE HORMONES

CG	Chorionic Gonadotropin
GH	Growth Hormone
LH	Luteinizing Hormone
PRL	Prolactin
PL	Placental Lactogen
FSH	Follicle-Stimulating Hormone

OTHER PEPTIDES

PLP-A	Prolactin-Like Protein A
PLF	Proliferin
PRP	Proliferin-Related Protein
MRP	Mitogen Regulated Protein

PREFIX TO HORMONES

h	human
o	ovine
b	bovine
r	rat
m	mouse

UNITS OF MEASURE

^{o}C	Degrees Centigrade
cpm	Counts per minute
g	Gram
mg	Milligram

UNITS OF MEASURE (cont'd.)

ug	Microgram
ng	Nanogram
M	Molar
mM	Millimolar
pmol	Picomole
N	Normal
cc	Cubic Centimetre
l	Litre
ml	Millilitre
ul	Microlitre
u	Unit
Ci	Curie
uCi	Microcurie
xg	X Gravitational Force
rpm	Revolutions per minute
cm	Centimetre
mm	Millimetre
um	Micrometre
nm	Nanometre
A_{580}	Optical Density Determined by Spectrophotometry at 580 nm
A_{260}	Optical Density Determined by Spectrophotometry at 260 nm

UNITS OF MEASURE (cont'd.)

A_{280}	Optical Density Determined by Spectrophotometry at 280 nm
mA	Milliamp
V	Volt
sec	Second
min	Minute
hr	Hour
wk	Week
bp	Base Pair
kb	Kilobase
mw	Molecular Weight
kd	Kilodalton
MYA	Million Years Ago
PAM	Accepted Point Mutation
UEP	Unit Evolutionary Period

ASSAYS

RRA	Radioreceptor Assay
RIA	Radioimmunoassay

REAGENTS

^{32}P-	denotes radioactive phosphorus labeled molecule
^{35}S-	denotes radioactive sulphur labeled molecule

REAGENTS (cont'd.)

TCA	Trichloroacetic Acid
EtBr	Ethidium Bromide
TEMED	N, N, N', N'-tetramethylethylene diamine
EDTA	Disodium Ethylene Diamine Tetraacetate
EtOH	Ethanol
dH_2O	distilled, deionized H_2O
TE	Tris-EDTA Buffer
$CHCl_3$	Chloroform
SDS	Sodium Dodecyl Sulphate
PEG	Polyethylene Glycol
CsCl	Cesium Chloride
CIP	Calf Intestinal Phosphatase
IPTG	Isopropyl-beta-thio-galactopyranoside
BCIG	Bromo-chloro-indoyl-galactoside
DTT	Dithiothreitol
BSA	Bovine Serum Albumin
NC	Nitrocellulose
DH	Denhardt's Solution
TBE	Tris Borate Electrophoresis Buffer
AGB	Agarose Gel Buffer

MISCELLANEOUS

DNA	Deoxyribonucleic Acid
cDNA	Complementary DNA
SSDNA	Single Stranded DNA
DSDNA	Double Stranded DNA
RFDNA	Replicative Form of DNA
RNA	Ribonucleic Acid
mRNA	Messenger RNA
tRNA	Transfer RNA
dNTP	Deoxynucleotide Triphosphate
ddNTP	Dideoxynucleotide Triphosphate
G	Guanine
A	Adenine
T	Thymine
C	Cytosine
UV	Ultra-violet
NH_2	Amino
pI	Isoelectric Point

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ABSTRACT

STRUCTURAL CHARACTERIZATION OF COMPLEMENTARY DNA CLONES TO RAT PLACENTAL LACTOGEN II MESSENGER RNA. K. L. Kirk, Dept. of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada.

Placental lactogen (PL), growth hormone (GH), and prolactin (PRL) form a set of mammalian hormones known as the PRL-GH gene family. The three hormones have evolved from a common ancestral gene by gene duplication. The PL's, PRL's, and GH's share similar biological, immunological and structural properties. The hormones, PRL and GH are produced by the anterior pituitary while PL is produced by the placenta. Human PL is well-characterized and has been found to be remarkably similar to hGH. Little is known about PL's in other species.

In the rat, two forms of rPL exist. The large molecular weight form, found at mid-pregnancy, is called rPL I while the small molecular weight form produced by the late pregnant placenta, is called rPL II. In order to facilitate studies on the structure and regulation of rPL II, cDNA clones to rPL II mRNA were isolated from late pregnant rat placenta.

In this study, the primary structure of rPL II was determined and compared to other members of the PRL-GH gene family. The rPL II cDNA clones were mapped with restriction

enzymes and subcloned into M13 vectors. The nucleotide sequence of the recombinant subclones was determined via the Sanger dideoxy chain termination method utilizing [^{35}S] and buffer gradient sequencing gels. The nucleotide sequence data was analyzed by computer.

The nucleotide sequence of the rPL II cDNA clones translated into a single open reading frame. The cDNA showed a slight preference for G or C in the codon third position, like PRL's but the termination codon and 3' untranslated region were unique. Rat PL II contains a hydrophobic signal sequence characteristic of secreted proteins. At the nucleotide level, rPL II is approximately 60% homologous to the PRL's but only 40% homologous to the GH's and hPL. Rat PL II is 40% homologous to the PRL's and only 20% homologous to the GH's and hPL at the amino acid level. Rat PL II contains four half-cystine residues like the GH's but the coding region terminates at the final half-cystine residue like the PRL's. The two tryptophan residues which are highly conserved in the PRL's are found in rPL II. Evolutionary analysis revealed that rPL II is more closely related to the PRL's, unlike the GH-like hPL. Further, rPL II evolved from the PRL's earlier in mammalian evolution than the gene duplication of hGH that produced hPL. Therefore, PL's have arisen at least twice in the evolution of placental mammals.

I. INTRODUCTION

A. The Placenta As An Endocrine Organ

During pregnancy, dramatic endocrine readjustments occur. At the turn of the century, investigators suggested that the placenta acted as a new endocrine organ.

Bouchacourt (1902) first postulated that the placenta acted as an endocrine organ during pregnancy since newborn infants of either sex occasionally secreted "witch's milk" from their mammary glands and women treated with sow placental extracts lactated. Halban (1904; 1905; 1909) later suggested that the endocrine changes that occurred during pregnancy could be attributed to the appearance of a new endocrine organ, the placenta, rather than to the altered function of pre-existing endocrine glands.

In 1913, Aschner reported the presence of luteotropic activity in placental extracts. Later, Ascheim and Zondek (1927) described large amounts of a potent gonadotropin in the urine of pregnant women. The substance, which they called "prolan", was capable of producing follicular growth and luteinization in immature mouse ovaries. The hormone, now known as chorionic gonadotropin (hCG), was the first placental protein hormone to be identified and characterized.

Human CG belongs to a class of glycoprotein hormones, which includes pituitary luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyrotropin (TSH). The hormones share similar structural, biological and immunological properties.

Human CG has a molecular weight of 36-40 K daltons (Got and Bourrillon, 1960). Approximately 30% of its weight is due to carbohydrate (Bahl et al., 1972). The hormone is made up of two dissimilar subunits , known as the α-subunit and β-subunit (Swaminathan and Bahl, 1970). The α-subunit is shared by all the members of the class of glycoprotein hormones. Although there are minor differences in the carbohydrate composition, the α-subunits are functionally interchangeable (Pierce et al., 1971). The specificity of the hormones lies in the unique β-subunits. The hCG β-subunit is unlike any of the other β-subunits.

The major function attributed to hCG is the stimulation of steroid production by the corpus luteum, placenta and the fetoplacental unit (Chatterjee and Munro, 1977a).

Placental gonadotropins have been reported in other primates (Simpson, 1945; Hampton and Hampton, 1965; Tullner and Hertz, 1966) but none have been isolated. Although there have been several reports of hCG-like placental hormones in subprimates (Forsyth, 1974), pregnant mare

serum gonadotropin (PMSG) is the only subprimate gonadotropin to be isolated and purified (Bourrillon and Got, 1959; Gospodarowicz, 1972).

Following the discovery of "prolan" or hCG by Ascheim and Zondek (1927), Madruzza (1927) suggested that the placenta contained substances other than gonadotropins, since implantation of placental homografts in virgin guinea pigs caused a lactational response. In 1936, Ehrhardt was the first investigator to demonstrate prolactin-like activity in human placental extracts.

Studies on the rodent during the 1930's revealed that the placenta played a crucial role in the second half of gestation. Pencharz and Long (1933) found that rats hypophysectomized before day 11 of pregnancy or ovariectomized at any time during gestation, aborted or resorbed the fetuses. Newton and Beck (1939) obtained similar results in mice. Selye et al. (1934; 1935) discovered that the placenta was essential for mammary gland development and post-partum lactation in rodents. In 1938, Astwood and Greep found that rat placental tissue maintained pseudopregnancy in rats.

In the following decades, the synthesis of steroid hormones by the placenta, alone, and in collaboration with the fetus, was well-characterized (Diczfalusy, 1953).

Studies on the role of the placenta in peptide hormone production subsided after the 1930's. The investigation of placental endocrinology was impeded due to the unique characteristics of the placenta. Classical techniques, such as ablation and replacement treatment, were not feasible even though the extirpation of other endocrine organs had led to the first evidence of the endocrine functions of the placenta. Also, as purified hormones became available, emphasis was placed on the study of the pituitary, adrenals, gonads and their target organs.

In the 1960's, interest in placental endocrinology was rekindled. Fukushima (1961) demonstrated growth hormone-like activity in human placental extracts. Subsequently, Kurosaki (1961), Ito and Higashi (1961) and Higashi (1961) found evidence of prolactin-like activity in human placental tissue, confirming Ehrhardt's (1936) earlier observations. However, it was the discovery of a substance in human term placenta and retroplacental blood which immuno-chemically cross-reacted with antiserum to the pituitary hormone, hGH, by Josimovich and MacLaren (1962), that led to the revival of interest in the function and distribution of mammalian placental hormones.

The substance isolated by Josimovich and MacLaren, had PRL-like activity in the pigeon crop sac assay and

stimulated epiphyseal growth in hypophysectomized rats with about 1% of the potency of hGH.

Kaplan and Grumbach (1964) confirmed the report and further demonstrated that the hGH-like antigen was present in the placental syncytiotrophoblast. Therefore, they called the protein "human chorionic growth hormone-prolactin" (hCGP). The hormone has also been called human chorionic somatomammotropin (hCS), human chorionic mammotropin (hCM), human placental prolactin, and human purified placental protein (PPH). In this thesis, the hormone will be called human placental lactogen.

By exploiting the immunological cross-reaction between human placental extracts and antisera to hGH, several investigators isolated and purified hPL to homogeneity (Kaplan and Grumbach, 1965; Friesen, 1965).

Human PL is a single chain polypeptide with a molecular weight of 21,600 daltons (Florini et al., 1966; Andrews, 1969; Li, 1970). The protein is composed of 191 amino acids. The complete amino acid sequence has been determined (Catt et al., 1967; Li et al., 1971; Niall, 1971; Niall et al., 1971; Sherwood et al., 1971). The amino and carboxyl termini are occupied by valine and phenylalanine, respectively. There are four half-cystine residues, at positions 53, 65, 181, and 188, which form two

intramolecular disulfide bonds. The protein contains no carbohydrate or lipid. Human PL is formed as a precursor and subsequently processed to the 21,600 dalton form observed in maternal serum (Boime et al., 1976; Birken et al., 1977).

Human GH and hPL are remarkably similar. Human GH and hPL share a common molecular weight and amino acid composition. Comparisons of the complete amino acid sequence of hPL and hGH have revealed that 85% (167/191) of the amino acid residues are identical (Niall et al., 1971, 1973; Sherwood, 1967; Sherwood et al., 1971; Niall, 1972; Li et al., 1971). Of the 24 remaining residues, 18 are "highly acceptable replacements" (Bewley and Li, 1974). The number and location of half-cystine residues for disulfide bond formation are identical. Although the chemical similarity extends throughout the entire polypeptide chain, there is an even greater degree of identity at the carboxy terminus. Wallis and Davis (1976) have observed that hPL resembles hGH to a greater extent than any other known mammalian GH resembles hGH. Human PL is also homologous to hPRL, although the homology is not as great as that seen between hPL and hGH (Li et. al., 1971; Bewley and Li, 1974).

Based on the comparison of the amino acid sequences of hPL, hGH, bGH and oPRL, Niall (Niall et al., 1971; Niall, 1972) and Aloj et al. (1972) have hypothesized that PRL, GH and PL form a family of hormones that have arisen from a common ancestor.

The three hormones are related immunochemically, functionally, structurally, and evolutionarily. However, they do differ in the temporal pattern of appearance and site of synthesis and secretion. Bewley and Li (1974) postulated that a common ancestral gene duplicated and gave rise to prolactin and another ancestral polypeptide, the latter subsequently gave rise to growth hormone and hPL through another gene duplication and subsequent mutation of each independent gene. It is more likely that the more primitive polypeptide was similar to prolactin, since prolactin is found throughout vertebrate species (Nicoll, 1980). Dayhoff et al. (1975), proposed that hPL arose by a relatively recent duplication of the hGH gene since it is so similar to hGH at the amino acid level.

In addition to hCG and hPL, several other peptides have been found in the human placenta and periplacental tissues (Table 1). Some of these peptides are analogous to pituitary hormones and hypothalamic releasing factors. Others have no known hormonal functions. The physiological

Table 1. Peptides Associated with Human Pregnancy

Peptides Hormones:	Pituitary/Hypothalamic Analog	Comments	References
Placental lactogen (hPL)	PRL/GH	22,000 dalton single chain	
Chorionic gonadotropin (hCG)	LH	23,000 daltons glycoprotein (α and β subunits)	
Placental growth hormone (hpGH)	GH	presence reported	Hennen et al. (1985a; b)
Decidual prolactin (hdPRL)	PRL	PRL mRNA localized to decidua	Clements et al. (1983)
Chorionic thyrotropin (hCT)	TSH	reportedly isolated	Hennen (1965)
Chorionic follicle-stimulating hormone (hCFSH)	FSH	activity detected in placental extracts	Tojo et al. (1975)
Adrenocorticotropin (ACTH)	ACTH	found as part of a large precursor	reviewed by Simpson and MacDonald (1981)
Chorionic β-lipotropin (hCβ-LP)	β-LP		
Chorionic β-endorphin (hCβ-endorphin)	β-endorphin		
α-Melanocyte stimulating hormone (α-MSH)	α-MSH		
Oxytocin (OX)	OX	presence reported	reviewed by Saxena (1971)
Relaxin	Relaxin		
Vasopressin (VP)	VP		
Renin			
Vasoactive intestinal peptide (VIP)	VIP	observed on placental ribosomes	Munro (1980)
Nerve growth factor (NGF)	NGF	reportedly isolated	Goldstein et al. (1978)
Uterotropic placental hormone (uTPH)	--	isolated from placenta and serum; caused uterine growth in mice	Beas et al. (1975)
Thyrotropin releasing factor (TRF)	TRF	substances cross-reacting immunologically with hypothalamic TRF and LHRF reported in cytotrophoblast	Gibbons et al. (1975)
Luteinizing hormone releasing factor (LHRF)	LHRF		
Non-Hormones:			
Placental alkaline phosphatase (PAP)	--	isolated from placenta; heat-stable, 116,000 dalton glycoprotein	Fishman and Ghosh (1967)
Pregnancy-associated plasma proteins (PAPP's)	--	found in pregnant plasma	Lin et al. (1974)
Pregnancy Zone protein (PZP)	--	found in non-pregnant women but increase during pregnancy; localized to placental blood vessel walls	Lin and Halbert (1976)

roles of these peptides in pregnancy are unknown. Gibbons et al. (1975) have suggested that releasing factors found in the cytotrophoblast may locally regulate peptide hormone secretion by the syncytiotrophoblast in an arrangement analogous to the hypothalamic control of pituitary peptide hormone secretion.

B. Placental Lactogens

Following the identification, purification and characterization of PL in primates, PL's were identified in a variety of subprimate species by classical bioassay methods. However, with the development of radioreceptor assays (RRA) using membrane receptors for PRL and lactogenic hormones from the mammary gland (Shiu et al., 1973) and GH from pregnant liver (Tsushima and Friesen, 1973), PL's were identified and quantitated in a large number of subprimates (Kelly et al., 1976). By utilizing RRA's, PL's were isolated from the rat (Robertson and Friesen (1975), sheep (Handwerger et al., 1976; Chan et al., 1976) and cow (Bolander and Fellows, 1976a). The occurrence of subprimate PL's is shown in Table 2.

In the subprimates, PL's have been purified from the cow, goat, sheep, rat, and mouse (Table 2). Rabbit PL was reportedly purified by Bolander and Fellows (1976b),

TABLE 2. Distribution of Placental Lactogens

Species	Qualitative* Assay	Response	Quantitative*	Purified
Human	pigeon crop sac	+	RRA	Josimovich and MacLaren (1962)
	in vitro mouse mammary gland	+	RIA	Friesen (1965)
	growth	+		
Monkey	pigeon crop sac	+	RIA	Shome and Friesen (1971)
	growth	+	RRA	
Baboon	in vitro mouse mammary gland	+	RIA	Josimovich et al. (1973)
	luteotropic (mice)	+		
Cow	in vitro mouse mammary gland	+	RRA	Bolander and Fellows (1976a); Beckers et al. (1980);
			RIA	Eakle et al. (1982); Murthy et al. (1982);
			Nb_2 lymphoma cell[1]	Arima and Bremel (1983)
Goat	in vitro mouse mammary gland	+	RRA	Grissom et al. (1975); Becka et al. (1977)
Sheep	in vitro mouse mammary gland	+	RRA	Handwerger et al. (1974); Martal et al. (1975);
	growth (rats)	+	RIA	Chan et al. (1976); Hurley et al. (1977);
				Reddy and Watkins (1978a)
Deer	in vitro mouse mammary gland	+		
Pig	in vitro mouse mammary gland	-	RRA(-)	
Horse	in vitro mouse mammary gland	-		
Rabbit	in vitro mouse mammary gland	-	RRA(+/-)	Bolander and Fellows (1976b)
Dog	in vitro mouse mammary gland	-	RRA(+/-)	
Ferrett	in vitro mouse mammary gland	-		
Guinea Pig	in vitro mouse mammary gland	+	RRA	
Hamster	in vitro mouse mammary gland	+	RRA	
Chinchilla	in vitro mouse mammary gland	+		
Vole	in vitro mouse mammary gland	+		
Rat	pigeon crop sac	+	RRA	Kelly et al. (1975)
	in vitro rat mammary	+	RIA[2]	Robertson and Friesen (1975)
	in vitro mouse mammary gland	+	Nb_2 lymphoma cell[3]	
	luteotropic	+		
Mouse	in vivo mouse mammary	+	RRA	Colosi et al. (1982)
	in vitro mouse mammary gland	+	RIA[4]	
	luteotropic	+		

*From Kelly et al. (1976); Kelly (1977); Talamantes et al. (1980)
[1]From Schellenberg and Friesen (1982)
[2]From Robertson and Friesen (1981)
[3]From Robertson et al. (1982)
[4]From Soares et al. (1982)

however rabbit placenta was not stimulatory in the in vitro mouse mammary gland assay (Forsyth, 1974; Talamantes, 1975) and no activity was detected in pregnant rabbit serum in the RRA-PRL (Kelly et al., 1973b).

Although hPL and other primate PL's cross-react with antisera to pituitary GH (Josimovich and MacLaren, 1962; Kaplan and Grumbach, 1964; Josimovich et al., 1973), none of the subprimate PL's studied so far has cross-reacted with antisera to pituitary GH. Gusdon et al. (1970) reported that placental extracts from the rat, dog, pig, horse, sheep, rabbit and cow cross-reacted with antiserum to hPL in a hemagglutination inhibition assay, however this report remains unconfirmed.

Of the few subprimate PL's isolated, most are single chain polypeptides with molecular weights of 20-23,000 daltons and 2-3 intrachain disulfide bonds. Bovine PL and the early forms of rat and mouse PL are exceptions. Bovine PL has a molecular weight of 35,000 daltons (Arima and Bremel, 1983) and the early forms of rodent PL are approximately 40,000 daltons (Robertson et al., 1982; Soares et al., 1982). The pI's of the subprimate PL's range from 5.7 to 8.8. Several isoforms of bPL (Arima and Bremel, 1983) and rat PL (Robertson et al., 1982) have been

reported. The amino acid composition of the subprimate PL's resemble the composition of pituitary PRL and GH.

Ovine and rabbit PL's contain 6 half-cystine residues and 2 tryptophan residues, like pituitary PRL (Hurley et al., 1977a; Bolander and Fellows, 1976b). Bolander and Fellows (1976a) initially reported that bPL contained 6 half-cystine residues and 2 tryptophans, like PRL's. Later, Arima and Bremel (1983) reported that bPL contained only 4 half-cystine residues, like GH. A partial amino terminal sequence of the early form of mouse PL has been determined (Linzer et al., 1985). Mouse PL II does not contain the two NH_2-terminal half-cystine residues seen in PRL's. Hurley et al. (1977) reported that the amino acids found at COOH-terminal of oPL were identical to oGH (Cys-Ala-Phe-OH).

Secretion of Placental Lactogens

The secretion of PL's varies during the course of gestation and patterns of release differ among species. Human PL is first observed in the syncytiotrophoblast at day 18 of pregnancy (Beck, 1970). The hormone is first seen in maternal serum between days 20 to 40 (Grumbach et al., 1968). The serum concentrations increase linearly until week 32 of gestation and then plateau for the

remainder of the pregnancy. Human PL is the most abundant protein synthesized by the third trimester placenta (Kaplan et al., 1968). The term placenta produces 1-2 grams of hPL per day. The concentration of hPL in serum at term is 5-10 micrograms per millilitre (Grumbach et al., 1968).

No diurnal variations in hPL secretion have been observed (Gaede and Norgaard-Pederson, 1974; Spellacy et al., 1971), although plasma levels undergo frequent spontaneous fluctuations (Vigneri et al., 1975). Twin pregnancies result in higher levels of hPL in the blood than simplex pregnancies (Spellacy et al., 1978).

The majority of hPL is secreted into the maternal circulation. Fetal plasma levels of hPL are 1% of maternal levels (Kaplan and Grumbach, 1965).

Kelly et al. (1976) found in a number of species (hamster, goat, sheep, monkey, human) that the serum concentration measured by RRA-PRL began to rise at or before midpregnancy and remained elevated until term. In other species, PL levels gradually declined after peaking just beyond midpregnancy (guinea pig) or showed two peaks of activity at mid- and late-pregnancy (rat, mouse). There have been conflicting reports on bPL concentrations in maternal serum, however using the Nb_2 lymphoma cell bioassay, Schellenberg and Friesen (1982) have demonstrated

that maternal serum bovine PL is absent or present in only very low concentrations (< 1 ng/ml). Fetal serum bPL levels were between 5 and 22 ng/ml at about six months gestation. Thus, fetal levels were considerably higher than maternal levels, suggesting that bPL may be involved in fetal growth. Chan et al. (1976) have shown that fetal peripheral serum concentrations of oPL were higher than maternal levels between days 50 and 80 of pregnancy.

The mechanisms which regulate PL secretion are not known, although it is probably not autonomous. The rate of PL secretion is not simply a function of placental mass since serum concentrations of hPL do not correlate with placental mass (Grumbach et al., 1968) and PL secretion in rats and mice is phasic (Kelly et al., 1976) while placental mass increases throughout pregnancy. There is indirect evidence that hPL secretion is regulated by specific control mechanisms.

Prostaglandins (Ylikorkala and Pennanen, 1973), epinephrine, norepinephrine (Belleville et al., 1978), ions (Choy and Watkins, 1976), and hormones (Talamantes et al., 1980) have been shown to modify hPL synthesis and release experimentally, yet the physiological regulation is not understood. Unlike pituitary PRL, PL's do not appear to be regulated by dopamine (Martal and Djiane, 1977).

Biological Activities of Placental Lactogens

The biological activities attributed to PL's appear to vary among species. PRL-like and GH-like activities have been demonstrated but interpretation of the studies of the biological actions of these hormones is further complicated by heterologous bioassays of crude preparations. The pituitary hormones, GH and PRL, vary functionally from species to species (Nicoll, 1982).

Pituitary PRL levels are low during pregnancy (Kelly et al., 1976) and the PRL-like PL's may be taking over specific functions of the pituitary hormone during pregnancy. The PL's may be involved in mammary gland development in preparation for postpartum lactation, regulation of ovarian steroidogenesis for pregnancy maintenance and promotion of fetal growth (Chatterjee and Munro, 1977a).

Human PL is mammotropic and lactogenic in experimental animals (Josimovich and MacLaren, 1962; Li, 1972; Chatterjee and Munro, 1977b; Ways et al., 1979; Friesen, 1966) and competes for the same receptors as hPRL in the rabbit mammary gland RRA (Shiu et al., 1973). However, hPL has not been shown to contribute to normal mammary development in humans. All of the subprimate PL's investigated

have demonstrated lactogenic activity in bioassays and the RRA-PRL (Table 2).

Human PL appears to be luteotropic in rats (Josimovich et al., 1963) and mice (Talamantes et al., 1977) but hPL alone does affect steroidogenesis in women during the luteal phase of the cycle (Stock et al., 1971). Monkey and baboon PL's exert luteotropic effects in mice (Josimovich et al., 1973; 1974). Rodent PL's have luteotropic properties (Blank et al., 1977). Administration of oPL to pseudo-pregnant rats prevents the loss of LH receptors in the corpus luteum and the $PGF_{2\alpha}$ induced drop in progesterone. (Chan et al., 1980). [^{125}I]-OPL binds specifically to corpus luteum membrane fractions (Chan et al., 1976). Therefore, oPL may affect ovarian function. However, in the pregnant ewe, no relationship has been found between progesterone levels and changes in oPL concentrations (Kelly et al., 1974a).

Human PL has growth-promoting activity in hypophysectomized rats but it is much less potent than hGH (Josimovich and Brande, 1964; Li, 1972; Friesen, 1965). The somatotropic activity of hPL in humans is unclear but it is generally agreed that hPL has very little growth-promoting activity in humans (Josimovich, 1968; McGarry and Beck, 1972).

Ovine PL exhibits much greater somatotropic activity in rats than hPL. In hypophysectomized rats, oPL had 1.5 times the somatotropic activity of bGH in terms of body weight gain (Chan et al., 1974; Handwerger et al., 1974) and tibial width increase (Blank et al., 1977). In the RRA-GH, oPL was equipotent to hGH (Chan et al., 1974; Martal and Djiane, 1977). Ovine PL binds in the RRA-GH, when human liver is the source of receptor (Carr and Friesen, 1976). No other non-primate GH's have been shown to bind to human receptors. Non-primate GH's are not growth-promoting in man. Ornithine decarboxylase (ODC) activity is stimulated by oPL in the liver of late fetal and neonatal rats (Hurley et al., 1979).

The PL's may play a role in the metabolic adjustments that occur during pregnancy. There is indirect evidence that like hGH, hPL may affect FFA (Talamantes, 1975), and glucose (Tyson et al., 1971b) levels in the maternal serum thereby promoting fetal growth. In sheep, oPL causes a decrease in plasma FFA's, glucose and amino nitrogen levels (Handweger et al., 1975). This is opposite to effects seen in humans following administration of hPL and hGH (Grumbach et al., 1968).

Hurley et al. (1977) have suggested that subprimate PL's have arisen from PRL, not GH like hPL, since they

appear to resemble PRL's more than GH's structurally and biologically.

C. Rat and Mouse Placental Lactogens

Placental lactogens in the rat and mouse will now be discussed in some detail. The foundation for PL research was laid with the early studies in the pregnant rodent. Pencharz and Long (1933) found that hypophysectomy during the second half of gestation did not terminate the pregnancy. Hypophysectomy in the first half of pregnancy or ovariectomy at any time led to abortion or fetal resorption. Newton and Beck (1939) obtained similar results in mice. In 1938, Astwood and Greep demonstrated that rat placental tissue stimulated the corpus luteum to secrete progesterone and thus, pseudopregnancy was maintained. They attributed the luteotropic activity to fetal placental tissue.

Selye et al. (1933) concluded that the pituitary gland was not essential for the maintenance of pregnancy, since in their experiments normal pregnancy and parturition took place in hypophysectomized rats. In subsequent experiments, Selye et al. (1934; 1935) suggested that the placenta was important for mammary growth in the second half of pregnancy in the rat since hypophysectomy and

fetectomy did not affect mammary gland development. Mammary development and transient lactation at parturition was observed by several investigators in rats (Pencharz and Long, 1933; Lyons, 1944; Leonard, 1945) and mice (Selye et al., 1933, 1934; Newton and Beck, 1939; Newton and Richardson, 1941; Gardner and Allen, 1942), following hypophysectomy at mid-pregnancy. In rats and mice, the placenta was known to be important for mammary development, since fetectomy or ovariectomy did not prevent mammary gland development unless a functional placenta was not retained (Newton and Beck, 1939; Lyons, 1944; Leonard, 1945).

In Lyon's experiments (Lyons, 1944), synergism between ovarian steroids and rat placental extracts, in stimulating mammary lobulo-alveolar growth and lactation, was demonstrated. Subsequent studies confirmed the role of the mid-pregnant rat placenta in mammary development. Canivenc (1951) demonstrated that day 12 placental tissue was luteotropic and mammogenic. The placental tissue from day 12 stimulated the proliferation of pigeon crop-sac epithelium. Ray et al. (1955) showed that day 12 rat placenta was luteotropic, mammotropic, lactogenic and weakly crop-sac stimulating.

Mayer and Canivenc (1950) reported that autografts of placenta onto the intestinal mesenteries of mid-term hysterectomized rats maintained mammary gland development.

Foulds (1949; 1954; 1956) observed accelerated mammary tumor growth in mice during pregnancy. Cerruti and Lyons (1960) found that mid-pregnant mouse placenta had mammotropic and lactogenic effects, similar to those in rats.

After hypophysectomy and fetectomy at day 12 of pregnancy, corpus luteum function was maintained in mice (Deanesly and Newton, 1941). Removal of the placenta led to corpus luteum degeneration with or without the presence of the pituitary.

Averill et al. (1950) found that pregnant rats abort or resorb the fetuses if rat placental extracts were injected. However, ovariectomy and hypophysectomy led to the termination of pregnancy and placental extracts could not overcome this effect. Averill and associates reported that the highest amount of luteotropic activity was present in day 12 placental tissue. These early studies demonstrated that the placenta played a critical role in mammary development and luteotropin production in the second half of gestation.

Following the isolation of hPL in 1962 (Josimovich and MacLaren, 1962), interest in rodent PL was revived. Matthies (1967) confirmed the work of the early investigators. Rat placenta possessed a lactogenic substance which he called rat chorionic mammotropin (rCM). The highest luteotropic and lactogenic activity appeared in maternal serum at day 12. Cohen and Gala (1969) corroborated Matthies report.

Desjardins et al. (1968) found that fetectomy on day 16 of pregnancy had no effect on the weight, DNA or RNA content of mammary glands removed on day 21. Complete abortion reduced mammary weight to control non-pregnant levels by day 21. Nagasawa and Yanai (1971) found a positive correlation in mice between the number of placentae and fetuses and the mammary development at day 19 of gestation. Placentas and fetuses were removed at day 8 of gestation. Grafts of mouse placenta over mammary glands of female virgin mice primed with estrogen and progesterone had mammotropic effects (Kohmoto and Bern, 1970).

As already mentioned, Shiu et al. (1973) discovered two peaks of activity in pregnant rodent serum in the RRA-PRL. The second peak, appearing in the serum at late pregnancy had not been reported previously. Matthies

(1974) and Talamantes (1975) later confirmed the report of Shiu and his associates.

Kelly et al. (1975) further characterized the two peaks of lactogenic activity in the serum and placental tissues of pregnant rats. Maximal activity in serum in the RRA-PRL occurred between days 11-13 and days 17-21 of pregnancy. In placental tissue, peak concentrations of lactogen were found between days 15 and 17. By gel filtration, the early and late peaks of activity had different molecular weights. The electrophoretic mobility was different too. Day 12 serum rPL had a half-time disappearance rate of 19.5 minutes whereas rPL in the serum from days 17-21 had a disappearance rate of 1.2 minutes.

The heterogeneity of rPL was perplexing. In the RRA-PRL, peak lactogenic activity occurred between days 15 and 17 (Kelly et al., 1975). Matthies (1967; 1974) reported that day 12 placental extracts had the highest luteotropic and lactogenic activity but he found no luteotropic activity after day 14. The heterogeneity could not be resolved in the RRA-PRL since the assay did not measure luteotropic activity. Kelly et al. (1975) suggested that two forms of rPL, with different bioactivities, could exist.

Robertson and Friesen (1975) partially purified the rPL in day 17-19 placenta. They reported a 1300-fold purification based on the bioactivity of the material in the RRA-PRL. The partially purified rPL was 41% as active as oPRL and 169% as active as hPL, in the RRA-PRL. The late pregnant rPL had a molecular weight of 22,000 by SDS-acrylamide gel electrophoresis and 18,000 by gel filtration on Sephadex G-100, whereas Robertson et al. (1980) reported that the predominant rPL in mid-pregnant rats had a molecular weight of 40,000-50,000 daltons.

In 1981, Robertson and Friesen developed a RIA to the rPL in late pregnant rat placenta. No PL's, PRL's or GH's looked at cross-reacted in the RIA. By RIA, only the 20,000 dalton rPL found in late pregnant rat serum was detected. The 40,000 to 50,000 dalton form from mid-pregnancy did not cross-react. Robertson and Friesen (1981) suggested that since the two forms differed in immuno-reactivity, they could be unrelated, distinct hormones.

Two Forms of rPL

Robertson et al. (1982) further characterized the two forms of rPL. They called the large molecular weight form, found at mid-pregnancy, rPL I while the small molecular

weight form in late pregnant rats, was called rPL II. The different molecular weights of the two forms of rPL were confirmed on SDS-PAGE (Robertson et al., 1982). Upon isoelectric focusing, rPL I had an isoelectric point of 4.5. The rPL II existed in three isoforms with PI values of 6.0, 6.2 and 6.4. Both rPL's were active in the Nb_2 lymphoma cell bioassay (Tanaka et al., 1980) for lactogenic hormones. The lactogenic effects of rPL II in the bioassay were blocked by antisera to rPL II. The levels of rPL I could be measured specifically in the Nb_2 lymphoma cell bioassay by adding antiserum to rPRL and rPL II because rPL I does not crossreact with either antisera. In this sensitive bioassay, rPL I was first detected in the serum at day 8 of pregnancy, peaked at day 12 and disappeared after day 15.

Robertson et al. (1982) compared the activities of the two rPL's in the rabbit mammary and pregnant rat liver RRA's. Rat PL I was 3 times more active than rPL II in the rat liver RRA whereas rPL II was 4 times more active than rPL I in the rabbit mammary RRA.

Thus, rPL was shown to exist in two forms with different patterns of appearance, half-time disappearance rates, molecular weights, electrophoretic mobilities, and immunoreactivity (Robertson et al., 1982). The two forms

may also differ in biological function. A summary of the differences between rPL I and rPL II is shown in Table 3.

Mouse PL

Mouse PL II was purified by Colosi et al. (1982) from day 14-18 placenta. An 1840-fold purification was reported. Mouse PL II is a single chain polypeptide with a molecular weight of 23,000 daltons. The isoelectric point is 7.1. The protein is relatively hydrophobic. The mPL II did not cross-react in RIA's for mPRL or mGH. In the RRA-PRL, mPL II was 150% more active than oPRL. In the pigeon crop-sac lactogenic assay, mPL II was equipotent to oPRL.

Using a RIA for mPL II, Soares et al. (1982) reported that the mPL present in mid-pregnant serum did not cross-react in the RIA, although it was detected by RRA-PRL.

Mouse PL II has been localized to the cytoplasm of the trophoblastic giant cells of mouse placentae at days 10, 12, 15 and 18 of gestation by immunocytochemical techniques using anti-mPL II antibodies (Hall and Talamantes, 1984).

In 1984, using the mPL II RIA, Soares et al. detected mPL II in the serum and placenta of pregnant Snell and Ames dwarf mice. Although these animals are deficient in GH and PRL, mPL II levels were similar to levels in normal mice. The PL in the dwarf mice was immunologically, electro-

TABLE 3

Characteristics of the two forms of rPL

Type of rPL	early rPL I	late rPL II
Days of gestation	8-14	12-21
Serum $t_{\frac{1}{2}}$ (min)	19.5	1.2
Mol. Wt.		
Sephadex G-100	40-50 K	20 K
SDS-PAGE	40 K	20 K
Isoelectric point	4.5	6.2
Activity		
RIA (rPL II)	non-reactive	active
Nb_2 lymphoma	active	active
$\frac{\text{Rabbit mammary RRA}}{\text{Rat liver RRA}}$ ratio	0.35	4.2

*from Robertson et al., 1982.

phoretically, biochemically and biologically identical to the mPL II found in normal pregnant mice.

The partial NH_2-terminal amino acid sequence of mouse PL II has been determined (Linzer et al., 1985) and is shown in Figure 1. The partial amino acid sequence of the secreted protein showed that mPL, unlike PRL's, does not contain the two NH_2-terminal half-cystine residues.

Secretion of Rat PL

Placental and serum levels of PL fluctuate throughout gestation in rats. Rat PL I is present in the serum from days 8 to 14 of gestation and rPL II from days 12 to 21 (Robertson et al., 1982).

Croze and Robertson (1985) studied the secretion of rPL by primary cell cultures of placenta-decidua (PD). The PD cells from day 10 of pregnancy secreted rPL I exclusively. Whereas day 13 PD cells secreted rPL II. Day 16 PD cells secreted only rPL II. Soares et al. (1985) studied the secretory products of rat trophoblast giant cells *in vitro* and found that rPL I and rPL II were secreted by the same cells. Day 10 trophoblast cells secreted rPL I, whereas day 11 trophoblast cells secreted rPL II, predominantly. Thus, production shifted between day 10 and day 11 of pregnancy. The cultured cells did not shift from

Figure 1. Amino-Terminal Amino Acid Sequence of mPL.

Leu-Pro-Asn-Tyr-Arg-Leu-Pro-Thr-Glu-Ser-Leu-Try-Gln-Arg-Val-Ile-Val-Val-Ser

1 10 19

From Linzer et al., 1985.

production of rPL I to rPL II *in vitro*, which led the authors to suggest that other factors are required for the change.

Regulation of rPL synthesis and secretion

The secretion of rPL I and rPL II is not simply a function of placental mass, since levels of the rPL's in serum increase and decrease during pregnancy (Shiu et al., 1973) as placental mass increases.

Indirect evidence indicates that neither form of rPL is regulated by dopamine, as pituitary PRL is. Kisch and Shelsnyak (1968) showed that administration of ergocornine to rats after day 7 of pregnancy inhibited pituitary PRL secretion. The pregnancy progressed normally suggesting rPL secretion was not affected.

Regulation of rPL I synthesis and secretion

In vitro, rat trophoblastic tissue, obtained from blastocyst outgrowths, secreted rPL I as early as day 6 of gestation (Glasser and McCormack, 1980). However, trophoblast cells which were adherent to decidual tissue *in vivo* failed to secrete rPL I until day 10. Based on this study, Jayatilak et al. (1985) suggested that decidual tissue, possibly decidual luteotropin, inhibited the

secretion of rPL I.

Regulation of rPL II synthesis and secretion

Klindt et al. (1981, 1982) monitored serum rPL II levels in late pregnancy by RIA. Minute to minute variations in the serum levels suggested that, like pituitary GH and PRL, rPL II was secreted in an episodic, pulsatile pattern. The authors postulated that dynamic control of rPL II secretion was occurring, perhaps by fetal, maternal or local factors.

The pituitary may inhibit rPL II secretion since hypophysectomy of rats at day 14 of pregnancy causes an increase in serum rPL II levels, above normal pregnant levels, by day 16 (Daughaday et al., 1979).

Fetal factors appear to stimulate rPL II secretion whereas ovarian factors are inhibitory. In 1981, Robertson and Friesen reported that serum rPL II concentrations increased exponentially as the fetal number increased, to a maximum of nine fetuses.

Robertson et al. (1984a) further investigated the role of the fetus and ovaries in the regulation of rPL II secretion. Fetectomy at day 14 of pregnancy decreased serum rPL II levels. Fetectomy and ovariectomy at day 14 led to an increase in serum rPL II concentrations. A

concurrent decrease in placental weight was observed. Therefore, the decrease in rPL II levels, seen with fetectomy alone, was not due to a decrease in placental mass. Rats, fetectomized and ovariectomized at day 14, were treated with pimozide and 17 β-estradiol. RPL II levels decreased, while rPRL levels increased. Hemi-fetectomy at day 17 led to a rise in rPL II levels whereas total fetectomy led to a rapid fall in rPL II. Hemi-fetectomy in conjunction with hemi-hysterectomy also led to a rapid fall in serum rPL II. From these ablation studies, Robertson et al. (1984a) concluded that the fetuses were required for the normal increase in rPL II levels in late pregnancy.

Robertson et al. (1984b) carried out further ablation experiments to study the regulation of rPL II secretion. Adrenalectomy and unilateral ovariectomy had no effect on serum rPL II concentrations. Hypophysectomy at mid-pregnancy caused an increase in rPL II levels in late pregnancy, confirming the observations of Daughaday et al. (1979). Bilateral ovariectomy at day 14 or 16 of pregnancy led to an increase in serum rPL II. Ovariectomy in combination with adrenalectomy did not decrease the rPL II levels to those seen after ovariectomy alone. Ovariectomy, combined with progesterone and estrone or 17 β-estradiol

treatment, resulted in an increase in rPL II levels as well as retention of the conceptuses. These studies suggested that ovarian factors inhibited rPL II secretion whereas adrenal and fetal factors were stimulatory.

Biological Roles of rPL I and rPL II

Like PRL, the rat PL's appear to play multiple roles during pregnancy involving regulation of the corpus luteum, mammary gland development and possibly, maternal metabolic changes and fetal growth.

In the human, the placental peptide, hCG, stimulates production of progesterone by the corpus luteum (Chatterjee and Munro, 1977a). Human PL does not appear to act as a luteotropin in humans. In the rat, a hormone similar to hCG has not been found although there have been several unconfirmed reports that a placental gonadotropin exists (Haour et al., 1976; Cheng, 1975).

The earliest studies in the rat by Pencharz and Long (1933) and Astwood and Greep (1938) demonstrated that a placental hormone played a crucial role in the regulation of corpus luteum function for the maintenance of the second half of pregnancy.

The luteotropic requirements of the rat corpus luteum change during its lifespan and include a variety of luteotropic factors. The initial formation of corpora lutea requires the stimulatory surges of pituitary LH and PRL on proestrous (McLean and Nikitovitch-Weiner, 1973). The newly formed corpus luteum is autonomous for a short time (Uchida et al., 1969). Then, the luteotropic requirements change and pituitary hormones become involved again.

Prolactin plays the key role in maintaining corpus luteum function for all of pseudopregnancy and the first half of normal pregnancy (MacDonald and Greep, 1968; Smith et al., 1976). Mating or cervical stimulation induces two daily surges of PRL. (Freeman et al., 1974); one surge, called nocturnal, occurs in the early morning and the second, called diurnal, occurs in the early evening (Freeman and Neill, 1972; Smith et al., 1975). In pseudopregnancy, the surges cease by day 11 or 12, the day before the next cycle begins, and pseudopregnancy is terminated (Smith and Neill, 1976). In normal pregnancy, diurnal and nocturnal PRL surges are last seen on days 9 and 11, respectively (Smith and Neill, 1976). The biphasic surges of PRL are essential for the maintenance of elevated progesterone until day 9, of pseudopregnancy (Lam and Rothchild, 1977; Rothchild et al., 1974) and day 7 of

normal pregnancy (Madhwa Raj and Moudgal, 1970; McNeilly et al., 1978; Shelesnyak, 1955) when the luteotropic requirement for PRL is lost and LH becomes the major luteotropic stimulus.

The LH is required for the support of the corpus luteum between days 8 and 11 (Ford and Yoshinaga, 1975). When hypophysectomy is performed after days 7, 8 or 9 of gestation, LH alone can maintain pregnancy (Yang et al., 1973; Alloiteau and Bouhours, 1965; Moudgal, 1969; Yoshinaga et al., 1972; Lyons and Ahmad, 1973).

MacDonald and Greep (1970) and Yoshinaga et al. (1972) have reported that LH requires the conceptus, possibly placental factors, to exert a luteotropic effect. Since the maintenance of LH receptors on the corpora lutea requires either PRL or rPL (Gibori and Richards, 1978) and the switch from corpora lutea dependence on PRL to LH occurs at the same time as rPL I levels increase (Shiu et al., 1973), rPL I may be required for the exertion of luteotropic actions by LH.

By day 12 of pregnancy, the pituitary is no longer required for the maintenance of progesterone secretion by the corpus luteum (Pencharz and Long, 1933). Rat PL I may act as the primary luteotropic agent at mid-pregnancy.

In the RRA-PRL, Glaser et al. (1984) found that rPL I binds preferentially to rat ovarian PRL receptors over rabbit mammary gland receptors. They also reported that rPL I was able to sustain ovarian progesterone production at levels high enough to ensure fetal survival.

There are other luteotropic substances which co-exist with rPL I in day 12 serum. However, PRL, LH and FSH are present at levels lower than 30 ng/ml (Cheng, 1976; Morishige et al., 1973; Merchant, 1974). Estrogen levels are also very low (0.3 ng/ml) in ovarian venous plasma (Yoshinaga et al., 1969). These hormones are not present in quantities sufficient to produce the luteotropic effects observed in day 12 serum (Blank et al., 1977) and synergism between these hormones has not been found (Takayama and Greenwald, 1973).

Recently, Jayatilak et al. (1985) characterized another PRL-like luteotropic factor secreted by decidual tissue which they called decidual luteotropin. Decidual luteotropin appears in the decidual tissue of pseudo-pregnant rats at day 6. Levels peak by day 9 and then decline until termination of the pseudopregnancy.

The decidual tissue of the human maternal placenta secretes PRL (Golander et al., 1978b; Riddick et al., 1978). However, the majority of the hPRL from the decidua

is secreted into the amniotic fluid, not the maternal circulation (Bigazzi et al., 1979a) and appears to play a role in the regulation of amniotic fluid volume.

A decidual luteotropin was purified from day 9 pseudopregnant rat decidual tissue extracts (Jayatilak et al., 1985). The protein, like PRL, has a molecular weight of 23,500 daltons. Decidual luteotropin is heat labile, trypsin sensitive and contains disulfide bond. Decidual luteotropin does not cross-react with antisera to rPRL or oPRL (Gibori et al., 1974).

Rat decidual luteotropin reaches the ovaries via the peripheral circulation and affects luteal and follicular function (Castracane and Rothchild, 1976). The factor binds to ovarian PRL receptors (Jayatilak et al., 1985). In vivo (Rothchild and Gibori, 1975) and in vitro (Terranova, 1980a, 1980b), decidual luteotropin increases the rate of progesterone secretion. Luteal production of progesterone is maintained by decidual luteotropin when PRL secretion is blocked (Gibori et al., 1974; Castracane and Rothchild, 1976; Basuray and Gibori, 1980).

The loss of corpora luteal dependence on PRL occurs 24 hours after implantation in pregnancy and 24 hours after the induction of decidualization in pseudopregnancy. At this time, decidual luteotropin appears (Gibori et al.,

1974; Castracane and Rothchild, 1976; Basuray and Gibori, 1980). However, decidual luteotropin is not capable of maintaining luteal progesterone production in the absence of the pituitary (Gibori et al., 1981). Between day 8 and day 11 of pseudopregnancy, decidual luteotropin and LH appear to synergize (Gibori and Kalison, 1982).

The physiological role of decidual luteotropin is unknown. Jayatilak et al. (1985) have suggested that decidual luteotropin exerts local effects upon placental function and fetal growth. The decidual tissue and trophoblast cells have PRL receptors (Williams et al., 1978; McWey et al., 1982; Herrington et al., 1980). Decidual luteotropin competes for PRL receptors, in decidual tissue, with oPRL in a dose-dependent manner (Kelly et al., 1975). Decidual luteotropin may inhibit the secretion of rPL I until its appearance at day 10 since trophoblastic tissue cultured without decidual tissue secreted rPL I as early as day 6 (Glasser and McCormack, 1980).

In addition to its luteotropic actions, rPL II has been implicated as the factor causing the cessation of the PRL surges at mid-pregnancy. Rat PL I levels are increasing at the time the PRL surge terminates (Kelly et al., 1975). The termination of the nocturnal PRL surge on

day 8 or 9 of pregnancy can be delayed if the implantation of the blastocyst is delayed (Yogev and Terkel, 1978). Tonkowciz and Voogt (1983a) reported a correlation between the delayed termination of the surges and the delayed rise in rPL I levels found when implantation was delayed. In hysterectomized pregnant rats, nocturnal PRL surges are extended suggesting the uterine-placental tissue inhibits PRL secretion (Voogt, 1980).

Removal of the conceptuses leads to a proportional decrease in rPL I levels and a concomitant extension of the PRL surges (Voogt et al., 1982). Tonkowicz et al. (1983b) described an inverse relationship between the first rPL I peak on the afternoon of day 11 and the cessation of the nocturnal PRL surges. Tonkowicz and Voogt (1984) confirmed the inverse correlation between rPL I and PRL levels on days 11 and 12. When rPL I levels in the serum were reduced by fetectomy and/or ovariectomy, the PRL surges continued beyond the normal time of termination.

Although injections of placental extracts into male rats inhibited the tonic secretion of PRL (Laherty et al., 1983), injections of rat placental extracts into pregnant rats are not capable of terminating the PRL surges (Smith and Neill, 1976). Voogt (1984) found that PRL secretion by anterior pituitary cells, co-cultured with Day 11

placentae, was inhibited. The author concluded that rPL I may exert a negative feedback directly upon the anterior pituitary.

Linzer and Talamantes (1985) studied the expression of mRNA's during pregnancy, while isolating and characterizing the cDNA clones of mouse pituitary PRL and GH mRNA's. Like in the rat, serum PRL and GH levels change markedly during mouse pregnancy. The PRL levels, at mid-gestation, decrease at least 10-fold and increase shortly before parturition (Soares and Talamantes, 1984). The GH levels increase transiently by a factor of 10. Linzer and Talamantes (1985) found that mPRL and mGH remained at a constant level from day 10 to day 18 of gestation. They concluded that the regulation of PRL and GH serum concentrations was a translational, post-translational or stability mechanism.

There is no evidence that rPL II is luteotropic although it may act as a luteolysin. No luteotropic activity has been found in rat placenta beyond mid-pregnancy (Averill et al., 1950; Matthies, 1967; Linkie and Niswender, 1973; Ray et al., 1955). When day 12 rat serum and placental extracts were size fractionated by gel filtration, only proteins, in the 25,000-50,000 dalton molecular weight range, had luteotropic activity (Matthies,

1967; Cohen and Gala, 1965; Linkie and Niswender, 1973). Rat PL II has a higher activity in the mammary gland RRA-PRL than rPL I (Robertson et al., 1982) and is more active in the mammary gland RRA-PRL than the pregnant rabbit liver RRA-PRL (Robertson et al., 1982) suggesting the mammary gland is its primary target organ. In addition, Tabarelli et al. (1982) found that injections of day 18 rat placental extracts did not cause gonadotropic or luteotropic responses in immature rats or mice.

Several investigators have suggested that rPL may have luteolytic effects. Malven (1969) has demonstrated that PRL is luteotropic and luteolytic in the rat depending upon the time in the oestrus cycle. During gestation, LH and PRL levels remain low (Cheng, 1976). Before parturition, PRL levels increase dramatically and LH levels increase slightly. Prolactin acts as a luteolysin prior to delivery (Rothchild et al., 1973; Malven and Sawyer, 1966).

Matthies (1967) has shown that rPL is luteolytic, depending upon the time of gestation and state of the ovaries. Matthies (1974) suggested that rPL, possibly in combination with PRL, could cause the lysis of refractive luteal tissue. Therefore, rPL II, which peaks during late pregnancy (Shiu et al., 1973; Kelly et al., 1975) could act as a luteolysin.

In addition to the early studies by Selye et al. (1934; 1935) and Pencharz and Long (1933) which suggested that rPL played a lactogenic/mammotropic role during pregnancy, there is a great deal of direct evidence that both forms of rPL act as mammotropins/lactogens (Shani et al., 1970; Anderson, 1975; Bussman et al., 1983; Peters and van Marle, 1976; Bussmann and Deis, 1984). Both rPL I and rPL II bind to rabbit mammary gland receptors in the RRA-PRL (Kelly et al., 1976). Rat PL II has a greater affinity for PRL binding sites in the mammary gland RRA-PRL than rPL I and prefers PRL binding sites in the mammary gland over those in the liver. Therefore, rPL II may act principally as a mammotropin/lactogen during pregnancy while pituitary PRL levels are low. Rat PL II, in the absence of PRL and progesterone, induces lactose synthesis (Bussman et al., 1983) and γ-glutamyl-transferase activity (Bussmann and Deis, 1984) in the pregnant rat mammary gland.

Recently, Nicoll et al. (1983; 1985) described a factor secreted by rodent liver which augmented the pigeon crop-sac response to PRL. The factor has been called "synlactin". In vitro, media containing factors from the livers of pregnant or lactating female rats augmented the PRL response whereas medium from male or virgin liver

slices and kidneys had no effect. The activity was not due to somatomedin-C/IGF-I. Prolactin and/or placental lactogens appear to stimulate the release of these factors.

Hebert et al. (1984) showed that conditioned medium from cultures of pregnant rat livers stimulated the incorporation of tritium-labeled thymidine into mammary gland tissue DNA. Liver slices from males and virgin females did not secrete any stimulatory factors.

The PRL/PL stimulated release of the liver factor, synlactin, may be analogous to the GH-stimulated release of somatomedins. GH stimulated factors promote body growth whereas PRL/PL stimulated factors, in concert with ovarian steroids, may be involved in promotion of mammary growth during pregnancy and maintenance of the gland during lactation (Nicoll et al., 1985).

The role of rPL's in the promotion of growth is unclear. Contopoulos and Simpson (1957; 1959) reported an increase of growth hormone-like activity in the serum of pregnant rats. Comparisons of the responses to thyroidectomy and hypophysectomy in pregnant and non-pregnant animals demonstrated that the growth-promoting activity in the serum from non-pregnant animals was decreased in either case whereas the activity was unchanged in pregnant animals.

In the rat tibia test, Matthies (1967) had observed no response to pregnant rat serum or placental extracts but Friesen et al. (1975) reported preliminary evidence which supported the earlier observations of Contopoulos and Simpson (1957; 1959). Kelly et al. (1976) looked at the activity of pregnant rat serum in RRA-GH. However, a serum factor which bound labelled GH specifically caused problems in the RRA-GH and made interpretation of the results difficult.

Subsequently, Daughaday et al. (1979) found activity in normal late pregnant rat serum in the RRA-GH. The GH-like activity was greater in hypophysectomized pregnant rats. The authors suggested that rPL could stimulate somatomedin release, perhaps by binding to PRL receptors in the maternal liver. There is evidence to support this since PRL binding sites in the liver increase during pregnancy in rats and mice (Kelly et al., 1974b; Sasaki et al., 1982a, 1982b).

In 1975, Francis and Hill reported that PRL stimulated the release of somatomedin from perfused rat liver. However, PRL-treated rats do not grow even though somatomedin levels increase (Bala et al., 1977; Russell et al., 1978). Daughaday and Kapadia (1978) found elevated serum somatomedin levels in pregnant rats following

hypophysectomy at day 14.

Fetal growth may be regulated by rPL. Wunderlich et al. (1979) found that administration of a low protein diet or diet containing alcohol during the last two trimesters of rat pregnancy, reduced the RNA content of the term placenta. The rPL levels, as determined by RRA of day 20 plasma and placental extracts, had decreased in parallel to the decrease in the placental ribosomal RNA content. This study led Munro (1980b) to suggest that the low protein intake or ethanol consumption had caused a reduction in fetal growth by inhibiting rPL synthesis. In another study, Jost (1961) showed that, in conjunction with adrenocortical steroids, rat placenta supported glycogen deposition in the fetal liver. The direct effects of rPL on fetal metabolism require further study.

Rat PL's may also play a role in the metabolic changes that occur during pregnancy. It has been shown that glucose uptake into maternal adipose tissue (Leake and Burt, 1969) and amino acid uptake into liver proteins (Burt et al., 1969) are increased during rat pregnancy. In 1968, Kinzey reported that dietary protein restrictions did not affect the luteotropic and mammotropic potency of midterm rat placenta. However, Henricks and Bailey (1976) observed dramatic effects which led to fetal resorption, when

protein intake was decreased after mid-pregnancy. These studies may indicate that rPL II, the PL found in late pregnant rats, may have a more important role than rPL I, in the metabolic adjustments that occur during pregnancy.

D. Molecular Biology Approach to the PRL-GH Family

With the recent development of recombinant DNA technology, many investigators have utilized the molecular biology approach to re-investigate the PRL-GH gene family. This approach has led to the discovery of a wealth of new information. There are several recent reviews on the study of the gene family (Moore et al., 1982a, 1982b; Kidd et al., 1983; Miller and Eberhardt, 1983).

Miller and Eberhardt (1983) have concluded that the family members are divided into two classes on the basis of the cDNA and genomic structure. There are PRL-like members and GH-like members. Human PL falls into the latter category. Subprimate PL's appear to be more closely related to PRL.

Human PL is the only PL that has been cloned and sequenced and the generalized features of PL's are based on the human hormone. However, several investigators of subprimate PL's maintain that they are more like PRL's than GH's and in that sense different from hPL. Little or no

information is available on the structure of subprimate PL's.

cDNA Structure

Codon Choice

The choice of nucleotide in the codon third position reveals that GH's and hPL have a strong preference for G or C in the third position (74-82%) whereas PRL's show little or no preference (50-63%) for G or C. Random codon selection would result in G or C being selected 42% of the time. The codon choice is not species related (Miller and Eberhardt, 1983).

Untranslated Regions

The cDNA sequence, if complete, reveals the structure of the 5' (proximal) end and 3' (distal) end untranslated regions of the mRNA. These regions include sites involved in ribosomal binding and translation regulation.

5' Untranslated Region

Human GH and PL share 26 out of 29 nucleotides at the proximal end of the 5' untranslated region of the mRNA (Miller and Eberhardt, 1983).

3' Untranslated Region

PRL's and GH's/hPL differ in the structure of the 3' untranslated regions. All known PRL mRNA's use the TAA (ochre) termination codon whereas all known GH's and hPL use the TAG (amber) stop codon (Miller and Eberhardt, 1983).

At the proximal end of the 3' untranslated region, PRL's use A or T in 59% of the first 43 nucleotide positions whereas GH's and hPL use G or C in 69% of the first 47 positions (Miller and Eberhardt, 1983).

All the PRL's share a high homology at the proximal end, 8 out of the first 10 nucleotides and around the AATAAA polyadenylation signal (Miller and Eberhardt, 1983). Bovine and human PRL share a short T-rich palindrome approximately 20 base pairs downstream from the stop codon. Aside from these regions, PRL's do not demonstrate any homology. The remainder of the region is 30% homologous or essentially random.

The GH's and hPL are not homologous at the proximal end. However, there are three regions that are highly homologous. One of these regions is near the AATAAA polyadenylation signal. There are two long C-rich palindromic dyads within the homologous regions (Miller and Eberhardt, 1983).

Sequence Homologies

Comparisons of the mRNA and amino acid sequences of several members of the PRL-GH gene family show that nucleotide sequence homology is greater than amino acid sequence homology in each case (Cooke et al., 1981). Interspecies comparisons of GH's and PRL's show similar nucleotide (~75%) and amino acid (~65%) homologies. Less homology was seen in intraspecies comparisons of PRL and GH, approximately 40% at the nucleotide and 25% at the amino acid level. An extremely high degree of homology is seen between hGH and hPL (Martial et al., 1979). At the nucleotide level, the mRNA's are 92% identical and 80% identical at the amino acid level. Therefore, hGH and hPL are more homologous to each other than to any other family members.

Evolutionary Analysis of the cDNA Structure

The comparison of cDNA structures within and between species yields information about mutational events at the nucleotide level which resulted in silent (unexpressed) or expressed mutations at the amino acid level.

Cooke et al. (1982) demonstrated that PRL's and GH's diverged approximately 400 million years ago (MYA). Miller and Eberhardt (1983) calculated the time of PRL and GH

divergence at 350 MYA. These values correspond well with the fossil record. Fish and tetrapods diverged approximately 400 MYA (Acher, 1976) and the existence of distinct PRL-like and GH-like molecules has been dated to this time. All vertebrates today have both pituitary GH and pituitary PRL.

Evolutionary Origin of hPL

Cooke et al. (1981) suggested that positive selective influences caused a rapid fixation of replacement substitutions in hPL and hGH. The two hormones are recently diverged since they display extensive homologies. Cooke et al. (1981) calculated that hPL and hGH diverged 10 MYA however they suspected that this value estimated the time of an intrachromosomal recombination, not the time of the actual gene duplication event. Miller and Eberhardt (1983) found that hGH and hPL diverged 64 MYA.

Both of the groups determined times of hPL and hGH divergence that were more recent than the estimated time of mammalian radiation (85-100 MYA) (Romero-Herrera et al., 1973; McKenna, 1969). This would suggest that hPL and hGH diverged after the radiation of mammalian species, even after the origin of placental mammals (~100 MYA) (Dickerson and Geis, 1980). Therefore, placental hormones resembling

pituitary PRL and GH may have evolved more than once.

Miller and Eberhardt (1983) suggest that the evolution of hPL and hGH is complicated by other factors. Human PL and hGH are two hormones with different functions, yet they are more similar to each other than hGH is to any other GH's. They suggest that hPL and hGH have evolved by concerted evolution. Concerted evolution occurs when two genes evolve together rather than independently. The rate of divergence is slowed. Gene duplications facilitate concerted evolution (Miller and Eberhardt, 1983).

Wallis (1981) assessed the molecular evolution of the PRL-GH gene family based on amino acid and mRNA information and concluded the rates of evolution were extremely variable. This could explain discrepancies in the calculated times of divergence reported by Cooke et al. (1981) and Miller and Eberhardt (1983).

Cooke et al. (1981) found that the 3' untranslated regions of the mRNA's were more highly divergent than the coding regions, except in the case of hGH and hPL. This is similar to other eukaryotic mRNA's.

Gene Structure

Human Genes

The genes for hGH, hPL and hPRL share several common structural features. The genes are made up of 5 exons (I, II, III, IV, V) interrupted by 4 introns (A, B, C, D). The exon/intron splice junctions are highly conserved (Miller and Eberhardt, 1983).

However, the intron size varies greatly between hPRL and hGH/hPL. The hPRL gene has large introns (Truong et al., 1984). The PRL gene is five times as large as the hPL/hGH genes. The hGH/hPL genes are approximately 2 kb in length whereas the hPRL gene is approximately 10 kb (Miller and Eberhardt, 1983; Truong et al., 1984).

There is a single copy of the hPRL gene per haploid genome (Truong et al., 1984). The hPRL sequences have been located on chromosome 6 (Owerbach et al., 1981).

In 1979, Fiddes et al., while performing a restriction enzyme analysis of human genomic DNA with a hGH cDNA probe, discovered that there were more hybridizing genes than were needed to code for hGH and hPL. Fiddes et al. (1979) suggested that there were multiple genes for hGH and hPL.

A total of five non-allelic hGH and hPL genes have been cloned and sequenced (DeNoto et al., 1981; Seeburg,

1982; Barrera-Saldena et al., 1983; Selby et al., 1984). The genes have been located on human chromosome 17 (Owerbach et al., 1980) on the long arm in the region 17q 22-24 (Harper et al., 1982). The genes are closely linked.

Other primates (chimpanzees, rhesus monkey, slow loris) also have multiple GH-PL genes (Moore et al., 1982b; Miller and Eberhardt, 1983). There are at least 4 genes and they appear to be closely related to the genes in the humans.

A regional map of the hGH/hPL gene cluster was published by Barsh et al. (1983). The genes stretch over a distance of approximately 50 kb. Using the nomenclature of Barrera-Saldena et al. (1983), the transcriptional orientation of the genes established by Barsh et al. (1983) is 5' hGH-N, hPL-1, hPL-4, hGH-2, hPL-3 3'. There have been reports of genes in reversed transcriptional orientation (Kidd and Saunders, 1982; Selby et al., 1984), a third hGH gene (Kidd and Saunders, 1982; Selby et al., 1984), a hGH/hPL hybrid gene (Moore et al., 1982a) and a pseudogene lacking Exon I (Moore et al., 1982b). However, Barsh et al. (1983), in their analysis, found no evidence of any of these genes.

The exons and exon/intron splice junctions of the genes are highly conserved (Parks, 1983). The introns show

greater than 90% homology with the exception of intron A of the hGH-2 gene and intron B of the hPL-3 and hPL-4 genes which are longer than the corresponding introns of hGH-N. The 5' flanking regions and untranslated regions are also very homologous (Parks, 1983).

The hGH-N gene is the only gene encoding hGH which is expressed in the pituitary (Parks, 1983). The mature hormone coded for by the gene, is 191 amino acids in length and has a molecular weight of 22,000 daltons. Approximately 90% of the pituitary GH is accounted for by this 22 K form.

A smaller peptide with a molecular weight of 20,000 daltons makes up the remaining 10% of pituitary hGH (Parks, 1983). The 20 K hGH is missing the amino acid residues 32-46 found in 22 K hGH (Lewis et al., 1980). Intron B of the hGH-N gene starts after residue 31 (Wallis, 1980). Wallis proposed that 20 K hGH is produced by the splicing of the 5' end of Exon I to a shorter Exon II beginning after codon 46. De Noto et al. (1981) found that the 20 K hGH is the result of alternative splicing of the hGH-N pre-MRNA based on their discovery of an appropriate intermediate mRNA. Therefore, hGH-N codes for both forms of hGH produced in the human pituitary.

The hGH-2 gene, also known as the hGH-V or variant gene (Barsh et al., 1983), encodes a protein product that is quite different from the hGH-N gene product. The proteins differ in amino acid composition and isoelectric point (Seeburg, 1982). No peptide corresponding to the hGH-2 gene product has been found *in vivo* (Parks, 1983). The product of this gene has been studied *in vitro* (Pavlakis et al., 1981; Hizuka et al., 1982). The hGH-2 peptide is immunologically non-reactive to hGH-N product antibodies but it is biologically active. The biological role of this form of hGH is unknown.

The hPL-1 gene is regarded as a non-functional pseudogene (Parks, 1983).

Both the hPL-3 and hPL-4 genes are expressed in the term placenta (Barrera-Saldena et al., 1983). There are 4 nucleotide differences in the coding regions of the genes which are silent at the amino acid level (Parks, 1983). At position -24 of the signal peptide, the genes encode a different amino acid. Although the ratio of hPL-3 to hPL-4 expression is highly variable in term placentas (Fitzpatrick et al., 1983), both non-allelic genes are responsible for the production of hPL during pregnancy.

Physiological Role of hPL

Studies in which maternal serum levels of hPL were monitored for the assessment of fetal health have revealed several cases of hPL deficiencies in otherwise normal pregnancies. There have been reports of incomplete (Gaede et al., 1978; Bradford and Hargreaves, 1978; Moshirpur et al., 1981) and complete (Nielson et al., 1979; Borody and Carlton, 1981) antenatal deficiency of immunoreactive hPL.

Wurzel et al. (1982) studied the genes of a child and his family in a case where no immunoreactive hPL was present in the maternal serum during pregnancy. Wurzel and associates found that the child, of normal height, had a homozygous 30 kb deletion of all of the hGH/hPL gene cluster downstream from the hPL-1 gene. Therefore, the hPL-4, hGH-2 (hGH-v) and hP-3 genes were deleted. Two other children with different hPL gene deletions but the same biochemical phenotype have subsequently been identified (Parks et al., 1983a; 1983b). These gene deletions demonstrate conclusively that hPL and the hGH-2 (hGH-v) gene product are not required for normal growth and development in humans (Parks, 1983).

Regulation of hGH and hPL Gene Expression

The study of the gene structure of hPL and hGH has raised the question of how genes which are so similar in the structural and flanking sequences can be regulated differentially and tissue-specifically.

Several investigators have studied the methylation and DNase I sensitivity of the genes. Transcriptionally active genes often show less methylation of cytosine residues and are more sensitive to DNase I digestion. Hjelle et al. (1982) could not demonstrate tissue specific under-methylation of the hGH genes or hPL genes, in the pituitary and placenta respectively. Barsh et al. (1983) found that sites in the 5' flanking region of the hGH-N gene were more sensitive to DNase I digestion in the pituitary than the placenta. They also found that the 3' flanking region of the hPL-3 and hPL-4 genes appeared to be more sensitive in the placenta than the pituitary. Therefore, the hGH and hPL genes may be in more active configurations in the tissues which express the genes specifically.

Subprimate Genes

The genes for rPRL and rGH have been cloned and characterized (Gubbins et al., 1980; Chien and Thompson, 1980; Barta et al., 1981; Maurer et al., 1981; Cooke and

Baxter, 1982; Page et al., 1981). The rat genes are similar to the human genes with 5 exons and 4 introns. The rPRL and rGH genes exist as single copies per haploid genome. The rPRL gene, like hPRL, is considerably larger than the GH gene.

The genes for PRL and GH in the cow have been isolated and characterized (Woychik et al., 1982; Camper et al., 1984). The genes, like the human genes, are made up of five exons and four introns. Each is present as a single copy per haploid genome. The bovine GH gene is approximately 1.8 kb (Woychik et al., 1982). The PRL gene has larger introns and is approximately 8 kb in length (Camper et al., 1984). A possible alternate exon/intron splice site was found within the signal peptide coding sequence for bPRL.

Subprimate PL Genes

The search for subprimate PL genes has provided further evidence that, unlike hPL, subprimate PL's are more closely related to PRL than GH.

In the rat, rGH cDNA does not hybridize to rat placental mRNA (Miller and Eberhardt, 1983). The rPL gene could not be located by hybridization of genomic DNA to a rGH cDNA probe under stringent conditions (Moore et al.,

1982b) even though the hPL gene(s) were first detected with a hGH cDNA probe under these conditions (Fiddes et al., 1979).

Attempts to isolate cDNA clones to bPL mRNA from bovine cotyledonary tissue with bGH and bPRL cDNA probes were unsuccessful (Hurley and Gorski, 1982). Using a different approach, bPRL and bGH cDNA clones were used to screen a bovine genomic DNA library. Several genomic clones which hybridized to bPRL under non-stringent conditions, were identified. The clones were distinct from bPRL and showed no hybridization to bGH. Camper et al. (1984) while isolating the bPRL gene, reported that a PRL-like gene, distinct from bPRL, hybridized to bPRL at low stringency. They did not characterize the gene.

In 1984, Schuler et al. identified a bPL cDNA clone selected from a bovine cotyledonary library with a bPL genomic clone. The bPL cDNA clone corresponded to a mature mRNA 1100 bp in length. The clone displayed hybridization homology to bPRL and bGH, although the hybridization to bGH was much weaker. Partial nucleotide sequence analysis demonstrated that the 3' end of the bPL mRNA was 75% homologous to bPRL at the nucleotide level and 39% homologous at the amino acid level.

Exon/Intron Boundaries and Intron Structure

The genes for all members of the family studied to date have an identical number of exons and introns in a similar arrangement. The exon/intron splice sites are highly conserved (Miller and Eberhardt, 1983). However, the intron sizes and sequences differ greatly, except in the case of hGH and hPL.

Wilson et al. (1977) have postulated that sequences under strong functional constraints will evolve less rapidly than those sequences that are less functionally important. This would suggest that conservation of the specific sequences involved in forming the exon/intron splice junctions confers a selective advantage. It also suggests that the genes arose from a common precursor with similar exon/intron boundaries (Miller and Eberhardt, 1983). The rate of intron divergence appears to be much more rapid than the rate of exon divergence, suggesting that introns are not functionally important in the expression of the genes.

Repetitive DNA

Several classes of dispersed middle repetitive DNA have been identified in and around the genes of the PRL-GH gene family members. The function of these repetitive

elements is unknown but several roles have been postulated. Repetitive elements may be involved in pre-mRNA processing (Jelinek et al. 1980), origin of replication (Jelinek et al., 1980) or intergenic elements which regulate temporal expression during development (Fritsch et al., 1980). The discovery of repetitive elements closely flanking and interspersed within the genes of the family may suggest they have been involved in the gene duplications (Miller and Eberhardt, 1983). The repetitive DNA sequences in the introns may act as "transposons" which can increase or decrease intron size. They may suggest insertional events which could lead to rapid divergence of the introns as well as rearrangements of the genes and exons (Barta et al., 1981).

Barsh et al. (1983) found three different classes of repeats interspersed throughout the hPL/hGH gene cluster. The authors postulated that the gene cluster evolved recently by homologous but unequal exchange between the middle repetitive Alu-family elements. Kidd and Saunders (1982) also reported the presence of Alu repeats within the introns and immediately flanking the 5' and 3' untranslated regions of the hGH/hPL genes.

Page et al. (1981) and Barta et al. (1981) have both described a 200 bp tandem repeat with transposon-like

features in Intron B of the rGH gene. They suggest that this repetitive element may account for the size disparity between Intron B of rGH and hGH. The presence of this element suggests how intron size could be varied during evolution.

Cooke and Baxter (1982) have proposed that the 5′ ends of rPRL and rGH have independent origins in view of the lack of homology and disparity in the lengths of Exon I of rPRL and rGH. They found three families of dispersed repetitive elements in and around the rPRL gene which may suggest an insertional event.

Schuler et al. (1983) described a restriction enzyme polymorphism near the rPRL gene caused by an Alu-like element. Gubbins et al. (1980) have also found repetitive elements within the introns of the rPRL gene, found elsewhere in the rat genome.

Flanking Regions

The DNA regions that lie adjacent to the 5′ and 3′ untranslated regions are called the 5′ and 3′ flanking regions. This DNA is not transcribed into RNA. The function of the 3′-flanking regions is unknown. The 5' flanking region contains the promoter as well as regions which bind hormone-receptor complexes to regulate gene

transcription and possibly regions responsible for tissue specific expression of genes.

Prolactin and growth hormone genes are expressed in the same tissues but their expression is often reciprocal. Frequently, growth hormone production decreases when prolactin production increases and vice versa, suggesting different specific mechanisms controlling gene expression (Cooke et al., 1981).

Sites within the 5′ flanking region have been implicated in the glucocorticoid induction of human and rat GH's (Miller et al., 1984; Robins et al., 1982) as well as the thyroid hormone induction of rGH (Miller et al., 1984).

Woychik et al. (1982) while studying the bGH gene discovered many areas within the 5′ and 3′ flanking and untranslated regions that were highly conserved between bovine, human and rat GH genes. In particular, a 38 bp stretch, 100 bp upstream from the transcription initiation site in the 5′ flanking regions, was 90% homologous among the GH's of the three species. The sequence is not found in other eukaryotic genes, suggesting a GH specific function. The sequence does not appear to be involved in glucocorticoid regulation.

Human GH/PL genes show extensive homology within the 5′ flanking regions (Miller and Eberhardt, 1983). This

may be due to the close linkage and recent duplications of the genes.

There is little sequence homology in the 3' flanking regions of the genes except for the hPL/hGH genes (Miller and Eberhardt, 1983).

Exon Domains

The concept of exons as functional domains was proposed by Gilert (1978). He suggested that exons are primitive genetic units that encode peptides with different functions.

Miller and Eberhardt (1983), considering the structure of the exons of the PRL-GH gene family and the variety of repetitive elements flanking the exons, have suggested Exon I may be a regulatory exon.

Several studies have suggested that the individual exons of the rPRL gene may be responsible for distinct biological activities. Exon II may be involved in receptor binding site or maintenance of the structure of the binding site since proteolytic removal of the amino acids encoded by Exon II reduced the receptor binding activity by 87% compared to the intact protein (Wong et al., 1981). Exon II encodes most of the signal peptide and a portion of the mature hormone that has been implicated in conferring

lactogenic activity (Miller and Eberhardt, 1983). Exons IV and V have antigenic sites which may confer mitogenic activity and/or conformational stability (Miller and Eberhardt, 1983).

There are four internal regions of homology in PRL, GH and hPL in Exons II, IV and V (Miller and Eberhardt, 1983). Two of these regions occur within Exon V. Niall et al. (1971) proposed that the homologous segments arose from the duplication of a small ancestral genetic segment. A previously existing intron which interrupted Exon V may have been removed at some time which would account for the two homologous regions in Exon V (Miller and Eberhardt, 1983).

The carbohydrate-regulating properties of GH appear to be partially localized within Exon III since the 20 K hGH variant lacks the diabetogenic activity of 22 K hGH.

Evolutionary Implications

Preliminary evidence suggests that subprimate genes have evolved differently than human genes. Human PL has evolved more recently from hGH, based on the homology and chromosomal locus. Subprimate genes (PRL and GH) exist as single copies whereas primate GH/PL genes are present in multiple copies. Subprimate PL's do not appear to be

closely related to GH's. Hurley et al. (1977) has suggested that subprimate PL's have a different evolutionary origin than hPL, and that they arose from the duplication of the PRL gene, not the GH gene, like hPL. However, the complete cDNA and gene structure of subprimate PL's are required to determine if PL's have arisen independently several times in mammalian evolution.

E. New Members of the PRL-GH Gene Family in Mice

Recently, several PRL-like genes have been described in the mouse which appear to be new members of the PRL, GH, and PL family of genes.

Mouse Proliferation (mPLF)

While studying growth related genes in serum stimulated mouse BALB/c 3T3 cells, Linzer and Nathans (1983) isolated a cDNA clone which appeared 3 hours after stimulation and reached maximum levels within 12-18 hours. Subsequent nucleotide sequence analysis revealed that the clone was very similar to PRL (Linzer and Nathans, 1984). They called this clone, isolated from proliferating cells, mouse proliferin (mPLF).

The cDNA translated into an open reading frame of 224 amino acid residues which encoded a 25 kd protein (Linzer and Nathans, 1984). The translation was terminated by the TGA stop codon. This codon is not used for termination in any of the known GH's or PRL's (Miller and Eberhardt, 1983). The polyadenylation signal was located at nucleotide 770.

Mouse PLF appears to have a signal peptide of 29 amino acids. There are 2 tryptophan residues and 6 cysteine residues in similar positions to those found in PRL's (Linzer and Nathans, 1984). There are 3 potential glycosylation signals, Asn-X-Ser, at positions 28-30 46-48 and 59-61. There are also potential sites for proteolytic cleavage within the mature protein at positions 120-122 (Lys-Lys-Lys), 145-146 (Lys-Lys) and 176-177 (Lys-Lys).

At the amino acid level, mPLF is 39% homologous to PRL, 37% homologous to bPRL and 22% homologous to (Linzer and Nathans, 1984).

Antisera to mPLF produced <u>in vitro</u> by an expression vector immunoprecipitated a heterogeneous population of glycoproteins (25-35 kd) in the 3T3 cell medium (Linzer and Nathans, 1984). When glycosylation was inhibited with

tunicamycin treatment, only a 22 kd form was present. This is the predicted size of the mature peptide.

Mitogen-Regulated Protein (MRP)

Nilsen-Hamilton et al. (1980; 1981) while studying the response of Swiss 3T3 cells to growth factors discovered that growth factors stimulated the production and release of a heterogeneously glycosylated protein of 34 kd into the medium. The unglycosylated form had a molecular weight of 21 kd. They called the protein, mitogen-regulated protein (MRP).

The synthesis and secretion of MRP was regulated in a similar manner to PRL. The growth factors, FGF, EGF, PDGF and tumor promoters stimulated DNA synthesis and transcription of the MRP message. Post-translational regulation occurred by lysosomal protease degradation.

The MRP is mPLF (Hamilton et al., 1986). Antibodies to MRP specifically immunoprecipitate mPLF. The nucleotide sequence of a cDNA clone to the 3' end of MRP mRNA is identical to mPLF.

Mouse PLF-2

Linzer et al. (1985) tested a number of mouse tissues for mPLF mRNA. They discovered that mPLF is specifically

expressed in the mouse placenta. The PLF isolated from BALB/c 3T3 cells was called mPLF-1 and the PLF isolated from placental tissue was called mPLF-2 because of several differences, which will be discussed later.

Mouse PLF-2 appears after day 8 of pregnancy, peaks at mid pregnancy and declines through day 18 (Linzer et al., 1985). Minced placental tissue secretes glycosylated PLF. The protein does not cross-react with antiserum to mPL.

The mature mPLF-2 message is 1 kb in length. Nucleotide sequence analysis of mPLF-2 mRNA showed that mPLF-1 and mPLF-2 differ by 5 single base substitutions that result in the alteration of 4 amino acids. All 5 changes occur at threonine or serine residues. The changes do not affect the potential glycosylation sites.

At the genomic level, there appear to be 3-5 copies of the PLF gene per haploid genome (Wilder and Linzer, 1986; Nathans et al., 1986). There are distinct PLF-1 and PLF-2 genes. Both types appear to be expressed in all cell types which secrete PLF. Therefore, expression of mPLF-1 or mPLF-2 is not tissue specific.

Mouse PLF is not lactogenic in the Nb_2 lymphoma cell bioassay (Linzer, personal communication) and the physiological role of these proteins is not known. Linzer et al. (1985) have suggested that PLF is secreted by

proliferating mouse cells and mouse placenta and may act as a growth factor, possibly autocrine, in cultured cells and pregnant animals.

Proliferin Related Protein

While searching for PLF expression in mouse tissues, Linzer and Nathans (1985) discovered a second set of clones in the mouse placenta which hybridized weakly to the PLF probe. The clones correspond to a 1.1 kb mRNA which appears later than PLF, peaks at day 12 and then gradually decreases to term. They called this mRNA, proliferin-related protein (PRP).

The encoded protein of 244 amino acids has a higher molecular weight than PLF. It has a calculated molecular weight of 27,956 daltons. The protein contains a potential signal peptide, 30 amino acid residues in length. There are 3 potential glycosylation signals, Asn-X-Thr/Ser, at positions 4-6, 19-21 and 61-63 of the predicted mature peptide. There are also 2 potential proteolytic cleavage sites at positions 35-36 (Arg-Lys) and 84-85 (Arg-Lys).

At the nucleotide level, PRP is 54% homologous to PLF. The two mRNA's share the same termination codon (TGA). At the 5' end, 92 out of the first 97 nucleotides are identical. At the amino acid level, mPRP is 32% homologous

to mPLF, 30% homologous to mPRL and only 16% homologous to mGH. Mouse PRP has 2 tryptophan residues in the positions they are found in most PRL's and 5 cysteine residues. The free sulfhydryl group could be involved in the formation of varying disulfide linkages. The amino acid sequence extends beyond the last cysteine residue as with the GH's. Mouse PRP has a region of 10 additional amino acids which does not align with any of the other family members. This may suggest that PRP is a more diverged member of the family.

F. Cloning the Rat Placental Lactogens

The complete primary structure of a subprimate PL has not yet been determined. Our laboratory decided to utilize recombinant DNA techniques in order to elucidate the structure of PL's in the rat. This approach was used because of the limited availability of rat placental tissue for protein purification for direct amino acid sequence analysis. In addition, this technique provides additional information about the genetic make-up of rPL.

Rat PL I and rPL II are probably not related through a common precursor since immunoprecipitation of translation products of poly A+ mRNA isolated from day 11 and day 19 shows that only rPL II is precipitated by the antibody to rPL II (Robertson et al., 1982).

Rat PL I

Attempts to isolate a cDNA clone to rPL I from Day 11 placental mRNA have not been successful in our laboratory. *In vitro* translation products contain no major proteins corresponding to rPL I. Since no antibodies to rPL I were available, selection of a minor component was not possible (T. Hatton, personal communication).

Rat PL II

Rat PL II cDNA clones were isolated from Day 18 placental mRNA libraries. The success was due to the availability of an antibody to rPL II and the prominence of the rPL II protein band at day 18 (M. L. Duckworth, personal communication).

Levels of rPL II in maternal serum are maximal at day 18 of rat pregnancy (Robertson and Friesen, 1981). Pooled day 18 placental poly (A+) mRNA was translated *in vitro* by rabbit reticulocyte lysate. The translation products consisted of several major proteins, between 20-30 Kd. Polyclonal anti-ovine PRL antisera, known to cross-react with rPL II and anti-rPL II antiserum, precipitated a major protein band of 25 K. Treatment of the translation mixture with dog pancreatic microsomes, followed by immunoprecipitation with anti-rPL II or anti-ovine PRL antisera,

resulted in the appearance of a 22 K protein. The size corresponded to the secreted protein observed in pregnant rat serum (M.L. Duckworth, personal communication).

Initially, cDNA clones to rPL II were isolated from a cDNA library made from day 18 rat placental mRNA. The library was made in the plasmid vector, pAT153, with the cDNA G-C tailed and inserted into pAT153's unique Pst I site.

The library was first screened kinetically for abundant mRNA's. Since rPL II mRNA was one of the most abundant mRNA's at day 18, clones which hybridized strongly to [^{32}P]-labeled single-stranded cDNA made from day 18 placental mRNA were selected. The selected clones were further characterized by hybrid selection. Only a single clone, pRP52, 270 bp long, hybridized to an mRNA that was translated and processed in vitro to a 25 Kd and a 22 Kd protein, respectively. Both protein bands were immunoprecipitated by anti-rPL II and anti-ovine PRL antisera.

Several other clones were hybrid selected but did not translate into products with properties consistent with rPL II. These other clones will be discussed later.

The plasmid library was rescreened with nick-translated (Rigby et al., 1977) pRP52. Out of 1280

recombinants, 9 additional rPL II clones were identified. From the abundance of rPL II clones in the library, it was calculated that rPL II mRNA made up 0.7% of the total day 18 placental mRNA population. Therefore, it is moderately abundant.

A second library was constructed in the phage vector, λGT10, following the method of Huynh et al. (1985) with the modifications for second strand synthesis as described by Okayama and Berg (1982). The cDNA was inserted into the unique EcoRI site of the vector with EcoRI linkers. The second library was constructed because the rPL II clone, pRP52, hybridized to a single transcript of 1 kb and none of the other clones isolated from the plasmid library were long enough to be considered full-length. The short length of the cDNA clones in the plasmid library was probably due to the S1 nuclease (Vogt, 1973) treatment used in the cDNA synthesis. The method used in making the second library did not require the use of S1 nuclease. Additional clones to rPL II were isolated from the lambda library following selection with pRP52A, the longest clone found in the plasmid library. The clones selected from the second library were subcloned into pAT153.

The rPL II mRNA induction pattern corresponded to the appearance of rPL II in maternal serum (Robertson and

Friesen, 1981). Based on RNA blot analysis of total RNA samples (White and Bancroft, 1982) extracted from placenta on different days of pregnancy and hybridization to [^{32}-P]-labeled rPL II cDNA, rPL II mRNA is shown to turn on abruptly at day 12 and increase until peaking at day 18.

The rPL II mRNA transcript appeared to be tissue specific since it was not detected by hybridization in any other adult female tissues studied, including decidua and fetal yolk sac membranes.

Rat PL II cDNA showed hybridization homology under stringent (65°C) conditions to hPRL and rPRL cDNA clones by Southern blot analysis. The rPL II cDNA did not show any hybridization, even under non-stringent (55°) conditions, to hPL or rGH cDNA clones. The rPRL cDNA clone hybridized weakly at 55°C to day 18 placental mRNA.

The rPL II clones were selected and designated as rPL II clones based on: the size of the translation product; the processing to a lower molecular weight, indicative of signal peptide cleavage, corresponding to the size of the secreted protein found in maternal serum; the precipitation by rPL II and oPRL antisera; the induction pattern; and the hybridization homologies.

Genomic DNA Information

Although the hPL gene was detected under stringent conditions with a hGH probe, the rPL genes have not been identified with rGH cDNA probes (Moore, et al., 1982b).

The rPL II gene is a large, single copy gene like rPRL (Cooke and Baxter, 1983), distinctly different from the rPRL gene. Genomic DNA Southern blot analysis of kidney genomic DNA digested with restriction enzymes showed unique digestion patterns and no overlapping fragments when hybridized to rPRL and rPL II cDNA probes. The rPL II gene is at least 10 kb, like the rPRL gene (M.L. Duckworth, personal communication).

Genomic DNA clones to rPL II have been isolated in our laboratory from a rat genomic library made up of Sau-3A/MboI fragments cloned into the lambda vector, EMBL 3, (kindly provided by Dr. M. Crerar, York University).

Two genomic clones have been isolated which are different from one another, but possibly overlapping. Restriction enzyme mapping shows that fragments of the clones correspond to fragments seen in rat kidney genomic DNA (P. Shah, personal communication).

Other Classes of Placental Clones

Our laboratory, while isolating cDNA clones to rPL II mRNA, discovered three other classes of cDNA clones which corresponded to abundant mRNA's at day 18 and translated *in vitro* to proteins of approximately 25 Kd (M.L. Duckworth, personal communication). These clones were called rPLP-A, pRP9/9A and pRP54. A fifth clone, pRP27, was also found which was present at day 18 but it is not an abundant message. All of these clones share similar protein products and hybridization homologies, yet they have unique genomic DNA restriction patterns, restriction enzyme maps and induction patterns. They all resemble PRL and, where studied in detail, have been found to be new members of the PRL-GH gene family.

Rat Prolactin-Like Protein (rPLP-A)

This clone corresponds to an abundant mRNA in placental tissue at day 18 of pregnancy. The *in vitro* translation product is 25 Kd. The protein is processed *in vitro* to a molecular weight of 27 Kd. The translation product is not precipitated by antisera to rPL II or oPRL. Out of 1280 recombinants, 12 clones were identified. Therefore, 0.9% of the total day 18 placental mRNA population is composed of rPLP-A.

The mRNA first appears in placental tissue at day 14, two days after rPL II. The levels peak by day 18 of pregnancy, like rPL II.

The rPLP-A clones hybridized to a single mRNA transcript of approximately 1 kb. Rat PLP-A has not been detected in any other rat tissues except in late pregnant decidua where very low levels are present.

Rat PLP-A hybridizes, under non-stringent conditions, to hPRL, rPRL, and rPL II. The clone does not hybridize to rGH or the GH-like hPL.

Hybridization of rPLP-A to restriction enzyme digested genomic DNA showed that rPLP-A hybridized to a large, single copy gene that was not similar to rPRL or rPL II.

The restriction enzyme map of rPLP-A is unique (L. Peden, personal communication).

Nucleotide sequence analysis yielded an open reading frame of 790 nucleotides which coded for a protein of a calculated molecular weight of 26,350 daltons (L. Peden, personal communication). A polyadenylation signal (AATAAA) is located 23 bp upstream from the remnant of the poly A tail in the 3′ untranslated region. The translation is terminated by the TAA stop codon, like all known PRL's (Miller and Eberhardt, 1983). There are two potential glycosylation signals (Asn-Tyr-Thr) at positions 10-12

and 144-146 which would explain the increased size in the translation product following processing.

There are also three potential proteolytic cleavage sites at positions 51-52 (Arg-Arg), 133-134 (Lys-Lys) and 174-175 (Lys-Lys).

It is not known if rPLP-A is a secreted peptide since the peptide has not yet been isolated. However, the first 31 amino acids of the translation could be a signal peptide. The amino acid residues are predominantly hydrophobic and statistical analysis of the amino acid positions (Von Heijne, 1983) would place the most likely cleavage site between residues 31 and 32. Based on this cleavage site, the secreted unglycosylated protein would have a molecular weight of 22,280 daltons.

Rat PLP-A is a new member of the PRL-GH gene family in the rat. From the deduced amino acid sequence, rPLP-A is highly homologous to human and rat PRL as well as mPLF and mPRP (Table 4). Amino acid positions 58-74 are especially homologous. Out of 17 residues, 14 are identical to hPRL.

Rat PLP-A has 5 cysteines. Like the GH's, there are no NH_2-terminal cysteines. The extra cysteine may allow for the formation of varied disulfide linkages. The two

TABLE 4

Amino Acid Homologies to rPLP-A

			% Identical
rPLP-A	vs.	hPRL	45
		rPRL	43
		mPLF-2	41
		mPRP	37
		rGH	29
		hGH	29
		hPL	29

tryptophans seen in the other family members are conserved. There are also two additional tryptophan residues.

Out of the 6 residues Kohmoto et al. (1984) considered essential for lactogenic activity, rPLP-A has only two of them. The other four are not present although one is replaced by a related amino acid. The physiological role of rPLP-A is unknown but it is probably a secreted placental protein with hormonal activities.

pRP9/9A

By hybrid select translation, these clones code for a protein of 25 Kd that is processed *in vitro* to a molecular weight of 24 Kd. The clones hybridize to two mRNA transcripts of 1.2 and 1.0 kb., but the two mRNA's code for the same protein. The clones, pRP9 and 9A, first appear at day 11 of pregnancy and peak in placental tissue by day 14. Both clones hybridize to rPRL, hPRL, rPL II, rPLP-A, pRP54 and pRP27. They do not show any hybridization homology to rGH or hPL. The restriction map and genomic DNA restriction pattern are unique.

pRP54

The clone, pRP 54, codes for a 20 K protein which appears to be the major placental protein at day 18. The

mature message is approximately 900 bp. The message first appears between day 14 and 15 of pregnancy and peaks at day 18. The clone, pRP54, hybridizes to hPRL, rPLP-A, pRP9 and weakly to rPL II. No hybridization is seen to pRP27, rPRL, rGH or hPL. The clone, pRP54, has a unique restriction map and genomic DNA pattern.

pRP27

The clone, pRP27, is not an abundant mRNA. The size of the protein product is unknown. The clone first appears at day 11 but is not expressed fully until day 14. The clone hybridizes to rPLP-A, pRP9, rPRL and hPRL but not to rGH, hPL, rPL II or pRP54. The restriction enzyme map is unique and preliminary nucleotide sequence analysis shows no homology to any of the PRL-GH gene family members.

PRL-like Transcripts in the Rat

In addition to the PRL-like placental clones, our laboratory has discovered an ubiquitous 1.7 kb. mRNA transcript in rat tissues which hybridizes to rPRL. These transcripts do appear to be polyadenylated (G. DiMattia, personal communication).

Two clones, K5 and K25, have also been isolated from a library of poly (A+) rat kidney mRNA (M. L. Duckworth,

personal communication). The clone, K5, corresponds to a 4 kb transcript and K25 to a 1 kb transcript. The clones hybridize to PRL. The clone, K5, is present in low levels in the rat lung, uterus and ovaries and high levels in day 18 placenta. The other clone, K25, is found in day 14, 16, 18 placenta and in very small amounts in day 18 decidua. Preliminary data suggest that the expression of K25 may be related to the gestational state of the animal.

These PRL-like transcripts may also represent new members of the rapidly expanding PRL-GH family of hormones.

II. AIMS OF THE STUDY

The human species is the only species in which PL has been cloned, sequenced and characterized. It is also the only species in which the PRL, GH, PL triad of hormones has been studied. Although subprimate PRL and GH genes have been characterized, genes for subprimate PL's have not yet been described.

Based on its biological actions, immunological cross-reactivity, hybridization homology and size, rPL II appears to be a member of the PRL-GH gene family, closely related to PRL, rather than the GH-like, hPL. However, the primary structure of rPL II is unknown.

The major aim of this study is to define the primary structure of rPL II. This will be accomplished by determination of the nucleotide sequence of cDNA clones to the rPL II mRNA. This approach has been utilized because rPL II is difficult to obtain in sufficient quantities and purity for direct amino acid sequencing. In addition, technically, nucleotide sequence analysis is simpler to perform, the sequence is unambiguous, and additional information at the genetic level can be revealed.

The mRNA structure and sequence, the deduced amino acid sequence and the protein will be compared to the other

members of the PRL-GH gene family in order to determine the structural, functional and evolutionary relationships of rPL II to the family.

III. METHODS AND MATERIALS

A. Experimental Design

The structure of rPL II mRNA, the deduced amino acid sequence and implied protein characteristics of rPL II were determined from the nucleotide sequence of cDNA clones to the mRNA.

The rPL II cDNA clones were mapped with restriction enzymes and subsequently subcloned into M13 RF DNA vectors, utilizing the elucidated restriction sites. The nucleotide sequence of the recombinant subclones was determined via the Sanger dideoxy chain termination method (Sanger et al., 1977) utilizing [^{35}S] and buffer gradient sequencing gels (Biggin et al., 1983). The nucleotide sequence data were analyzed by computer.

B. Background to the Methodology

In the late 1970's, two methods for nucleotide sequence determination were developed. Maxam and Gilbert (1977) developed the chemical cleavage method. Sanger et al. (1977) developed the dideoxy chain termination method. The dideoxy chain termination method was used to elucidate the primary structure of rPL II and will be described here.

Sanger dideoxy sequencing is based on the use of deoxynucleotide (dNTP) analogs, dideoxynucleotides (ddNTP's), which cause chain termination at specific nucleotides where they are incorporated into an extending DNA strand.

A single-stranded DNA template annealed to a primer containing a 3'-hydroxyl group, is accurately copied by the Klenow fragment of E. coli DNA polymerase I in the 5' to 3' direction, in the presence of dNTP's. Since the Klenow enzyme can incorporate ddNTP's as well as dNTP's, addition of ddNTP's results in chain termination because the incorporated ddNTP's lack the 3'-hydroxyl group required for the formation of the next phosphodiester bond in the chain.

When four separate reactions are carried out in the presence of all four dNTP's and only a single, known ddNTP, newly synthesized strands are specifically terminated. The fragments all share a common initiation point. By using a radioactively labeled dNTP, the generated fragments can be visualized by autoradiography. The DNA is electrophoresed on a denaturing polyacrylamide gel that separates the fragments by size. By using very thin gels, DNA fragments differing by a single base can be resolved (Sanger and Coulson, 1978).

The Sanger dideoxy sequencing method requires a SSDNA template. At first, this was a major drawback to the methodology, since isolation of SSDNA is a laborious procedure. Recently, however, the life cycle of a filamentous bacteriophage, M13, was exploited as a biological means to acquire the SSDNA template.

M13 is a filamentous bacteriophage with a closed circular single-stranded DNA genome (Denhardt et al., 1978). The phage infects bacterial cells via the F pilus. In the bacterial host, the phage is stripped of its protein coat and converts to a double-stranded replicative form (RF). The DS DNA replicates and forms SSDNA which is processed and packaged into the mature viral form. The SSDNA virus is extruded from the infected cells. The host cells are not lysed.

The M13 RF DNA is used as a cloning vehicle. Double-stranded cDNA is inserted into the M13 RF vector. The DNA is used to transform competent host cells and the DS DNA is amplified by the host cell. DS DNA can be isolated from the bacterial cells and SSDNA can be extracted from the viral particles in the medium.

C. General Methods

Restriction Enzyme Digestion of DNA

Materials

Restriction enzymes were purchased from Boehringer Mannheim (Montreal, PQ), Bethesda Research Laboratories (Gaithersburg, MD) and New England Biolabs (Beverly, MA).

Method

Purified plasmid and M13 vector DNA were cleaved with various sequence specific restriction enzymes.

Restriction digests were performed in the appropriate restriction enzyme buffers in a water bath of the required temperature (30-65^{o}C) according to Maniatis et al. (1982). The reactions consisted of plasmid or vector DNA (0.5-2.0 ug), restriction enzyme, restriction enzyme buffer and distilled H_2O. The reaction volume was 10-25 ul. An excess of enzyme (2-10 units/ug of DNA) was used but the volume of enzyme never exceeded 1/10 of the total reaction volume. Digestions were performed for 3-18 hrs.

Double digestions of DNA were done simultaneously when buffers were compatible. However, in cases where salt requirements differed, the DNA was first cut with the enzyme requiring a lower salt buffer. Following digestion with the first enzyme, salt was added and the DNA was

cleaved with the second enzyme. If digestion was not successful using the latter two methods, the order of digestion was reversed. Phenol extraction and ethanol precipitation were performed prior to digestion with the second enzyme, in these cases.

Electrophoresis of DNA

DNA samples were electrophoresed for a variety of reasons. Agarose mini-gels were used to monitor restriction enzyme digestions and to evaluate DNA preparations. For accurate size analysis for restriction enzyme mapping, large horizontal agarose gels and vertical polyacrylamide gels were used.

Materials

Ultra pure DNA grade agarose and the DNA size markers, ØX 174 RF DNA-Hae III digest and λDNA-Hind III digest, were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Polyacrylamide, bis-acrylamide, TEMED and ammonium persulfate were supplied by BioRad (Mississauga, Ont.). The ethidium bromide and bromophenol blue were purchased from Sigma Chemical Co. (St. Louis, MO). The xylene cyanol FF was obtained from Kodak (Toronto, Ont).

Reagents

50XTAE: 242g Tris, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0)/l

5X agarose gel loading buffer (5XAGLB): 50% glycerol, 1.5% Ficoll 400, 10 mg/ml bromophenol blue in 1XTAE

10XTBE: 108 g Tris, 55 g boric acid and 40 ml 0.5M EDTA (pH 8.0)/l

5X polyacrylamide gel loading buffer (5XPAGLB): 50% sucrose (w/v), 2.5XTBE, 0.25% bromophenol blue and 0.25% xylene cyanol.

Method

In general, the DNA samples were mixed with 1/5 volume of the appropriate 5X loading buffer in H_2O. The amount of DNA was sufficient to allow for 10 ng/band. The samples were heated at 68°C for 10 min prior to loading. The samples were loaded onto the gel, electrophoresed and stained with 0.5 ug/ml EtBr. The DNA was visualized with a UV transilluminator. Photographs (Polaroid Type 57 film) were taken with a Polaroid MP-4 camera.

Agarose Gel Electrophoresis

Mini-Gels

DNA samples (1-4 ul) in a 5-10 ul volume containing

1XAGLB were run on 10-20 ml horizontal 1% agarose mini-gels. The gels were run at 80 mA for 30-60 min in 1XTAE containing 0.5 ug/ml EtBr.

Large Gels

Restriction enzyme digestions (20-25 ul) with DNA fragments 7-0.4 Kb length fragments were electrophoresed against DNA size markers on 1-1.2% 150-300 ml agarose gels. The gels were run in 1XTAE at 30V o/n or 100V for 6 hrs. The gels were stained in 1XTAE containing 0.5 ug/ml EtBr for 20 min, following the run.

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels were prepared according to Maniatis et al. (1982). Restriction enzyme digestions (20-25 ul) in 1XPAGLB were run against DNA size markers. The gel concentration was chosen by considering the fragment sizes expected. The gels were run in 1XTBE for 7 mA o/n or 100 V for 4 hrs. The gels were stained with EtBr (0.5 ug/ml) in 1XTBE for 20 min, following the run.

Phenol Extraction

One half to equal volume of redistilled phenol, saturated with 10 mM Tris pH 7.4 / 0.1 mM EDTA at room

temperature, was vortexed for 30 sec. into the DNA sample. Following separation at room temperature (10-15 min), the contents were centrifuged for 2 min. The upper, aqueous phase was removed and transferred to a clean tube.

Chloroform-Isoamyl Alcohol Extraction

DNA samples were mixed for 30 sec. with an equal volume of chloroform-isoamyl alcohol (24:1 w/v). The emulsion was centrifuged for 2 min. The upper, aqueous phase was removed and transferred to a clean tube.

Ether Extraction

Diethyl ether (5X volume) was vortexed into the DNA samples for 5 min. The phases were separated by centrifugation for 2 min. The upper layer was removed and discarded. The residual ether was evaporated with a gentle air stream applied by Pasteur pipette. The procedure was repeated 3-4X.

Isoamyl Alcohol Extraction

A 3X volume of isoamyl alcohol was shaken into the DNA sample for 30 sec. Following phase separation (3 min), the upper phase was removed and discarded. The extraction was repeated 3-4X.

Ethanol Precipitation

A 1/10 volume of 3M NaAC pH 5.2-5.5 was mixed into the DNA solution. Then, 2.5 volumes of cold (-20°C), 95% EtOH were mixed in. The DNA was precipitated overnight at 20°C, or for 2-4 hrs at -70°C. The tube was centrifuged for 10-15 min in an Eppendorf microcentrifuge. The supernatant was poured off and discarded. The pellet was washed with 1 ml of cold (-20°C), 70% EtoH. The tube was recentrifuged for 2-5 min. The wash was repeated, if required. The DNA pellet was dried *in vacuo* for 5-10 min and resuspended in the appropriate volume of TE buffer.

D. Source of the rPL II cDNA Clones

The cDNA clones to rPL II mRNA : pRP52, 52A, 52-3, and 52B were kindly provided by Dr. M. L. Duckworth (Department of Physiology, University of Manitoba).

E. Restriction Enzyme Mapping of the rPL II cDNA Clones

The rPL II cDNA clones were initially excised from the plasmid vector with restriction enzymes and sized by electrophoresis against DNA size markers. Then, the clones were mapped for restriction enzyme sites to determine the orientation in the vector and to facilitate subcloning into the M13 vectors.

Digestions were performed with a series of restriction enzymes (see General Methods), in parallel with pAT153 DNA, for 17-24 hours. The digestion products were

electrophoresed against known DNA size markers and pAT153 fragments. The sizes of the cDNA fragments were computed from a standard curve of the migration distance versus the log of the number of base pairs of the reference DNA fragments. The location of the restriction enzyme sites was determined.

F. Subcloning rPL II cDNA Clones into M13 Vectors

Host Cells

The JM101 host cells were E. coli, strain K12. (Genotype: Δ (lac-pro) supE thiF' pro+ lac i^{qz} ΔM15 traD36.)

Materials

Difco Bacto-agar, Bacto-tryptone and Bacto-yeast extract were purchased from BDH (Poole, UK).

Media

Distilled deionized H_2O (dH_2O) was used for preparing the bacterial media.

Minimal Glucose Media Plates (30 plates/l)

Fifteen g of agar were mixed into 500 ml of H_2O. In a separate flask, 500 ml of 2X salt solution (20X stock :

210g K_2HPO_4; 20 g $(NH_4)_2SO_4$ and 10 g Na Citrate/1 H_2O were made. The two solutions were autoclaved separately.

After cooling (~55°C), the solutions were mixed under sterile conditions. Then, 1 ml of 1M $MgSO_4$ (sterile), 500 ul of 1% thiamine-HCl (sterile) and 10 ml of 20% glucose (sterile) were mixed in. The plates were poured and stored at 4°C.

2XTY Broth

Sixteen g of tryptone, 10 g of yeast extract, 5 g of NaCl and 2 ml of 4N NaOH /1 dH_2O. The solution was autoclaved and stored at room temperature.

TYE Media Plates (30 plates/1)

Ten g of tryptone, 5 g of yeast extract, 8 g of NaCl and 15 g of agar/1 dH_2O. The solution was autoclaved, cooled and plates poured. The plates were stored at 4°C.

H Top Agar

Ten g of tryptone, 8 g of NaCl and 8 g of agar/1 H_2O. The solution was autoclaved, dispensed aseptically into 50 ml aliquots and stored at room temperature.

Methods

Maintenance of JM101 F' Host Cells

The JM101 host cells were maintained on minimal glucose medium plates to maintain the F' phenotype since the F-pilus is required for M13 infection. Because of a chromosomal deletion, cells lacking the F' plasmid do not contain the proline gene required for survival on the minimal medium.

A loopful of an overnight culture was streaked to individual colony density on a minimal glucose plate. The plate was incubated at 37°C for 2 days. The plate was stored at 4°C. The cells were passaged, in this manner, at least once a month to maintain the F' phenotype.

Overnight Culture of JM101 Host Cells

A single colony from a minimal glucose plate was inoculated into 5 ml of 2XTY broth. The cells were grown overnight at 37°C in a shaking incubator.

Preparation of JM101 Competent Cells

The calcium-dependent procedure for bacteriophage DNA infection (Mandel and Higa, 1970) was employed for the introduction of recombinant M13 RF DNA.

A 1:100 dilution of an overnight culture was made in 2XTY broth. The cells were grown for 1-½ hrs in a 37°C shaking incubator to mid-log phase ($A_{580} < 0.4$).

In 50 ml Corning centrifuge tubes, 40 ml of cells were centrifuged at 1 K for 5 min at 5°C (I.E.C. PR6000). The supernatant was poured off and the pellet resuspended in 20 ml of cold, sterile 50 mM $CaCl_2$. The resuspension was incubated on ice for 20 min. The cells were recentrifuged for 5 min at 1 K at 5°C. The supernatant was poured off and the cells resuspended in 4 ml of cold, 50 mM $CaCl_2$ (i.e. 1/10 of the original volume). The cells were stored on ice in the 4°C cold room for a maximum of 4 days. Transformation efficiency was highest in the first 24 hours.

M13 Vectors

M13 mp8, mp9 (Messing and Vieira, 1982) and mp18 (Norrander et al., 1983) were used as vectors for subcloning to rPL II cDNA.

Messing and Vieira (1982) engineered M13 vectors which contain a cloning cassette of unique restriction enzyme sites for the insertion of cDNA in an intergenic region. The cassette does not interfere with the essential viral functions. In M13 mp8 and mp9, the cloning sites are

reversed to facilitate asymmetric cloning. M13 mp18, a vector similar to M13 mp8 and mp9, carries additional unique sites within the cloning cassette (Norrander et al., 1983).

Utilizing the M13 mp8 and mp9 vectors, rPL II cDNA fragments were cloned forcibly in some cases. Since the cloning sites of the two vectors are mirror images, fragments were inserted in opposite orientations permitting nucleotide sequence analysis of both strands in opposite directions.

Preparation of M13 RF DNA

M13 mp8 and mp9 RF DNA was isolated, in parallel preparations, from a large scale alkaline lysis preparation (modification of Birnboim and Doly, 1979) and purified on two CsCl gradients. The RF DNA was used as the cDNA cloning vehicle.

Growth and Collection of Cells

Five hundred ml of prewarmed (37°C) 2XTY broth were inoculated with 5 ml of a JM101 overnight culture. The culture was grown for 1-1½ hrs at 37°C in a shaking incubator. The culture was infected with 0.5-1 ml of M13 phage stock (see Infection of JM101 Host Cells) and grown

for an additional 4-6 hrs (A_{580} = 0.3). The cells were harvested in 500 ml bottles spun in a JA10 rotor (Beckman J2-21 centrifuge) for 10 min at 6 K rpm at 4°C. The supernatant was poured off and the pellet placed at -20°C overnight.

Lysis

The thawed pellet was resuspended in 9 ml of lysis buffer (25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose) with a Pasteur pipette. One ml of fresh lysozyme (20 mg/ml) was added and the mixture was incubated on ice for 15 min. Ten ml of fresh 0.4 N NaOH / 2% SDS was gently swirled into the solution. The samples were incubated on ice for an additional 15 min. In a 30 ml Corex tube, the samples were centrifuged in a JA20 rotor (J2-21) at 15 K rpm at 4°C for 20 min. The clear supernatant was transferred to a 50 ml Corning centrifuge tube and extracted with an equal volume of phenol/chloroform shaken into the solution for 5 min. The emulsion was separated by centrifugation at 3 K rpm (PR6000), at 4°C for 10 min. The upper, aqueous phase was transferred to a 150 ml Corex bottle and precipitated with 2.5 volumes of cold, 95% EtoH and 1/10 volume 3M NaAc pH 4.8. The solution was placed at -20°C o/n.

The precipitate was collected by centrifugation in a JS7.5 rotor (J2-21) at 7 K rpm at 4°C for 30 min. The ethanol was poured off and the pellet was washed with 25 ml cold, 70% EtoH. The sample was respun for 10 min. The ethanol was poured off and the pellet dried *in vacuo* for 15 min. The DNA was resuspended in 20 ml of 10 mM Tris pH 8.0/1mM EDTA.

CsCl Gradients

CsCl (KBI-technical grade), 1 g/ml, was dissolved in the DNA solution in a 50 ml Corning centrifuge tube wrapped in foil. When dissolved, 2.0 ml of EtBr (10 mg/ml-fresh, filtered) was added. The mixture was placed in 40 ml Beckman polyallomer quick-seal tubes. The tubes were filled with mineral oil, balanced and heat-sealed. The phage DNA was separated from the *E. coli* DNA by centrifugation in a Ti60 rotor (Beckman Model L5-65 ultracentrifuge) at 35 K rpm for 60 hrs at 20°C.

In the dark, the lower phage band was visualized by long-wave UV light. The top of the tube was pierced. The phage band was recovered by insertion of a 3 ml syringe fitted with an 18 gauge needle just below the band. The phage DNA was slowly drawn off. The EtBr was removed with 3-4 isoamyl alcohol extractions. The volume was increased

to approximately 5 ml with sterile H_2O. The DNA was precipitated with 2.5 volumes of 95% EtoH and a 1/10 volume 3M NaAc pH 5.2 in 30 ml siliconized Corex tubes. The solution was placed at -20°C overnight.

The DNA was recovered by centrifugation at 12 K rpm at 4°C for 20 min. The pellet was washed with 10 ml of 70% EtoH and desiccated in vacuo for 15 min. The pellet was resuspended in 20 ml of 10 mM Tris pH 8.0/1 mM EDTA and prepared for a second gradient.

The second CsCl gradient was carried out in exactly the same way as the first. The recovered DNA pellet was resuspended in 250 ul of 10 mM Tris pH 7.4/0.1 mM EDTA. A 1:100 dilution of DNA in sterile H_2O (3 ul + 297 ul) was made and the preparation was quantified by spectrophotometry (A_{260}). The ratio of A_{260}/A_{280} was calculated as an indication of purity.

The DNA was diluted to 1 ug/ul and stored at -20°C in 20 ul aliquots.

Cleavage of the M13 RF DNA

M13 RF DNA (2.0 ug) was digested with the appropriate restriction enzyme(s) and buffer(s) in a total volume of 20 ul. Single digestions were completed in 3 hrs and double digestions in approximately 6 hrs to prevent DNA

degradation, by exonuclease contaminants in the restriction enzyme solutions.

Complete digestion was verified on a 1% agarose mini-gel. The cleaved DNA (100 ug/ul) was stored at 4°C for up to 1 wk.

Calf Intestinal Phosphatase (CIP) Treatment

M13 RF DNA cleaved with a single restriction enzyme or having two "blunt ends" was treated with CIP to prevent self-ligation.

Materials

CIP was purchased from Boehringer Mannheim (Montreal, PQ).

Method

One hour before the completion of restriction enzyme digestion of the M13 vector DNA, 2.5 units (~1 ul) of CIP was mixed into the reaction. The DNA was incubated at 37°C for 1 hr.

The completed reaction was incubated at 65-70°C for 10 min. One hundred ul of 10 mM Tris pH 7.5/0.1 mM EDTA was added. The DNA sample was extracted 2X with phenol (50 ul)

and 3-4X with diethyl ether (500 ul). The final concentration of M13 RF DNA was 10 ng/ul.

Preparation of the rPL II cDNA for Subcloning

The cDNA in the plasmid (2.0 ug) was digested with the appropriate restriction enzyme(s) and buffer(s) for 3-6 hrs in a total volume of 20 ul.

Complete digestion was verified on a 1% agarose mini-gel. The cleaved DNA (100 ug/ul) was stored at 4°C for up to 1 wk.

Ligations

Materials

T4 DNA ligase was obtained from Boehringer Mannheim (Montreal, PQ).

Reagents

10X Ligation Buffer (10XLB): 1M Tris pH 7.5, 1M MgCl , 150 mM DTT and 10 mM rATP in sterile H_2O.

Methods

Self-Ligation of M13 RF DNA

As a control for the ligation efficiency, M13 RF DNA cleaved with a single restriction enzyme was self-ligated.

Double cut or CIP treated vector DNA will not religate.

Twenty ng of cleaved M13 RF DNA were mixed with 0.5 ul of 10XLB and 1 unit (0.5-1.0 ul) of T4 DNA ligase. The reaction was brought up to a final volume of 5 ul with sterile H_2O. The reaction was incubated at 14-16°C overnight. The final concentration of M13 RF DNA was 4 ng/ul.

Ligation of M13 RF DNA with rPL II cDNA Fragments

A 3:1 molar ratio of rPL II cDNA fragments to M13 vector was used in the ligations. Twenty ng of M13 RF DNA and 100 ng of cDNA were mixed with 0.5 ul of 10XLB and 1 unit (0.5-1.0 ul) of T4 DNA ligase. The reaction was brought to a final volume of 5 ul with sterile H_2O. The reaction was incubated at 14-16°C o/n. The final concentration of M13 RF DNA was 4 ng/ul. The amount of enzyme was doubled for "blunt end" ligations since ligation of "blunt ends" is much less efficient than the ligation of "sticky ends".

Transformation of JM101 Host Cells

When M13 infected JM101 host cells are plated out on a lawn of uninfected cells, transformants are characterized by plaques or regions of retarded growth. Since plaque

formation does not differentiate between cells infected with native M13 and those infected with recombinant M13 bearing foreign cDNA, an additional colorimetric selection method is used.

The F′ JM101 host cells carry a defective β-galactosidase gene. Introduction of native M13 into the host cells results in a functional β-galactosidase gene by complementation since M13 carries the missing portion of the β-galctosidase gene. The gene can be induced by introduction of the inducer, IPTG. Addition of the lactose analogue, BCIG, to the medium results in plaques with a blue perimeter since the β-galactosidase cleaves the BCIG to a blue dye, bromochloroindoxyl.

Insertion of foreign cDNA into the M13 cloning sites renders the β-galactosidase gene non-functional. Therefore, plaques from the infection with recombinant M13 phage are colorless and can be differentiated from the blue non-recombinant plaques.

Materials

Bromo-chloro-indolyl-beta-galactoside(BCIG) was purchased from Sigma Chemical Company (St. Louis, MO). Isopropyl-beta-thio-galactopyranoside (IPTG) was obtained from Boehringer Mannheim (Montreal, PQ).

Method

The following amounts of DNA were used to transform the competent host cells: 2 ul of 1 ng/ul M13 vector (control for transformation efficiency); 5 ul of 1 ng/ul cut and religated M13 vector (control for ligation efficiency); 5 ul of 4 ng/ul "blunt end" ligation; 2 ul and 5 ul of 1 ng/ul "sticky end" ligation; and 2 ul of a 1:100 dilution, in 10 mM Tris pH 7.4/1 mM EDTA, of SSDNA (isolated from medium of infected cells) or DSDNA (isolated from cellular pellet of infected cells).

The appropriate amount of DNA was mixed with 300 ul of JM101 competent cells in 10 ml, sterile culture tubes and incubated on ice for 40 min. The cells were heat-shocked in a 42°C H_2O bath for 3 min.

At room temperature, 200 ul of fresh JM101 (1:10 dilution of an overnight culture in 2XTY grown for 1½-2 hrs, to mid-log phase, at 37°C in a shaking incubator), 20 ul of IPTG (20 mg/ml in sterile H_2O) and 20 ul of BCIG (20 mg/ml in dimethylformamide) were added to each tube. The tubes were vortexed. Three ml of molten, well-mixed H top agar (~55°C) were added and carefully mixed in. The mixture was poured onto TYE plates and spread over the surface. The plates were incubated at 37°C overnight. Plaques were observed on the following day.

Uncut vector and religated vector were expected to give more than 500 blue plaques per plate.

Selection of Recombinant Clones

The colorimetric plaque assay was employed for the selection of recombinant clones. However, in some cases, the assay was not an adequate means of selection. Some restriction enzyme digestions of the rPL II cDNA led to the insertion of pAT153 DNA fragments into the M13 vector. Therefore, plaques were colorless yet did not contain rPL II cDNA recombinants.

Colorimetric Plaque Assay

In cases where the plaque assay was an adequate means of selection, 20 colorless plaques of each subclone were picked.

Plaque Hybridization

Recombinant clones which could not be selected solely on the basis of the colorimetric plaque assay were identified using the Benton and Davis (1977) plaque hybridization method. The phage DNA in the plagues was transferred to nitro-cellulose (NC) filters and hybridized to nick-translated (Rigby et al., 1977) rPL II cDNA probes.

Materials

Nitrocellulose (NC) discs (BA 85; 0.45 um; 82.5 mm) were purchased from Schleicher and Schuell (Keene, NH). The nick translation kit was purchased from Amersham (Oakville, Ont). The α-[^{32}P]-dCTP (3000 Ci/mMol) was obtained from New England Nuclear (Lachine, PQ).

Reagents

1XSSC: 0.15 M NaCl; 0.015 M NaCitrate pH 7.0

4XDH: A modification of Denhardt's solution (Denhardt, 1966). 8 g Ficoll 400; 8 g PVP-360; and 8 g BSA / l

SSC+DH: 6XSSC; 1XDH; and 0.1% SDS

SSC+DH+: 6XSSC; 1XDH; 0.1% SDS; 1 ug/ml poly(A); and 50 ug/ml denatured salmon sperm DNA

stopping buffer: 20 mM EDTA; 0.2% SDS; and 2 mg/ml denatured salmon sperm DNA

Methods

DNA Transfer

The plates were chilled for at least 2 hrs at 4^{o}C. A NC disc was placed on a replica plating block and marked with the number of the plate and 3 asymmetric lines. The plate was placed on top of the NC and removed immediately,

with the disc adhering to it. While the NC was wetting, the bottom of the plate was marked with corresponding asymmetric lines. The filter was stabbed through with a fine needle, dipped in black India ink, in 3 places. The disc was carefully lifted off the plate.

The transferred DNA was denatured and fixed by immersion, phage side up, into a solution of 0.1 N NaOH/1.5 M NaCl, for at least 30 sec. The filter was neutralized by 2 immersions in 0.2 M Tris pH 7.5 and a final immersion in a solution of 2XSSC. The solution volume was 50 ml/filter. The filters were inverted in each subsequent immersion. The filters were air dried at room temperature on a sheet of Whatman 3 MM paper and baked for 2 hrs in vacuo at 80°C.

Prehybridization

The filters were wet in a plastic box containing 250 ml of 6XSSC (room temperature). The filters were prehybridized in a sealable plastic bag (Dazey Seal-a-Meal) in a volume of 6 ml/filter in a 65°C shaking water bath as follows: 6XSSC for 30 min; SSC+DH (prewarmed and degassed) for 3 hrs; and SSC+DH+ (prewarmed and degassed) for at least 1 hr.

When time was limited, the filters were wet in 6XSSC

and prehybridized in the final solution (SSC+DH+) for 3-4 hrs.

Nick Translation

Nick translations were performed following the kit instructions with minor modifications. Fifty to 200 ng of recombinant plasmid were combined with 4 ul of solution #1 (nucleotide buffer solution containing dNTP's in Tris/HCl pH 8.0, $MgCl_2$ and 2-mercaptoethanol) and 7-10 ul of [^{32}P]-dCTP (70-100 uCi). The reaction volume was brought up to 20 ul with sterile dH_2O. Two ul of solution #2 (enzyme solution containing DNase I and DNA polymerase I in Tris/HCl pH 7.5, $MgCl_2$, glycerol and BSA) was vortexed into the reaction and the contents spun down. The reaction was incubated at 14-16°C for 90 min. The reaction was stopped with the addition of 20 ul of stopping buffer and 4 ul of 10 mg/ml yeast tRNA. The mixture was vortexed, spun down and incubated at 65-68°C for 15 min.

A 1 ul aliquot was removed for TCA precipitation.

The reaction (46 ul) was separated by chromatography on a 3 ml column of Sephadex G-100. The elution buffer was 10 mM Tris pH 7.4/1 mM EDTA. The nick translated DNA, in the first peak, was collected in 2 drop fractions and

pooled (~1 ml). The fractions from the second peak of unincorporated dNTP's were discarded.

A 1 ul aliquot of the pooled fractions was removed for TCA precipitation.

Trichloroacetic Acid (TCA) Precipitation

In two 10 ml glass tubes, the 1 ul aliquots of nick translated DNA, 1 ul prior to and 1 ul following column chromatography, were vortexed into a solution of 3 ml cold 10% TCA, 0.1 ml 10 mg/ml BSA and 0.5 ml 0.2 M NaPPi. The samples were chilled on ice for 15 min.

The precipitate was collected by filtration through Whatman GF/C filters (2.4 cm) using a sintered glass filter-chimney apparatus (Millipore). The samples were pipetted onto the filters, under suction, and rinsed with 20 ml of cold 5% TCA. The filters were dried on tin foil, under a heat lamp. The filters were counted in Omnifluor (New England Nuclear: 4 g/l of toluene) by liquid scintillation spectrometry.

Hybridization

The nick translated probe was denatured in a boiling H_2O bath for 5 min. The required amount (500,000 cpm/ml)

was mixed into a prewarmed (65°C), degassed solution of SSC+DH+ (3 ml/filter).

The prehybridization fluid was removed from the bag of filters and the probe solution poured in. The bag was resealed and submerged in a 65°C shaking H_2O bath. The hybridization was incubated for 17-24 hrs.

Washing

The hybridization fluid was removed from the bag of filters and stored at -20°C for 1 week, to be reused in subsequent hybridizations.

The filters were washed stringently, in plastic boxes in a shaking H_2O bath, as follows: 4X at room temperature for 10 min in 2XSSC, 0.1% SDS (25 ml/filter); and 2X at 65°C for 90 min in 1XSSC, 0.1% SDS (25 ml/filter).

Audioradiography

The washed filters were wrapped in plastic wrap (numbered side up). Pieces of tape were marked with radioactive ([^{32}P]) India ink at the location of the asymmetric lines. The filters were numbered with the 'hot' ink. The ink was allowed to dry and covered with Scotch tape. The labeled filters were placed on a sheet of Whatman 3MM paper in a cassette (Picker), covered with a

sheet of film (Kodak X-Omat AR) and a screen (Dupont Lightning Plus) and exposed at -70°C for 2 hrs. Exposures were increased if 2 hrs was not sufficient to detect positive plaques.

The developed films were aligned with the filters, using the marks left by the radioactive ink. The films were marked, with a red felt pen, in the positions of the asymmetric dots on the filters. The films were inverted and placed on a light box. The agar plates were aligned with the marks on the film and positive plaques corresponding to colorless plaques were identified.

Preparation of SSDNA Template

Infection of JM101 Host Cells

A 1:100 dilution of a JM101 overnight culture in 2XTY broth was grown for 1-1½ hrs at 37°C in a shaking incubator.

Aliquots of 1 ml of the fresh culture were dispensed into sterile, 10 ml culture tubes. Each tube was inoculated with the viral particles from a single plaque, by stabbing a sterile, wooden toothpick into the centre of a plaque. (The fresh culture could also be infected with titred phage stock.) The tubes were shaken at 37°C for 4½ hrs.

The infected cultures were transferred to 1.5 ml tubes (Eppendorf) and centrifuged for 15 min in a Beckman microcentrifuge. The supernatant (= phage stock) was transferred to a clean tube. If the phage stock was not processed immediately, it was stored at 4°C for a maximum of 24 hrs and recentrifuged to remove bacterial cells, prior to SSDNA isolation. For long term storage of the phage stock 10ul of $CHCl_3$ was vortexed in. The stock was stored at 4°C and titred prior to re-infection of the host cells.

The pellet (= infected cells with DSDNA) was stored at -20°C.

SSDNA Isolation

Materials

Carbowax PEG 8000 (previously called 6000) was obtained from Fisher Scientific, Ltd. (Fair Lawn, NJ).

Method

The SSDNA isolation was performed at room temperature and all solutions were, also, at room temperature.

Following centrifugation of the phage stock, 200 ul of 2.5 M NaCl-20% PEG were added to each supernatant and mixed. The tubes were allowed to stand on the bench for 10-15 min. The viral precipitate was collected via a 10 min centri-

fugation. The supernatant was removed and the residual PEG solution sucked off the pellet with a drawn out capillary attached to a 5 ml syringe. The tubes were respun for 10 sec and any remaining PEG removed.

The viral pellet was resuspended in 100 ul of 10 mM Tris pH 7.4/0.1 mM EDTA by vortexing for 10 sec, letting stand for 5 min and vortexing again for 10 sec. The viral protein coat was removed by phenol extraction (50 ul), followed by chloroform-isoamyl alcohol extraction (~100 ul). The DNA was EtOH precipitated at -20°C with 10 ul of 3 M NaAc pH 5.5. and 250 ul of 95% EtOH.

The DNA precipitate was pelleted, washed and dried *in vacuo*. The DNA was resuspended in 25 ul of 10 mM Tris pH 8.0/0.1 mM EDTA.

A 1-2 ul aliquot of the SSDNA was electrophoresed on a 1% agarose mini-gel, with M13 SSDNA, to monitor the template DNA recovery. Recombinant SSDNA had a reduced electrophoretic mobility. The remainder of the sample was stored at -20°C.

The SSDNA isolation was scaled up when more template was required.

DSDNA Isolation

In some cases, the DSDNA was isolated from the pellet of infected cells in order to characterize the recombinant subclone DNA by restriction enzyme analysis. The small scale alkaline lysis method (Maniatis et al., 1982) was used.

G. DNA Sequence Analysis

The efficiency of the Sanger dideoxy method has been improved by utilizing several modifications.

An M13 specific universal primer (Duckworth, et al., 1981) was used. The 17mer primer can be used for any DNA fragment cloned into M13 vectors since it is complementary to a region in the 3′ direction from the unique cloning sites. Therefore, the primer is extended 5′ to 3′, by the Klenow enzyme, using as template the cloning site containing the insert cDNA.

Buffer gradient gels and [^{35}S] as described by Biggin et al. (1983) were used to increase the efficiency of the sequencing method. The buffer gradient gels (5X buffer gradient) reduce the vertical band spacing with a high buffer concentration at the bottom of the gel. By using [^{35}S] instead of [^{32}P] as the radioactive label, the band resolution is increased. Sequence reactions can also be

stored for additional gel runs due to the longer half-life of the [^{35}S]. By combining the buffer gradient gels and the [^{35}S] label, the amount of DNA sequence information derived from a single gel was increased.

Annealing M13 Primer to SSDNA Template

Materials

The M13 specific 17mer primer was obtained from Collaborative Research Laboratories (Lexington, MA).

Method

For each SSDNA template, 7 ul of DNA was placed in a 0.5 ml Eppendorf tube and mixed with 2 ul of primer (0.1 pmol/ul of sterile H_2O, stored at -20°C) and 1.5 ul of 100 mM Tris pH 8.0/50 mM $MgCl_2$. The contents of the tube were spun down in a microfuge and incubated in a 60°C oven for 1 hr. The annealed primer-template was stored at -20°C, if it was not used immediately. The reaction was scaled up when more primer-template was required.

Sequencing Reactions

Materials

The deoxynucleotides (dNTP's) were obtained from Sigma Chemical Co. (St. Louis, MO). The dideoxynucleotides

(ddNTP's) were purchased from P. L. Biochemicals, Inc. (Milwaukee, WI). The Klenow fragment, large fragment *E. coli* DNA polymerase I was obtained from Amersham (Oakville, Ont.). The α-[^{35}S]-dATP, specific activity 500 Ci/mMol, was purchased from New England Nuclear (Lachine, PQ).

Reagents

All H_2O was sterile, deionized, double-distilled.

ddNTP stock solutions: 10 mM in 5 mM Tris pH 7.5/0.1 mM EDTA

ddNTP working solutions: 0.10 mM ddGTP; 0.025 mM ddATP; 0.25 mM ddTTP; and 0.05 mM ddCTP

dNTP stock solutions: 100 mM and 0.5 mM in H_2O

'cold' dNTP mix: 0.5 mM of each dNTP in H_2O

N^o solutions:

	G^o	A^o	T^o	C^o
0.5 mM dGTP	1 ul	20 ul	20 ul	20 ul
0.5 mM dTTP	20 ul	20 ul	1 ul	20 ul
0.5 mM dCTP	20 ul	20 ul	20 ul	1 ul
50 mM Tris pH 8.0	5 ul	5 ul	5 ul	5 ul

Formamide dye mix: 95% deionized formamide; 10 mM EDTA; 3% bromophenol blue; and 3% xylene cyanol FF. (The solutions were dispensed in 50 ul aliquots and stored at -20°C).

Single Track (T-track) Sequence Analysis

Initially, annealed primer-template was subjected to a single sequencing reaction (T) in order to evaluate the template quality and for screening recombinant clones.

T-track Sequence Reactions (20 clones)

In a siliconized 10 ml glass tube, 21 ul of [^{35}S]-dATP were dried *in vacuo* for 20 min. The isotope was resuspended in 25 ul of ddTTP and 25 ul of T solution. The annealed primer-templates were distributed in 2 ul aliquots into 20 capless, 1.5 ml Eppendorf tubes. Two ul of the resuspended isotope was added to each tube. The tubes were placed in a Beckman microcentrifuge.

The Klenow enzyme was diluted to 0.1-0.5 units/ul with cold 10 mM Tris pH 8.0, thoroughly mixed, spun down and immediately distributed in 2 ul aliquots (using a siliconized pipette tip) on the lip of each tube. The enzyme was immediately spun into the tube (time = 0). At time = 17 min, 2 ul of the cold dNTP mix was added to the lip of each tube. At time = 20 min, the mix was spun into the tube. At time = 32 min, 5 ul of the formamide dye mix was distributed to the lip of each tube. At time = 35 min, the tube was spun and the reaction stopped with the dye mix.

The caps were replaced on the tubes. The reactions were denatured by heating for 3-5 min in a boiling H_2O bath. The samples were loaded immediately onto a gel.

When the samples were not electrophoresed immediately, or sample remained after loading, the tubes were stored at -20°C for 1 wk. The DNA was denatured in the boiling H_2O bath prior to gel loading.

The GATC Sequence Reactions

Two ul of each annealed primer-template was dispensed into 4 capless 1.5 ml Eppendorf tubes (labeled G, A, T, C). In 4 siliconized 10 ml tubes (labelled G, A, T, C), 5 ul of [^{35}S]-dATP was dried *in vacuo*. The isotope was resuspended in 5 ul each of the corresponding ddNTP and N solution.

The resuspended isotope was dispensed in 2 ul aliquots into each of the 4 corresponding tubes containing the primer-template. The tubes were placed in a Beckman microcentrifuge (arranged according to G, A, T, C).

The Klenow enzyme was diluted to 0.1-0.5 units/ul with cold 10 mM Tris pH 8.0, thoroughly mixed, spun down and immediately distributed in 2 ul aliquots (using a siliconized pipette tip) to the lip of each tube. The enzyme was immediately spun into the tube (time = 0). At time = 17 min, 2 ul of the cold dNTP mix was added to the

lip of each tube. At time = 20 min, the mix was spun in. At time = 32 min, 5 ul of the formamide dye mix was distributed to the lip of each tube. At time = 35 min, the reaction was stopped with the dye mix.

The caps were replaced onto the tubes. The reactions were denatured by heating for 3-5 min in a boling H_2O bath. The samples were loaded immediately onto a gel.

When the samples were not electrophoresed immediately, or sample remained after loading, the tubes were stored at -20°C for 1 wk. The DNA was denatured in the boiling H_2O bath prior to gel loading.

Some recombinant clones displayed regions of secondary structure, due for example to homopolymer tails. Subsequent sequencing reactions were incubated at 30-50°C in a H_2O bath, following addition of the Klenow enzyme. The number of units of Klenow enzyme per reaction was also increased.

Sequencing Gels

T-track analysis was performed on 'regular' gels whereas GATC sequence reactions were electrophoresed on buffer gradient plus.

Materials

The glass plates (3mm x 20cm x 40cm; 1 plate/pair

'rabbit eared' with a 16.5 cm x 2cm notch) and vertical slab gel apparatus were purchased from Raven Scientific Ltd. (Haverhill, UK). Replacement glass plates were obtained from Kildonan Glass (Winnipeg, MB). Spacers (1cm x 40cm) and combs (20 well) were cut from 0.35 mm thick Plastikard sheets obtained from Slater's Ltd. (Matlock Bath, Derbyshire, UK). Gibco (Burlington, ON) supplied the BRL sequencing gel sealing tape. The dimethyldichlorosilane siliconizing solution and analytical grade Amberlite MB-1 monobed resin were supplied by BDH (Poole, UK). Ultra pure Schwarz-Mann urea was purchased from Canadian Scientific Products (London, ON).

Preparation of the Gel Plates

The gel plates were cleaned scrupulously to prevent bubble formation. The plates were washed with soapy H_2O and 95% EtOH. When dry, the plates were cleaned with Windex. The top, notched plate was siliconized to aid in gel pouring and prevent tearing of the gel. In a fume hood, 5-10 ml of siliconizing solution was wiped over the plate and allowed to dry for 10-20 min. The plate was rinsed with H_2O, 95% EtoH and cleaned with Windex. The side spacers were clamped into the plates and the bottom and the sides were sealed with the gel sealing tape.

Preparation of the Sequencing Gel Solutions

Regular Gel Solution

Regular gel solutions (6% acrylamide/8M urea) for T track analysis were made up in 300 ml batches. This was sufficient solution for 6 gels. Urea (144 g), acrylamide (17.1 g) and bis-acrylamide (0.9 g) were brought to a volume of 250 mL with H_2O and dissolved by vigorous stirring on low heat for 30 min. The solution was deionized with 10 g of Amberlite MB-1. The resin was stirred gently with the solution for 30 min, then removed by filtration through a sintered glass filter. The solution was then brought to 300 ml with 30 ml of 10XTBE buffer and H_2O, and fine particles were removed by filtration through a nitrocellulose filter (Millipore HA 0.45 um). The gel mix was stored at 4°C in the dark for up to 1 wk.

Buffer Gradient Gel Solutions

GATC sequence reactions were analyzed on 5X buffer gradient gels (6% acrylamide/8M urea). Solutions for 8 gels were made once a week. The first solution, 0.5XTBE, was made up of urea (153.6 g), acrylamide (8.2 g) and bis-acrylamide (0.5 g). The reagents were brought to a volume of 300 ml with H_2O and stirred vigorously on low heat for 30 min. When the solids had dissolved, 15 g of Amberlite MB-1 resin were added. The solution was stirred

gently for 30 min. The resin was removed with a sintered glass filter. The solution was brought to a final volume of 320 ml with 16ml 10XTBE buffer and H_2O. The gel mix was filtered through a nitrocellulose filter (Millipore HA 0.45 um) and stored at 4°C in the dark for 1 wk.

The second solution, 2.5 XTBE, was made up of urea (28.8 g), acrylamide (3.4 g), bis-acrylamide (0.2 g) and sucrose (6.0 g). The reagents were brought to a volume of 40 ml with H_2O and stirred vigorously on low heat for 30 min until dissolved. Then, 4 g of Amerlite MB-1 resin were added. The solution was stirrd gently for 30 min. The resin was removed with a sintered glass filter. The solution was brought to a final volume of 60 ml with 15 ml 10XTBE buffer, 600 ul 10% bromphenol blue and H_2O. The gel mix was filtered through a nitrocellulose filter (Millipore HA 0.45 um) and stored at 4°C in the dark for a maximum of 1 week.

Pouring the Sequencing Gels

Regular Gel

For one regular gel, 35 ul TEMED and 350 ul fresh 10% ammonium persulfate were mixed into 50 ml of the gel solution. The solution was taken up in a 50 ml syringe.

With the plates tilted at a 30° angle from the horizontal and sloped toward one side, the solution was poured in one side. As the bottom filled, the plates were straightened. The plates were filled to the top. Then, the plates were rested horizontally on the bench, the comb, cut from the same piece of Plastkard as the spacers, inserted and the sides clamped. The top of the gel was covered with a sheet of plastic wrap to aid in the polymeriztion and prevent desication of the wells. The gel was polymerized overnight, although 1 hr was sufficient when necessary.

Buffer Gradient Gel

For one gel, 59 ul TEMED and 148 ul fresh 10% ammonium persulfate were mixed with 30 ml of the 0.5XTBE solution. In another beaker, 14ul TEMED and 35 ul fresh 10% ammonium persulfate were mixed with 7 ml of the 2.5XTBE gel solution. In a 50 ml syringe, 23 ml of the 0.5XTBE solution were taken up and set aside. In a 20 ml pipette, 4 ml of the 0.5XTBE solution was taken up, followed gently by 6 ml of the 2.5XTBE solution. The two phases were mixed slightly with 1 or 2 bubbles. With the plates tilted at a 45° angle from the horizontal and sloped toward one side, the solution in the 20 ml pipette was poured down one side in a 5 cm stream. As the bottom filled, the plates were straightened and

lowered to a 15° angle to halt the flow. The solution in the 50 ml syringe was poured in a steady stream on the same side as the first solution and then fanned across to the centre. The plates were filled to the top. Then, the plates were rested horizontally on the bench and the comb, cut from the same piece of Plastikard as the spacers, was inserted and the sides clamped. The top of the gel was covered with a sheet of plastic wrap. The gel was polymerized overnight.

Electrophoresis of the Sequencing Reactions

The comb was carefully removed and the plates and well slots rinsed with H_2O. The tape at the bottom of the gel was slit. The plates were clamped to the gel apparatus, with the notch against the upper buffer chamber. The buffer chambers were filled with 1XTBE buffer, made from the same stock as the 10XTBE in the gel solution since conductivity varied with each batch, and the wells flushed with a Pasteur pipette to remove unpolymerized acrylamide and any urea which had leached from the gel.

The wells were flushed again just prior to loading. The heat-denatured samples (2-4 ul) were taken up in a finely drawn out, precalibrated, capillary tube, and layered into the wells. The order of loading was GATC. The samples were immediately run into the gel (5 min at 28 mA).

The power was then shut off and 0.6 cm aluminum plates were clamped to the front of the apparatus. The aluminum plates, slightly shorter than the gel, ensured even heat distribution during electrophoresis. The upper buffer chamber was topped up and electrophoresis continued at 28 mA.

T-track reactions, on regular gels, were run until the bromophenol blue dye front had reached the bottom of the gel (1-1/2-2 hrs). The complete GATC sequence reactions on buffer gradient gels were run until the bromophenol blue dye front, corresponding to fragments of approximately 20 bp had reached the bottom in 2-1/2-3 hrs. Longer clones were electrophoresed until the xylene cyanol dye front had reached the bottom of the gel (5-6 hrs.)

Autoradiography

Upon completion of the run, the plates were removed from the gel apparatus and the tape taken off. With a spatula, the top, notched plate was pried gently off the gel. The gel, resting on the bottom plate, was covered with a piece of plastic mesh and fixed in a solution of 10% acetic acid/10% methanol for 20 min. The gel was lifted out of the fixative, drained and covered with a sheet of 3MM paper (Whatman). The gel adhered to the paper and peeled off the bottom glass plate. The gel was then placed on another sheet of 3MM paper and covered with plastic wrap.

The gel was dried on a slab gel dryer (BioRad) under vacuum for 1 hr. Heat (80°C) was applied for the final 30 min. The plastic wrap was removed. The dried gel was placed in a cassette, covered with a sheet of film (Kodak X-Omat XAR) and exposed overnight at 20°C. Longer exposures were performed when necessary.

Reading the Gels

The sequence of nucleotides of the clones was read from the autoradiogram of the sequencing gels. Starting with the smallest fragments, corresponding to first band at the bottom of the autoradiogram, the gel was read upwards and the track in which each band appeared recorded. In this way, the complementary strand of the template was read 5' to 3' from the primer.

H. Computer Analysis of the Nucleotide Sequence

The sequence was analyzed by the DNA/protein sequence analysis software, distributed by International Biotechnologies, Inc. (New Haven, CT) and written by James M. Pustell (Pustell and Kafatos, 1982a, 1982b, 1984), on an IBM-PC.

The secondary structure of the predicted protein was derived by a secondary structure analysis program,

described by Garnier et al. (1978) and kindly provided by Dr. W. G. Baldwin (Department of Chemistry, University of Manitoba), on an Apple II C.

IV. RESULTS

To obtain the complete sequence of the cDNA to rPLII mRNA, four cDNA clones from Day 18 placental libraries were sequenced.

The initial clone, pRP52, was found to represent only the 3′ third of the mRNA. The sequence translated into a single open reading frame, including a TGA stop codon. Comparison to rPRL mRNA (Cooke et al., 1980) showed extensive homology to the 3′ end of the mRNA translation. The rPRL was used as a reading frame reference in subsequent translations.

To obtain a more complete cDNA, pRP52A was selected and analyzed. Sequence analysis showed pRP52A to be a more 5′ clone, however it did not extend to the expected methionine codon at the translation intitiation site. Clones pRP52-3 and pRP52B were also analyzed. Although they both provided more 5′ information, they also did not contain the initiator methionine.

A. Restriction Enzyme Analysis

Restriction enzyme digestions were performed on all the clones for estimation of the cDNA insert size. The

estimated length and actual length (determined by sequence analysis) of the cDNA inserts are shown in Table 5.

PRP52A was extensively mapped for restriction enzyme sites because it was the longest clone. A polyacrylamide gel of digested pRP52A is shown in Figure 2. A summary of the enzymes with which pRP52A was analyzed is shown in Table 6.

B. M13 Subcloning

A summary of the subcloning of the cDNA into the M13 vectors is shown in Table 7. The subclones which were generated covered both strands and the restriction enzyme sites used for subcloning were overlapped.

C. Nucleotide Sequencing

A representative buffer gradient sequencing gel of a pRP52A subclone is shown in Figure 3. A summary of the sequencing strategy is shown in Figure 4. The sequence of the message and anti-message strands was read completely. The sequence information from the four cDNA clones; pRP52, 52A, 52-3 and 52B, not including the poly A tail, totalled 807 bp. The length of the mature message is estimated to be 1 kb (Duckworth et al., 1984). Therefore, 80% of the message has been sequenced.

TABLE 5

The estimated length vs. the length determined by sequence analysis of the inserts of the cDNA clones

RPLII cDNA CLONE	ESTIMATED LENGTH (BP)	LENGTH DETERMINED BY SEQUENCE ANALYSIS (BP)
pRP52	300	308
pRP52A	740	693
pRP52-3	330	376
pRP52B	500	433

The cDNA insert was cleaved from the unique cloning site of the plasmid vector, pAT153. The inserts in pRP52, 52A and 52-3 were released via PstI digestion, while that in pRP52B was released via EcoRI digestion. The digested DNA was sized according to its electrophoretic mobility against DNA size markers.

Figure 2. 3.5% polyacrylamide gel of PRP52A and PAT153 digested with HincII and HincII/PSTI.

LANE	SAMPLE
1	ØX174 DNA - HaeIII digest
2	pRP52A - PstI/HincII digest
3	pAT153 - PstI/HincII digest
4	pRP52A - HincII digest
5	pAT153 - HincII digest

The HincII site in pRP52A was localized using the information on this gel.

Figure 2

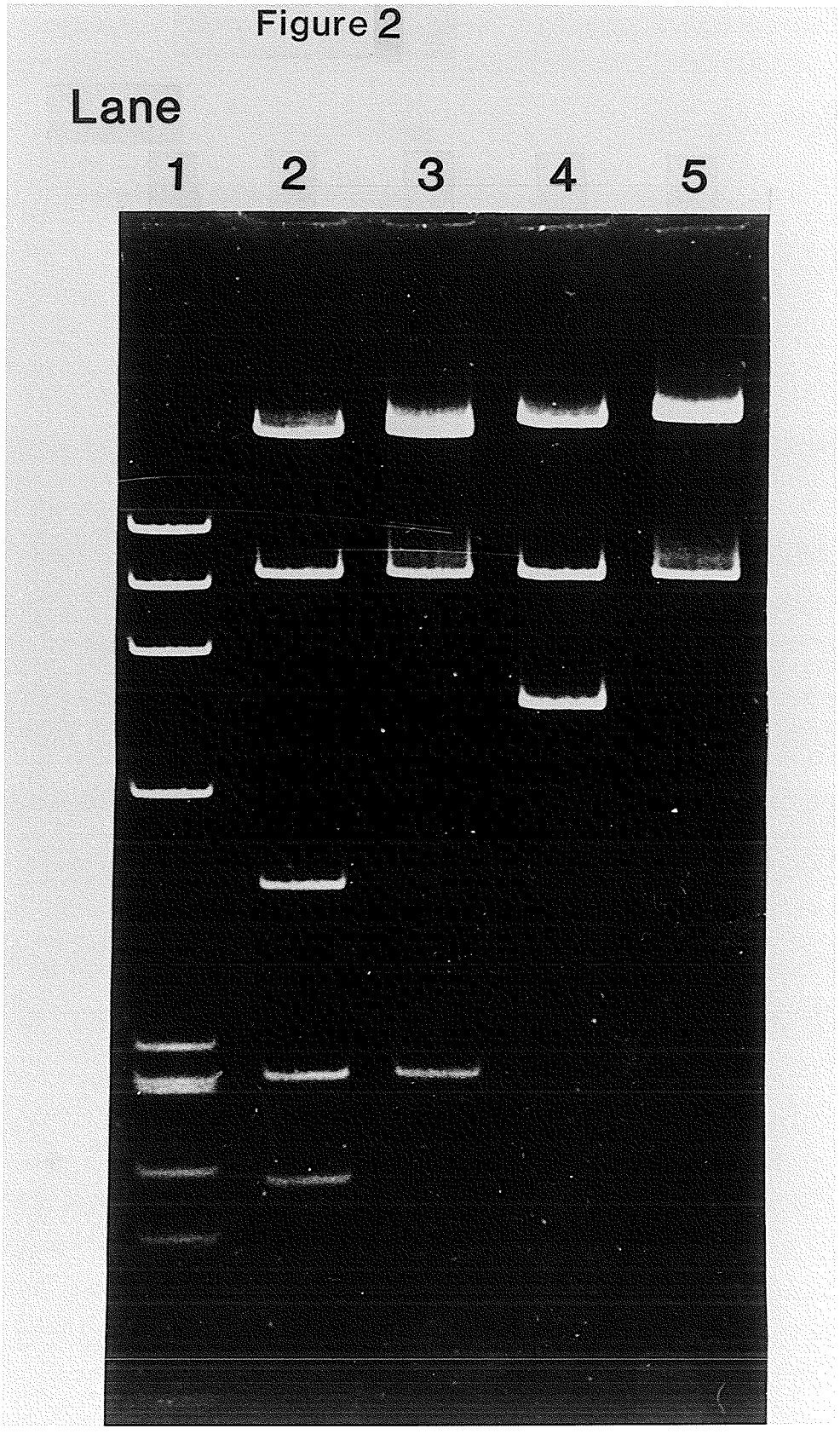


TABLE 6

Summary of Restriction Enzyme Analysis of pRP52A

SITES FOR:	NO SITES FOR:
AluI	AccI
EcoRI*	BamHI
FnuDII	BclII
HhaI	BglII
HincII*	ClaI
PvuII*	HaeII
Sau3A	HindIII
XbaI	HinfI
	HpaI
	HpaII
	MspI
	SacI
	SalI
	SmaI
	TaqI

Plasmid pRP52A was digested with a series of restriction enzymes to determine which enzymes cleaved the cDNA. The starred enzymes were used in subcloning the pRP52A into M13 vectors.

Table 7. Summary of the cDNA Subcloning into M13 Vectors

The subclones of the four cDNA clones are shown. 52A* and pAT* indicate the nick-translated [^{32}-P]-cDNA probes used in the plaque hybridizations.

The whole inserts of clones 52 and 52-3 were cloned randomly into M213mp8 because of the short length of the inserts. The recombinant clones were selected via the colorimetric plaque assay. Both orientations of the clones were selected.

The longest clone, 52A, following extensive restriction mapping, was subcloned forcibly into the complementary vectors, M13 mp8 and mp9. The recombinants were selected by plaque hybridization.

Clone 52B was initially subcloned as a single fragment. Further subclones were generated by utilizing the restriction enzyme site for RsaI located by computer analysis of the sequence. The fragments were subcloned into M13mp18 because the SmaI and EcoRI sites in mp8 and mp9 overlap. SmaI was chosen for creating the blunt end site for the RsaI end insertion since HincII can be contaminated with exonucleases. The exonucleases can degrade the beta-galactosidase gene in M13 and render the plaque assay useless. The forced clones were selected via the plaque assay.

TABLE 7 SUMMARY OF cDNA SUBCLONING INTO M13 VECTORS

Clone	Subclone	M13 Vector	Restriction enzyme site(s) cleaved for subcloning		Selected by plaque hybridization to:	Orientation of synthesized strand
			Vector	Clone		
52						
	52I	mp9	PstI	PstI	-	5' - 3'
	52II	mp9	PstI	PstI	-	3' - 5'
52A						
	S.1	mp9	PstI/EcoRI	PstI/EcoRI	52A*	5' - 3'
	S.2	mp9	PstI/EcoRI	PstI/EcoRI	52A*	3' 5'
	Bmp9b	mp9	PstI/HincII	PstI/HincII	52A*	5' - 3'
	Bmpa	mp9	PstI/HincII	PstI/HincII	52A*	3' - 5'
	Smp8X	mp8	PstI/EcoRI	PstI/EcoRI	52A*	3' - 5'
	Smp8Y	mp8	PstI/EcoRI	PstI/EcoRI	52A*	5' - 3'
	Bmp8X	mp8	PstI/HincII	PstI/HincII	52A*	3' - 5'
	Bmp8Y	mp8	PstI/HincII	PstI/HincII	52A*	5' - 3'
	PHX	mp8	PvuII/HincII	PvuII/HincII	52A*	3' - 5'
	PHY	mp8	PvuII/HincII	PvuII/HincII	52A*	5' - 3'
	PHZ	mp8	PvuII/HincII	PvuII/HincII	52A*/pAT*	3' - 5'
52-3						
	52-3Y	mp8	PstI	PstI	-	5' - 3'
	52-3X	mp8	PstI	PstI	-	3' - 5'
52B						
	LX	mp8/9	EcoRI	EcoRI	52A*	5' - 3'
	LY	mp8/9	EcoRI	EcoRI	52A*	3' - 5'
	X-29	mp18	SmaI/EcoRI	RsaI/EcoRI	-	5' - 3'
	F-35	mp18	SmaI/EcoRI	RsaI/EcoRI	-	3' - 5'

Figure 3. Autoradiograph of 35-S-labelled DNA fragments generated by dideoxy chain termination reactions.

A representative 6% acrylamide / 8M urea buffer gradient sequencing gel run to the xylene cyanol dye front. The sequence of the pRP52A subclone, S1, begins at base 593 of the cDNA and proceeds in the 3' direction of the sense strand. The XbaI site (TCTAGA) is crossed by the clone.

Figure 3

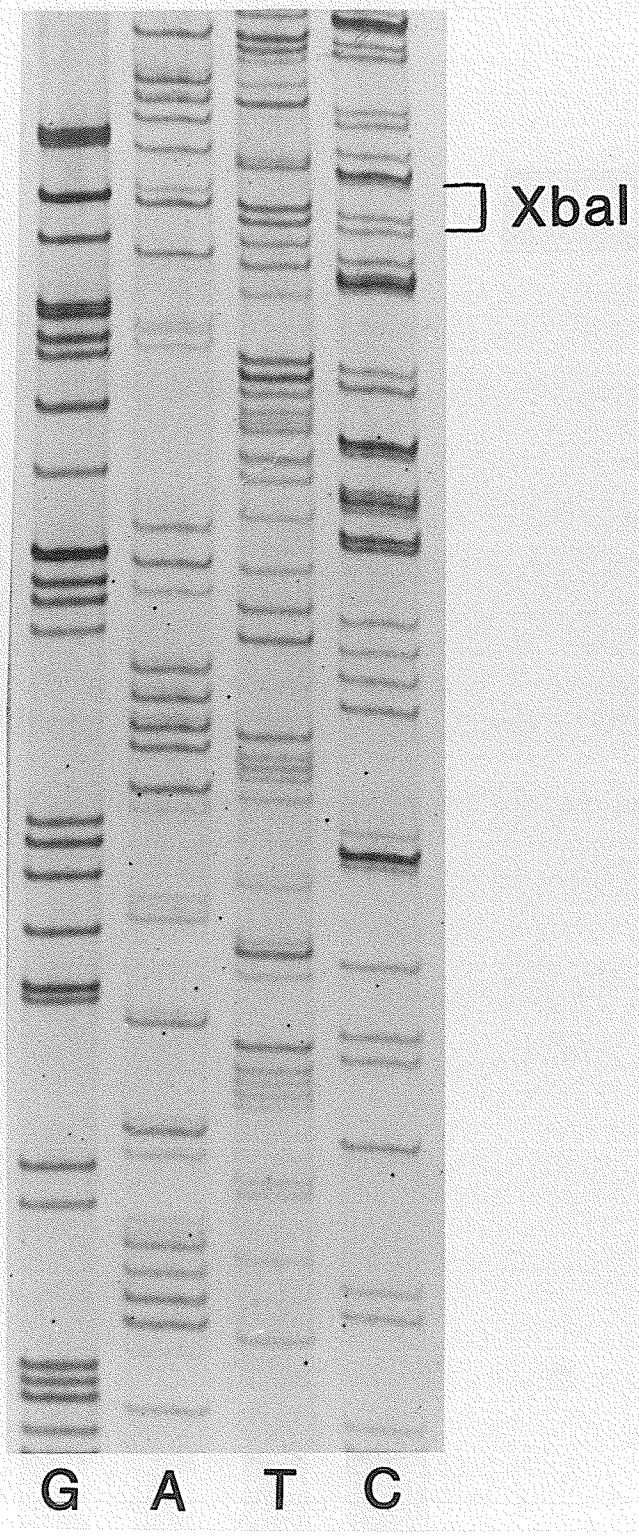
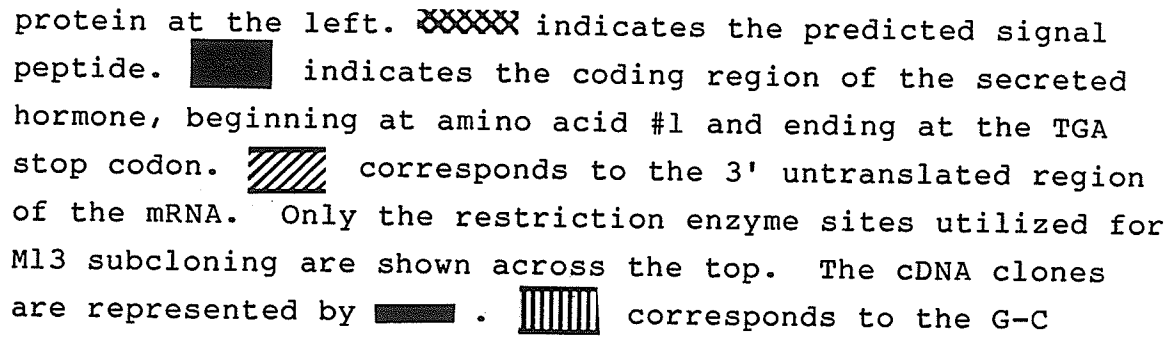


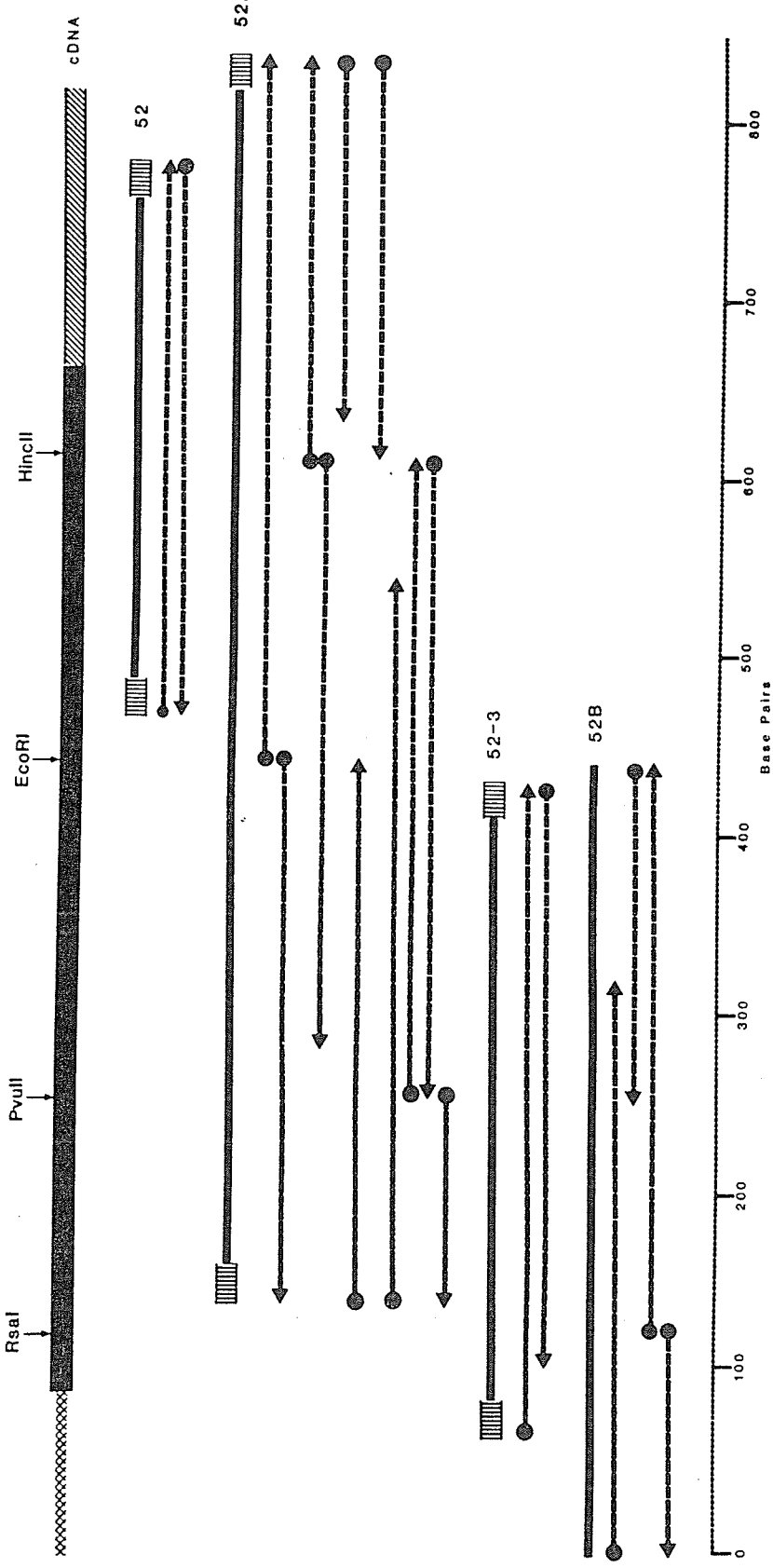
Figure 4. Sequencing Strategy and Restriction Map of rPLII cDNA.



A composite of the cDNA clones is represented linearly across the top of the figure. The cDNA is shown in the mRNA sense strand with the 5' end of the mRNA or NH2 end of the protein at the left. XXXXX indicates the predicted signal peptide. ■ indicates the coding region of the secreted hormone, beginning at amino acid #1 and ending at the TGA stop codon. ▨ corresponds to the 3' untranslated region of the mRNA. Only the restriction enzyme sites utilized for M13 subcloning are shown across the top. The cDNA clones are represented by ▬ . ▥ corresponds to the G-C homopolymer tails used for insertion of 52, 52A and 52-3 into the PstI site of pAT153. The arrows indicate the restriction enzyme fragments of the cDNA clones subcloned into the M13 vectors. The sequencing proceeded in the direction of the arrows for the distance indicated by the length of the arrow. The cDNA is numbered in base pairs along the bottom.

Figure 4

Sequencing Strategy and Restriction Map of rPLII cDNA



D. Computer Analysis

A complete restriction map of the rPLII cDNA sequence is shown in Figure 5.

The complete nucleotide sequence and predicted amino acid sequence of rPLII is shown in Figure 6. The nucleotide sequence translated into a single open reading frame. The mature protein is 191 amino acid residues in length. There are no consensus glycosylation signals, Asn-X-Ser/Thr (Bahl and Shah, 1977) within the translation. There is a single basic dipeptide, Arg-Arg, at positions 167 and 168; this sequence could be a site of proteolytic cleavage, which is often utilized in the processing of peptide hormones (Docherty and Steiner, 1982).

The codon usage in rPLII, as shown in Tables 8 and 9, is non-random.

The amino acid composition of the primary sequence of rPLII is shown in Table 10. The protein, including the portion of the signal peptide, has a calculated mw of 24,954 daltons and an estimated pI of 6.47. The predicted secreted protein has a calculated mw of 21,693 daltons and an estimated pI 6.60. The secreted form has four cysteine residues which may be involved in the formation of two disulfide bonds.

The hydropathy of the protein is analyzed in Figure 7. The molecule is most hydrophobic at the carboxyl end of the

Figure 5. Complete Restriction Enzyme Map of rPLII cDNA.

The locations of potential restriction enzyme cleavage sites determined by computer are shown. The sites confirmed by restriction enzyme digestion and electrophoretic analysis are underlined. The sequence is a composite of the four cDNA clones. The enzymatically added G-C tails (20bp/end) are not shown. Clone 52 extends from base #161-#814. Clone 52A extends from base #161-#814. Clone 52-3 extends from base #74-#410. Clone 52B extends from base #1-#433. The remnant of the poly A tract seen in 52A is shown at the 3' end.

FIGURE 5

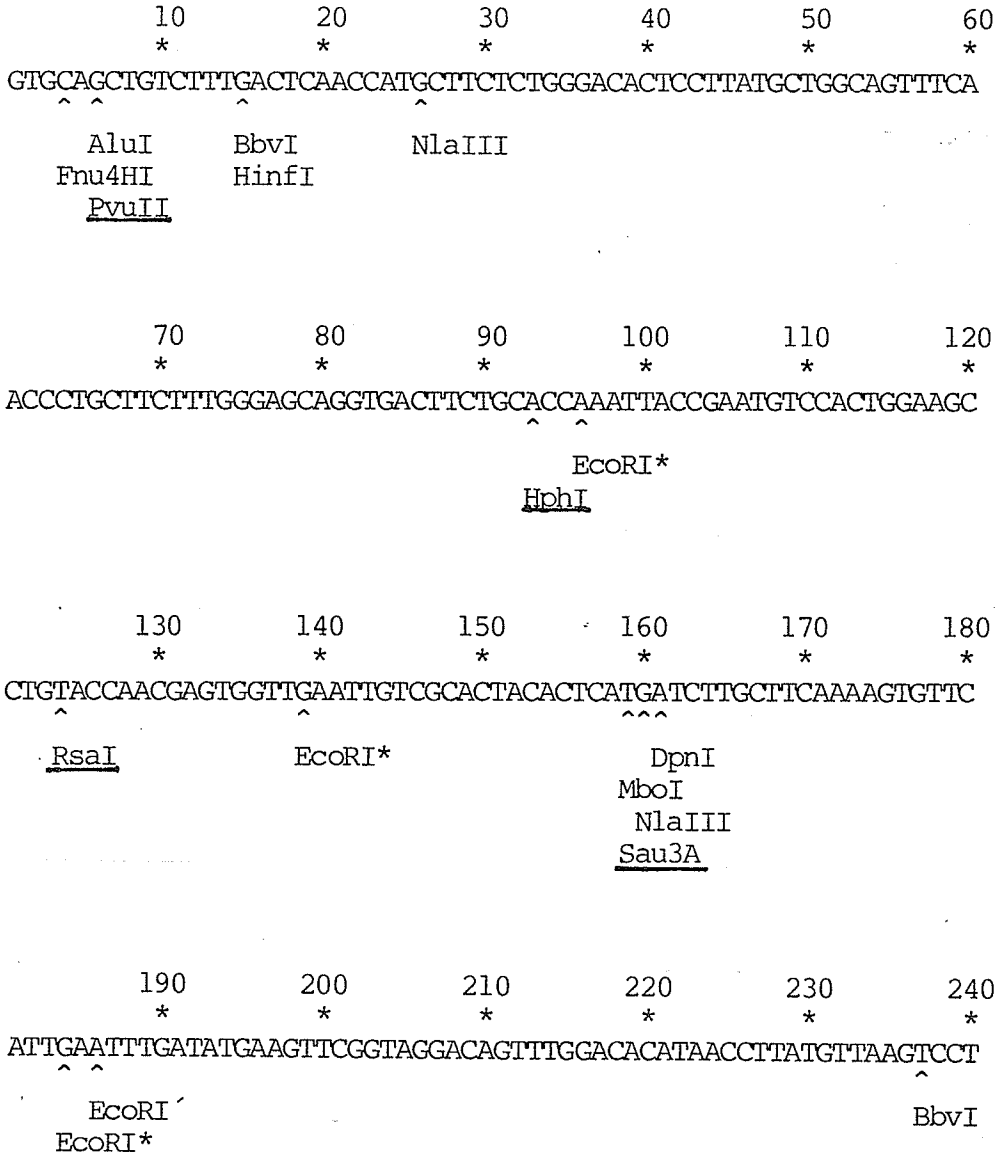

10 20 30 40 50 60
GTGCAGCTGTCTTTGACTCAACCATGCTTCTCTGGGACACTCCTTATGCTGGCAGTTTCA
AluI
Fnu4HI
PvuII
BbvI
HinfI
NlaIII
70 80 90 100 110 120
ACCCTGCTTCTTTGGGAGCAGGTGACTTCTGCACCAAATTACCGAATGTCCACTGGAAGC
EcoRI*
HphI
130 140 150 160 170 180
CTGTACCAACGAGTGGTTGAATTGTCGCACTACACTCATGATCTTGCTTCAAAAGTGTTC
RsaI
EcoRI*
DpnI
MboI
NlaIII
Sau3A
190 200 210 220 230 240
ATTGAATTTGATATGAAGTTCGGTAGGACAGTTTGGACACATAACCTTATGTTAAGTCCT
EcoRI
EcoRI*
BbvI

FIGURE 5 CONT'D

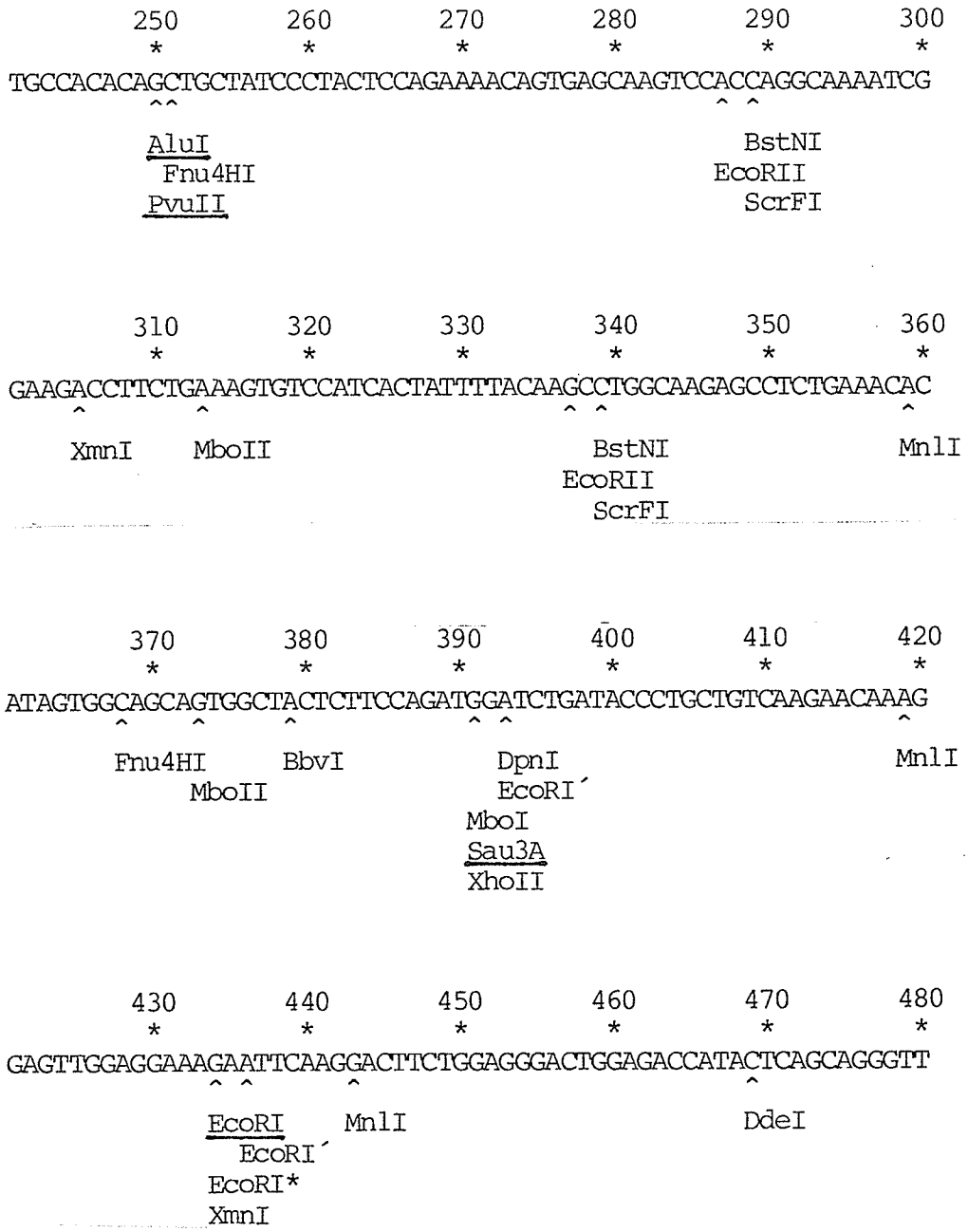


FIGURE 5 CONT'D

```
          490         500         510         520         530         540
           *           *           *           *           *           *
CAACCTGGAGCTGTTGGAAGTGATTATACTTTCTGGTCTGAGTGGTCAGATTTGCAGTCA
 ^ ^   ^                                     ^           ^
   BstNIAluI                                 DdeI        EcoRI´
  EcoRII
    ScrFI

          550         560         570         580         590         600
           *           *           *           *           *           *
TCTGATAAATCCACTAAGAATGGTGTTCTTAGTGTCCTGTATCGGTGCATGCGCAGGGAT
          ^   ^           ^                     ^^^
         DdeI              DdeI                  HhaI
        EcoRI´                                   HinPI
                                                 MstI
                                                 NlaIII
                                                 SphI

          610         620         630         640         650         660
           *           *           *           *           *           *
ACACATAAAGTTGACAATTTTCTCAAGGTCTTGAAATGCCGCGATATTTATAACAACAAC
            ^ ^                     ^ ^                     ^
           EcoRI*                   Fnu4HI                  FokI
          HincII                     ThaI

          670         680         690         700         710         720
           *           *           *           *           *           *
TGCTGAGTAGCCATCCCTGTCCTTTGTCTCTGAGAAGGTCCCTCATGCTCTAGACCTTCA
 ^                           ^           ^ ^         ^ ^ ^
 DdeI                        DdeI      AvaII          MnlI
                                       NlaIV       NlaIII
                                      Sau96I        XbaI

          730         740         750         760         770         780
           *           *           *           *           *           *
GGGCACTAATAAATCTCTACCTCTTTGGTGCCCTTCCTGATTTAGTTTGTATCTTTCTTA
   ^         ^                 ^ ^^ ^
  Bspl286 EcoRI´              BanI Bspl286
                               MnlI
                               NlaIV

          790         800         810
           *           *           *
AAAATAAACTCACTCTTTGGAAATGCTAAAAAAA
```

Figure 6. The Nucleotide Sequence and Predicted Amino Acid Sequence of the mRNA Coding for rPLII.

The amino acid sequence was deduced from the standard genetic code. The nucleotide sequence translated into a single open reading frame of 221 amino acids. Initiation of translation is assumed to begin prior to the first codon of the signal peptide shown (designation of the signal peptide and assignment of amino acid #1 will be described in the discussion) and to continue until the TGA (OPAL) stop codon (indicated by ——). The poly A addition signals, AATAAA (Proudfoot and Brownlee, 1976) indicated by ▬▬▬ , are found 25 and 83 bp. upstream from the poly A tail (indicated by XXXX). The sequence includes 30 amino acids of the predicted signal peptide, 191 amino acids of the hormone coding region and 145 bp. of the 3' untranslated region of the corresponding mRNA.

FIGURE 6

NUCLEOTIDE SEQUENCE AND PREDICTED AMINO ACID SEQUENCE OF THE mRNA CODING FOR RPL-II

```
-30                                  -20
 *                                    *
GTG CAG CTG TCT TTG ACT CAA CCA TGC TTC TCT GGG ACA CTC CTT ATG CTG GCA GTT TCA
Val Gln Leu Ser Leu Thr Gln Pro Cys Phe Ser Gly Thr Leu Leu Met Leu Ala Val Ser

-10                                   1                                   10
 *                                    *                                   *
ACC CTG CTT CTT TGG GAG CAG GTG ACT TCT GCA CCA AAT TAC CGA ATG TCC ACT GGA AGC
Thr Leu Leu Leu Trp Glu Gln Val Thr Ser Ala Pro Asn Tyr Arg Met Ser Thr Gly Ser

                                    20                                  30
                                     *                                   *
CTG TAC CAA CGA GTG GTT GAA TTG TCG CAC TAC ACT CAT GAT CTT GCT TCA AAA GTG TTC
Leu Tyr Gln Arg Val Val Glu Leu Ser His Tyr Thr His Asp Leu Ala Ser Lys Val Phe

                                    40                                  50
                                     *                                   *
ATT GAA TTT GAT ATG AAG TTC GGT AGG ACA GTT TGG ACA CAT AAC CTT ATG TTA AGT CCT
Ile Glu Phe Asp Met Lys Phe Gly Arg Thr Val Trp Thr His Asn Leu Met Leu Ser Pro

                                    60                                  70
                                     *                                   *
TGC CAC ACA GCT GCT ATC CCT ACT CCA GAA AAC AGT GAG CAA GTC CAC CAG GCA AAA TCG
Cys His Thr Ala Ala Ile Pro Thr Pro Glu Asn Ser Glu Gln Val His Gln Ala Lys Ser

                                    80                                  90
                                     *                                   *
GAA GAC CTT CTG AAA GTG TCC ATC ACT ATT TTA CAA GCC TGG CAA GAG CCT CTG AAA CAC
Glu Asp Leu Leu Lys Val Ser Ile Thr Ile Leu Gln Ala Trp Gln Glu Pro Leu Lys His

                                   100                                 110
                                     *                                   *
ATA GTG GCA GCA GTG GCT ACT CTT CCA GAT GGA TCT GAT ACC CTG CTG TCA AGA ACA AAG
Ile Val Ala Ala Val Ala Thr Leu Pro Asp Gly Ser Asp Thr Leu Leu Ser Arg Thr Lys

                                   120                                 130
                                     *                                   *
GAG TTG GAG GAA AGA ATT CAA GGA CTT CTG GAG GGA CTG GAG ACC ATA CTC AGC AGG GTT
Glu Leu Glu Glu Arg Ile Gln Gly Leu Leu Glu Gly Leu Glu Thr Ile Leu Ser Arg Val

                                   140                                 150
                                     *                                   *
CAA CCT GGA GCT GTT GGA AGT GAT TAT ACT TTC TGG TCT GAG TGG TCA GAT TTG CAG TCA
Gln Pro Gly Ala Val Gly Ser Asp Tyr Thr Phe Trp Ser Glu Trp Ser Asp Leu Gln Ser

                                   160                                 170
                                     *                                   *
TCT GAT AAA TCC ACT AAG AAT GGT GTT CTT AGT GTC CTG TAT CGG TGC ATG CGC AGG GAT
Ser Asp Lys Ser Thr Lys Asn Gly Val Leu Ser Val Leu Tyr Arg Cys Met Arg Arg Asp

                                   180                                 190
                                     *                                   *
ACA CAT AAA GTT GAC AAT TTT CTC AAG GTC TTG AAA TGC CGC GAT ATT TAT AAC AAC AAC
Thr His Lys Val Asp Asn Phe Leu Lys Val Leu Lys Cys Arg Asp Ile Tyr Asn Asn Asn

TGC TGA GTAGCCATCCCTGTCCTTTGTCTCTGAGAAGGTCCCTCATGCTCTAGACCTTCAGGGCACTAATAAATCTCT
Cys ——                                                              ■■■■■■

ACCTCTTTGGTGCCCTTCCTGATTTAGTTTGTATCTTTCTTAAAAATAAACTCACTCTTTGGAAATGTT polyA
                                                                    ■■■■■■
                                                                    ◇◇◇◇
```

Table 8. The Codon Usage in rPLII mRNA.

The frequency and percentage of each codon in the entire mRNA sequence, including the codons coding for the portion of the signal peptide, is shown.

--- designates the translation terminators TAA (OCHRE), TAG (AMBER) and TGA (OPAL).

Table 8

THE CODON USAGE IN rPLII mRNA

TTT Phe	2	.9%	TCT Ser	6	2.7%	TAT Tyr	3	1.4%	TGT Cys	0	.0%
TTC Phe	4	1.8%	TCC Ser	3	1.4%	TAC Tyr	3	1.4%	TGC Cys	5	2.3%
TTA Leu	2	.9%	TCA Ser	5	2.3%	TAA ---	0	--	TGA ---	1	--
TTG Leu	5	2.3%	TCG Ser	2	.9%	TAG ---	0	--	TGG Trp	5	2.3%
CTT Leu	9	4.1%	CCT Pro	4	1.8%	CAT His	3	1.4%	CGT Arg	0	.0%
CTC Leu	3	1.4%	CCC Pro	0	.0%	CAC His	4	1.8%	CGC Arg	2	.9%
CTA Leu	0	.0%	CCA Pro	4	1.8%	CAA Gln	7	3.2%	CGA Arg	2	.9%
CTG Leu	11	5.0%	CCG Pro	0	.0%	CAG Gln	4	1.8%	CGG Arg	1	.5%
ATT Ile	4	1.8%	ACT Thr	9	4.1%	AAT Asn	3	1.4%	AGT Ser	4	1.8%
ATC Ile	2	.9%	ACC Thr	3	1.4%	AAC Asn	5	2.3%	AGC Ser	2	.9%
ATA Ile	2	.9%	ACA Thr	6	2.7%	AAA Lys	7	3.2%	AGA Arg	2	.9%
ATG Met	5	2.3%	ACG Thr	0	.0%	AAG Lys	4	1.8%	AGG Arg	3	1.4%
GTT Val	7	3.2%	GCT Ala	5	2.3%	GAT Asp	9	4.1%	GGT Gly	2	.9%
GTC Val	3	1.4%	GCC Ala	1	.5%	GAC Asp	2	.9%	GGC Gly	0	.0%
GTA Val	0	.0%	GCA Ala	5	2.3%	GAA Glu	5	2.3%	GGA Gly	6	2.7%
GTG Val	7	3.2%	GCG Ala	0	.0%	GAG Glu	8	3.6%	GGG Gly	1	.5%

Table 9. The Codon Usage in rPLII mRNA Coding for the Mature Hormone.

The frequency and percentage of each codon in the mRNA sequence is shown.

---designates the translation terminators TAA (OCHRE), TAG (AMBER) and TGA (OPAL).

Table 9

THE CODON USAGE IN rPLII mRNA CODING FOR THE MATURE PROTEIN

TTT Phe	2	1.0%	TCT Ser	3	1.6%	TAT Tyr	3	1.6%	TGT Cys	0	.0%
TTC Phe	3	1.6%	TCC Ser	3	1.6%	TAC Tyr	3	1.6%	TGC Cys	4	2.1%
TTA Leu	2	1.0%	TCA Ser	4	2.1%	TAA ---	0	--	TGA ---	1	--
TTG Leu	4	2.1%	TCG Ser	2	1.0%	TAG ---	0	--	TGG Trp	4	2.1%
CTT Leu	6	3.1%	CCT Pro	4	2.1%	CAT His	3	1.6%	CGT Arg	0	.0%
CTC Leu	2	1.0%	CCC Pro	0	.0%	CAC His	4	2.1%	CGC Arg	2	1.0%
CTA Leu	0	.0%	CCA Pro	3	1.6%	CAA Gln	6	3.1%	CGA Arg	2	1.0%
CTG Leu	8	4.2%	CCG Pro	0	.0%	CAG Gln	2	1.0%	CGG Arg	1	.5%
ATT Ile	4	2.1%	ACT Thr	7	3.7%	AAT Asn	3	1.6%	AGT Ser	4	2.1%
ATC Ile	2	1.0%	ACC Thr	2	1.0%	AAC Asn	5	2.6%	AGC Ser	2	1.0%
ATA Ile	2	1.0%	ACA Thr	5	2.6%	AAA Lys	7	3.7%	AGA Arg	2	1.0%
ATG Met	4	2.1%	ACG Thr	0	.0%	AAG Lys	4	2.1%	AGG Arg	3	1.6%
GTT Val	6	3.1%	GCT Ala	5	2.6%	GAT Asp	9	4.7%	GGT Gly	2	1.0%
GTC Val	3	1.6%	GCC Ala	1	.5%	GAC Asp	2	1.0%	GGC Gly	0	.0%
GTA Val	0	.0%	GCA Ala	4	2.1%	GAA Glu	5	2.6%	GGA Gly	6	3.1%
GTG Val	5	2.6%	GCG Ala	0	.0%	GAG Glu	7	3.7%	GGG Gly	0	.0%

Table 10. Amino Acid Composition of rPLII.

A. The amino acid composition of the entire protein coding region, including the portion of the predicted signal peptide. The 221 amino acids yield a calculated m.w. of 24,954 daltons and an estimated pI of 6.47.

B. The amino acid composition of the secreted protein. The 191 amino acids yield a calculated m.w. of 21,693 daltons and an estimated pI of 6.60.

Table 10

A

Non-polar:		Number	Percent
Ala	A	11	4.977
Val	V	17	7.692
Leu	L	30	13.575
Ile	I	8	3.620
Pro	P	8	3.620
Met	M	5	2.262
Phe	F	6	2.715
Trp	W	5	2.262

Polar:		Number	Percent
Gly	G	9	4.072
Ser	S	22	9.955
Thr	T	18	8.145
Cys	C	5	2.262
Tyr	Y	6	2.715
Asn	N	8	3.620
Gln	Q	11	4.977

Acidic:		Number	Percent
Asp	D	11	4.977
Glu	E	13	5.882

Basic:		Number	Percent
Lys	K	11	4.977
Arg	R	10	4.525
His	H	7	3.167

Total AAs = 221
Calculated Molecular Weight = 24954.860
Estimated pI = 6.47

B

Non-polar:		Number	Percent
Ala	A	10	5.236
Val	V	14	7.330
Leu	L	22	11.518
Ile	I	8	4.188
Pro	P	7	3.665
Met	M	4	2.094
Phe	F	5	2.618
Trp	W	4	2.094

Polar:		Number	Percent
Gly	G	8	4.188
Ser	S	18	9.424
Thr	T	14	7.330
Cys	C	4	2.094
Tyr	Y	6	3.141
Asn	N	8	4.188
Gln	Q	8	4.188

Acidic:		Number	Percent
Asp	D	11	5.759
Glu	E	12	6.283

Basic:		Number	Percent
Lys	K	11	5.759
Arg	R	10	5.236
His	H	7	3.665

Total AAs = 191
Calculated Molecular Weight = 21693.150
Estimated pI = 6.60

Figure 7. Hydropathy Plot of rPLII.

The hydropathy of the predicted prehormone was plotted by computer. The plot is based on a running average over nine amino acids of the hydropathic indices of those amino acids. The positive values indicate hydrophobicity. The negative values indicate hydrophilicity. The figure is a fold-out found at the back of the thesis.

signal peptide and within the core of the secreted protein.

A prediction of the secondary structure of the secreted protein is shown in Figure 8. Rat PLII is predicted to have a 36% alpha-helical structure.

Figure 8. Secondary Structure Prediction of rPLII

The calculation of information sums for each amino acid residue in each of the four conformation states: alpha-helix (A-HELIX); extended chain (XTNDD); reverse turn (TURN) and coil (COIL) is shown. The conformation with the highest sum, indicated by the underlining, is the predicted state.

The amino acids in the deduced rPL II protein are represented by the one-letter abbreviations recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (1968).

One-Letter Amino Acid Code

A	ALA	G	GLY	M	MET	S	SER
C	CYS	H	HIS	N	ASN	T	THR
D	ASP	I	ILE	P	PRO	V	VAL
E	GLU	K	LYS	Q	GLN	W	TRP
F	PHE	L	LEU	R	ARG	Y	TYR

Figure 8. Secondary Structure Prediction of rPL II.

POSN	AA	'INFORMATION SUMS' FOR A-HELIX	XTNDD	TURN	COIL	POSN	AA	A-HELIX	XTNDD	TURN	COIL
1	A	-130	-63	123	130	33	F	146	-14	-168	-156
2	P	-157	-48	106	163	34	D	120	-54	-54	-35
3	N	-241	-26	32	181	35	M	63	-67	-8	69
4	Y	-225	70	124	139	36	K	3	-98	50	127
5	R	-204	139	151	93	37	F	-54	-69	102	91
6	M	-197	158	127	124	38	G	-81	-62	97	79
7	S	-209	143	121	160	39	R	1	-11	76	43
8	T	-216	123	178	157	40	T	34	3	43	42
9	G	-213	63	162	134	41	V	49	18	-85	10
10	S	-159	18	154	140	42	W	42	25	-1	7
11	L	-93	13	99	105	43	T	19	33	53	37
12	Y	-45	75	39	14	44	H	15	25	37	66
13	Q	16	137	-16	-60	45	N	14	44	65	76
14	R	54	144	-19	-82	46	L	20	48	39	55
15	V	72	143	-55	-75	47	M	38	63	42	34
16	V	70	143	-60	-85	48	L	45	43	34	35
17	E	58	100	-37	-44	49	S	21	13	131	85
18	L	37	28	-36	15	50	P	-9	-38	71	28
19	S	11	-22	1	60	51	C	-83	24	-86	-2
20	H	5	-55	17	51	52	H	-8	30	-3	26
21	Y	5	-45	19	59	53	T	44	58	53	32
22	T	14	-67	-7	27	54	A	105	82	-5	-20
23	H	50	-75	2	16	55	A	130	112	-17	-45
24	D	95	-134	14	-15	56	I	114	72	94	27
25	L	127	-147	-31	-5	57	P	91	2	111	93
26	A	140	-168	-15	20	58	T	-18	-62	93	187
27	S	174	-147	-34	35	59	P	-36	-73	116	218
28	K	201	-113	-40	-23	60	E	-109	-90	121	216
29	V	212	-127	-80	-68	61	N	-118	-66	197	226
30	F	234	-99	-123	-131	62	S	-91	-27	231	205
31	I	224	-53	-151	-148	63	E	-57	30	133	146
32	E	188	-40	-202	-199	64	Q	10	67	49	105

Figure 8. (Continued)

'INFORMATION SUMS' FOR

POSN	AA	A-HELIX	XTNDD	TURN	COIL	POSN	AA	A-HELIX	XTNDD	TURN	COIL
65	V	64	43	-45	40	97	T	154	23	-137	-48
66	H	132	-10	-53	36	98	L	47	-2	-16	60
67	Q	193	-33	-66	5	99	P	-22	-13	-1	103
68	A	233	-88	-45	-20	100	D	-137	-59	-24	104
69	K	246	-138	-20	-13	101	G	-196	-57	102	129
70	S	229	-182	11	7	102	S	-204	-22	186	165
71	E	221	-175	-22	-19	103	D	-172	6	201	145
72	D	183	-154	1	0	104	T	-133	78	128	122
73	L	147	-77	-46	30	105	L	-63	93	39	60
74	L	82	-12	-76	40	106	L	27	63	-8	0
75	K	66	67	-80	-8	107	S	87	13	-34	-35
76	V	47	118	-75	-23	108	R	137	-56	1	-52
77	S	41	133	-4	50	109	T	165	-102	48	27
78	I	41	142	59	77	110	K	190	-163	85	32
79	T	62	158	28	52	111	E	204	-120	71	8
80	I	96	152	4	32	112	L	228	-77	-36	-5
81	L	172	108	-18	20	113	E	261	-30	-77	-64
82	Q	218	77	-61	-40	114	E	231	-30	-117	-39
83	A	221	12	-95	-55	115	R	236	-11	-144	-2
84	W	205	-65	6	-8	116	I	221	-33	-141	27
85	Q	188	-108	69	-10	117	Q	206	-48	-81	40
86	E	136	-130	98	56	118	G	165	-87	-8	59
87	P	93	-128	36	108	119	L	165	-97	-6	85
88	L	-33	-62	-121	95	120	L	158	-97	-16	75
89	K	-47	-33	-50	52	121	E	127	-90	-12	1
90	H	40	0	-38	13	122	G	70	-67	-28	-11
91	I	141	57	-91	-43	123	L	65	-2	-51	-5
92	V	234	108	-150	-100	124	E	23	50	-27	41
93	A	290	107	-180	-135	125	T	15	83	-37	82
94	A	315	117	-215	-160	126	I	6	177	-36	62
95	V	294	93	-220	-145	127	L	-23	203	-33	45
96	A	238	47	-195	-110	128	S	-49	183	-24	60

Figure 8. (Concluded).

'INFORMATION SUMS' FOR

POSN	AA	A-HELIX	XTNDD	TURN	COIL	POSN	AA	A-HELIX	XTNDD	TURN	COIL
129	R	-56	169	-9	8	161	S	-31	148	-59	-25
130	V	-36	118	-30	45	162	V	-53	158	-65	-75
131	Q	-60	87	34	45	163	L	-58	173	-61	-80
132	P	-92	47	11	58	164	Y	-65	130	4	-71
133	G	-191	3	-113	74	165	R	-51	129	56	-62
134	A	-180	7	30	130	166	C	-18	119	64	-67
135	V	-196	-22	180	185	167	M	8	78	72	-71
136	G	-231	-57	242	224	168	R	16	39	81	-77
137	S	-214	-17	256	245	169	R	31	-16	61	-52
138	D	-190	-19	261	185	170	D	20	-74	66	-20
139	Y	-145	-5	224	179	171	T	9	-107	53	22
140	T	-118	13	163	127	172	H	37	-100	52	31
141	F	-81	31	87	84	173	K	63	93	63	22
142	W	-25	45	149	77	174	V	89	-42	10	-3
143	S	-11	53	181	110	175	D	105	-59	16	-15
144	E	3	50	78	126	176	N	97	-31	-23	-39
145	W	-3	10	141	157	177	F	91	6	-43	-86
146	S	-56	-22	166	160	178	L	82	13	-96	-100
147	D	-105	-34	121	140	179	K	81	27	-65	-78
148	L	-108	3	9	95	180	V	87	63	-35	-58
149	Q	-135	22	-11	30	181	L	82	113	-31	-50
150	S	-141	-12	21	65	182	K	83	177	-10	-103
151	S	-134	28	36	50	183	C	80	164	29	-140
152	D	-125	6	76	35	184	R	81	114	46	-112
153	K	-117	-13	90	62	185	D	70	41	86	-50
154	S	-124	-47	126	147	186	I	21	37	94	-3
155	T	-111	-37	83	107	187	Y	-50	10	144	19
156	K	-102	-33	25	47	188	N	-106	-16	182	71
157	N	-96	-51	60	48	189	N	-131	-21	197	86
158	G	-88	-32	32	19	190	N	-131	-21	202	81
159	V	-36	43	-20	-25	191	C	-118	19	184	58
160	L	-13	113	-36	-10						

V. DISCUSSION

The nucleotide sequence of rPL II mRNA and the predicted amino acid sequence encoded by the mRNA have been determined. The nucleotide sequence translated into a single open reading frame of 221 amino acids. Approximately 80% (807/1000 bp) of the mature rPL II mRNA nucleotide sequence was contained within the cDNA clones studied. The deduced amino acid sequence did not encompass the translation initiator methionine residue, nor the nucleotide sequence of the 5′ untranslated region of the mRNA, since a full-length clone was not available.

A. cDNA Structure

Rat PL II is distinct from the other rat placental clones, rPLP-A, pRP9, pRP54, and pRP27 based on the comparison of restriction enzyme maps (data not shown).

Codon Usage

The degeneracy of the genetic code usually results from variation in the identity of the third nucleotide of the codon. The choice varies between A,T,G, or C. Codon choice is quantified by the determination of the

number of codons ending in A or T versus those terminating with G or C. Because of the characteristics of the genetic code and the rarity of certain amino acids, random codon selection would results in 42% of the codons ending in G or C in general vertebrate DNA (Sueoka, 1961).

The codon usage in rPL II mRNA is non-random. Of the 807 nucleotides in the cloned cDNA, 45% (359/807) are G or C. Of the 220 codons encoded by the mRNA, 45% end in G or C.

A comparison of the codon usage in members of the PRL-GH gene family is shown in Table 11. In the PRL-GH family of genes, there is a wide range of preference for G or C in the codon third position (Miller and Eberhardt, 1983). The GH's and hPL show a strong preference (73-82%) for G or C whereas PRL's show a slight preference (52-64%) for G or C. Rat PL II has a lower preference for G or C than the GH's. The codon usage of rPL II mRNA appears to be closer to the PRL's. Rat PLP-A, like the PRL's, utilizes G or C in the third position in 58% of the codons. Like rPL II, mRNA's for the mouse proteins, mPRP, mPLF-1 and mPLF-2, show a slight preference (46-47%) for G or C. Miller and Eberhardt (1983) have used the codon preference to characterize GH-like and PRL-like members of the PRL-GH gene family. In this case, rPL II appears to be more

Table 11. The relative abundances of codons ending in G or C in various mRNAs.

The stop codons have not been included in the calculations.

Data derived from the published sequences: hPL, Shine et al. (1977); hGH, Martial et al. (1979); bGH, Miller et al. (1980); mGH, Linzer and Talamantes (1985); rGH, Seeburg et al. (1977); mPRP, Linzer and Nathans (1985); rPLP-A, Peden, personal communication; mPLF-1, Linzer and Nathans (1984); mPLF-2, Linzer et al. (1985); rPRL, Cooke et al. (1980); mPRL, Linzer and Talamantes (1985); bPRL, Miller et al. (1981); hPRL, Cooke et al. (1981).

TABLE 11

Relative Abundances of Codons Ending in G or C in Various MRNA's

	Percent G+C in Codon Third Positions					
	signal		coding		total	
	#	%	#	%	#	%
rPL II	16/30	53	82/190	43	98/220	45
hPL	18/26	69	149/191	78	167/217	77
hGH	14/26	54	144/191	75	158/217	73
bGH	21/26	81	156/191	82	177/217	82
mGH	19/26	73	147/190	77	166/216	77
rGH	17/26	65	143/190	75	160/216	74
mPRP	22/30	73	91/214	43	113/244	46
rPLP	22/31	71	110/196	56	132/227	58
mPLF-1	17/29	59	88/195	45	105/224	47
mPLF-2	17/29	59	89/195	46	106/224	47
rPRL	21/28	75	97/197	49	118/225	52
mPRL	18/29	62	101/197	51	119/226	53
bPRL	23/30	77	118/199	59	141/229	62
hPRL	23/28	82	122/199	61	145/227	64

closely related to PRL's. However, the significance of codon preference is not known. Cooke et al. (1981) have concluded that bias in codon usage in the PRL-GH gene family is not due to evolutionary relationships.

Termination Codon

The rPL II coding sequence is terminated by the opal (TGA) stop codon. The choice of termination codon has been used by Miller and Eberhardt (1983) as a feature which differentiates the PRL-GH gene family into two groups, PRL-like and GH-like hormones. All known PRL's use a single termination codon, ochre (TAA) whereas GH's and hPL terminate with the amber (TAG) stop codon.

The various termination codons used by the PRL-GH gene family are shown in Table 12. The mouse clones, mPRP, mPLF-1 and mPLF-2 use the same termination codon as rPL II and form a third group within the family in terms of termination codon choice unlike PRL's and hPL/GH's. Rat PLP-A, like the PRL's, is terminated by the ochre (TAA) codon.

3' Untranslated Region

A comparison of the 3' untranslated regions of rPL II, rPRL and rGH is made in Figure 9. The 3' untranslated

Table 12. Choice of termination codons in the PRL-GH gene family.

The termination codons were obtained from the published sequences: hPL, Shine et al. (1977); hGH, Martial et al. (1979); bGH, Miller et al. (1980); mGH, Linzer and Talamantes (1985); rGH, Seeburg et al. (1977); mPRP, Linzer and Nathans (1985); rPLP-A, Peden, personal communication; mPLF-1, Linzer and Nathans (1984); mPLF-2, Linzer et al. (1985); rPRL, Cooke et al. (1980); mPRL, Linzer and Talamantes (1985); bPRL, Miller et al. (1981); hPRL, Cooke et al. (1981).

TABLE 12

Choice of Termination Codons in the PRL-GH Gene Family

Amber (TAG)	Ochre (TAA)	Opal (TGA)
hPL	rPRL	rPL II
hGH	mPRL	mPLF-1
bGH	bPRL	mPLF-2
mGH	hPRL	mPRP
rGH	rPLP-A	

Figure 9. Comparison of the 3' untranslated regions of rPL II, rGH and rPRL mRNA.

The rPRL sequence is from Cooke et al. (1980). The rGH sequence is from Seeburg et al. (1977). The sequences are aligned without gaps. Identical bases in rGH and rPRL, to rPL II, are marked by asterisks. The AATAAA poly A addition signals are underlined.

Figure 9. Comparison of the 3' untranslated regions of rPL II, rGH and rPRL mRNA.

```
rGH      GCACACACTGGTGTCTCTGCGGCACTCCCCCGTTACCCCCCTGTACTCTGGCAACTGCCACCCCTACACTTTGTCCTAAT
         * *  **    ****  *            **  * *               *  **       *  ** *  *    *
rPL II   GTAGCCATCCCTGTCCTTTGTCTCTGAGAAGGTCCCTCATGCTCTAGACCTTCAGGGCACTAATAAATCTCTACCTCTTT
         *    *** * *  * * *  *     * * ** * * *   ****   **  * **  *** *     *  * ** *
rPRL     GCCTACATTCATTCCATGTACCATCCGGGATGTTCTTAAAAGTCTATTTCTCAAAGGTTCTATTTGCATTACAACTTTCA

rGH      AAAATTAATGATGCATCATATpolyA
                 *  **  * *  *
rPL II   GGTGCCCTTCCTGATTTAGTTTGTATCTTTCTTAAAAATAAACTCACTCTTTGGAAATGTTpolyA
         *       *   **
rPRL     GCACATGCTTAAGA
```

region of rPL II, when compared without gaps to the 3' untranslated region of rPRL, is identical in 36 of the 94 bases (38%). When compared without gaps to the 3' untranslated region of rGH, 28 of the 101 bases (28%) are identical. The homology in the 3' untranslated region is less extensive than the homology within the coding region.

The PRL's demonstrate a preference for A or T in the 3' untranslated region. In the first 43 bases, 59% of the bases are A or T (Miller and Eberhardt, 1983). Rat PL II uses A or T in 20 of the first 43 bases (47%).

The GH's demonstrate a preference for G or C in the proximal region of the 3' untranslated area (Miller and Eberhardt, 1983). In the first 47 bases, 69% of the bases are G or C. Rat PL II uses G or C in 25 of the first 47 base positions (53%). Therefore, rPL II does not appear to have the same base preference as GH's or PRL's.

Miller and Eberhardt (1983) also characterize several palindromic structures within the 3' untranslated regions of PRL's or GH's. These palindromes are not found within the 3' untranslated region of the rPL II mRNA.

B. Post-translational Processing of rPL II

Signal Peptide

Rat PL II is known to be a secreted protein. The

protein (~20-22K mw) is found in pregnant rat serum (Robertson et al., 1982).

Secreted proteins share a highly conserved sequence of amino acid residues, found at the amino terminus of the nascent polypeptide chains, which is cleaved prior to secretion (Blobel and Dobberstein, 1975). Therefore, polypeptide hormones, including those of the PRL-GH family, are synthesized as prehormones and subsequently cleaved to the secreted mature forms.

In vitro translation of rPL II mRNA produced a 25 Kd protein which was processed *in vitro* by dog pancreatic microsomes to a molecular weight of 22 Kd. The molecular weight of the processed form corresponds to the size of the rPL II found in pregnant rat serum (Robertson et al., 1982). Therefore, it appears that rPL II is formed as a precursor with a signal peptide that is processed prior to secretion.

Since no amino acid sequence of the mature rPL II protein is available, the authentic cleavage site for the secreted protein is not known. However, based on several criteria, a potential cleavage site has been assigned.

Signal peptides are predominantly composed of hydrophobic residues (Chou and Fasman, 1978). The hydropathy plot of the deduced amino acid sequence of rPL II revealed

that a highly hydrophobic region occurs at the amino terminus of the protein (Figure 7 in Results). Within the first 30 amino acid residues of the translation product, 14 of the residues are hydrophobic (Figure 10). A core occurs in which 10 out of 12 residues are hydrophobic.

Recently, the amino terminal amino acid sequence of the secreted mouse PL II protein was determined by Edman degradation (Linzer et al., 1985). When Ala-31 of rPL II is aligned with Leu-1 of mouse PL II, 12 of the succeeding 19 amino acid residues are identical (Figure 11). At 3 more positions, the amino acid residues are related. Therefore, a signal peptide cleavage site was assigned between Ser-30 and Ala-31 of rPL II.

The assigned cleavage site between Ser-30 and Ala-31 of rPL II, determined by comparison with mPL II, was checked to see if it satisfied the empirical rules for a signal peptide cleavage site. There are no charged residues within the proposed signal sequence (Von Heijne, 1983). The glutamine at position -4 which fulfills the requirement for a helix breaking residues commonly found at this position (Watson, 1984). Threonine and serine, at positions -2 and -1 respectively, meet the requirement for amino acid residues with small, uncharged side chains at the carboxyl end of the signal peptide (Von Heijne, 1983).

Figure 10. Hydrophobic Residues at the Amino Terminal of rPL II.

The IUPAC-IUB Commission on Biochemical Nomenclature one-letter notation for amino acids (1968) has been used.

Ala	A	Gln	Q	Leu	L	Ser	S
Arg	R	Glu	E	Lys	K	Thr	T
Asn	N	Gly	G	Met	M	Trp	W
Asp	D	His	H	Phe	F	Tyr	Y
Cys	C	Ile	I	Pro	P	Val	V

Hydrophobic residues (A, V, L, I, P, M, W) are denoted by asterisks. The hydrophobic core is underlined.

Figure 10. Hydrophobic Residues at the Amino Terminal of rPL II.

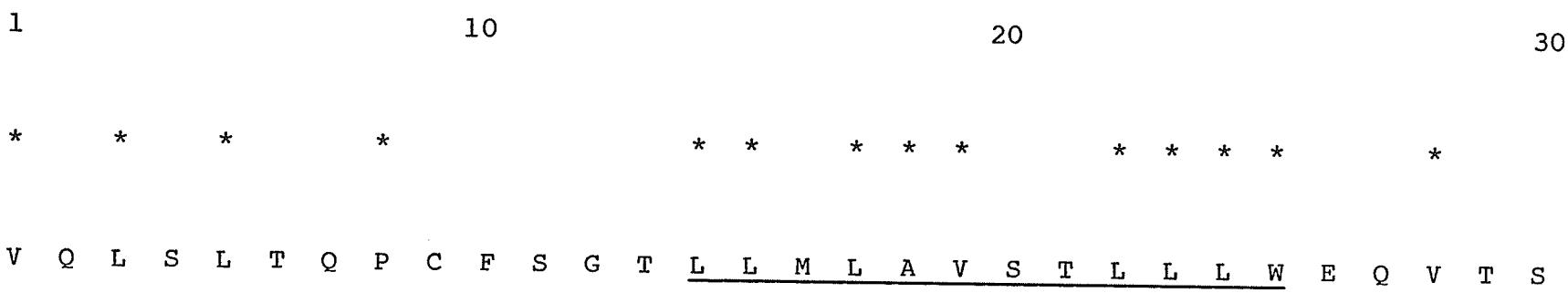
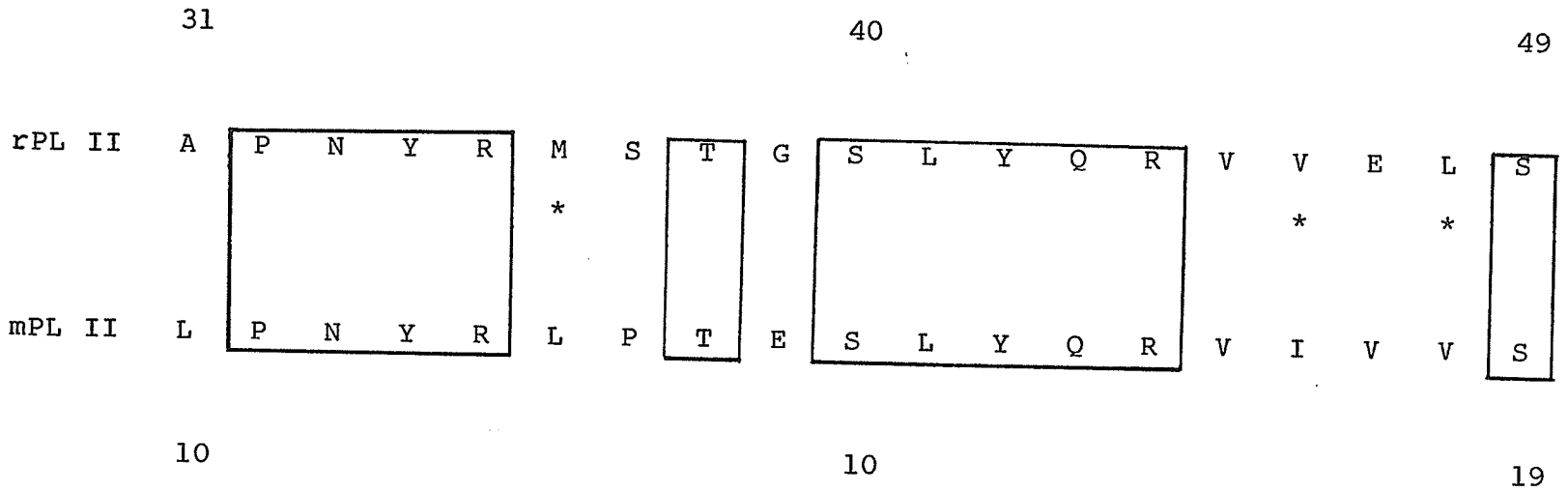


Figure 11. Alignment of rPL II with mPL II.

The amino acid sequence of mPL II is taken from Linzer et al. (1985). Ala-31 of the translation of rPL II is aligned with Leu-1 of the mPL II protein. The one-letter notation for amino acids, as described in Figure 10, is used. Identical residues are shown by the boxed areas. Related residues (V=L=I=M; S=T; Q=N; E=D; R=K; Y=F) are denoted by asterisks.

Figure 11. Alignment of rPL II with mPL II.



Serine is the most commonly occurring residue at position -1. Following Von Heijne's (1983) method for assignment of the site with the highest processing probability by statistical analysis, the predicted site concurs with the calculated site (Figure 12).

The predicted cleavage site corresponds with the *in vitro* translations of rPL II mRNA. The calculated molecular weight of the presumed precursor is 24,954 daltons versus the *in vitro* translation product of 25 kd. The mature hormone has a calculated molecular weight of 21,693 daltons versus 22 kd in the *in vitro* translation with dog pancreatic microsomes. The entire mature rPL II protein has been sequenced via the cDNA and would consist of 191 amino acid residues. The signal peptide is made up of at least 30 amino acids.

The predicted signal sequence of the rPL II protein is longer than those of most other members of the PRL-GH gene family although rPLP-A has a predicted signal peptide of 31 amino acids (Peden, personal communication) and PRL's, in general, have longer signal peptides than GH's and hPL (Table 13). Therefore, rPL II is more PRL-like than GH-like in terms of the length of its signal peptide.

The sequence of the predicted rPL II signal peptide also shows a high degree of homology to the signal peptides

Figure 12. Statistical Assignment of the Signal Peptide Cleavage Site.

The first quadruplet containing at least three hydrophobic residues is found between the threonine at position -18 and the methionine at position -15. (Denoted by the boxed area in the figure.) Therefore, the "window" as defined by Von Heijne (1983), containing the cleavage site occurs between valine at position -3 and methionine at position 6. (Denoted by the arrows.) The calculated processing probabilities, shown below the residues within the window, demonstrated that cleavage between serine and alanine, as determined by comparison with mPL II, is the most probable statistically (Von Heijne, 1983).

Figure 12. Statistical Assignment of the Signal Peptide Cleavage Site.

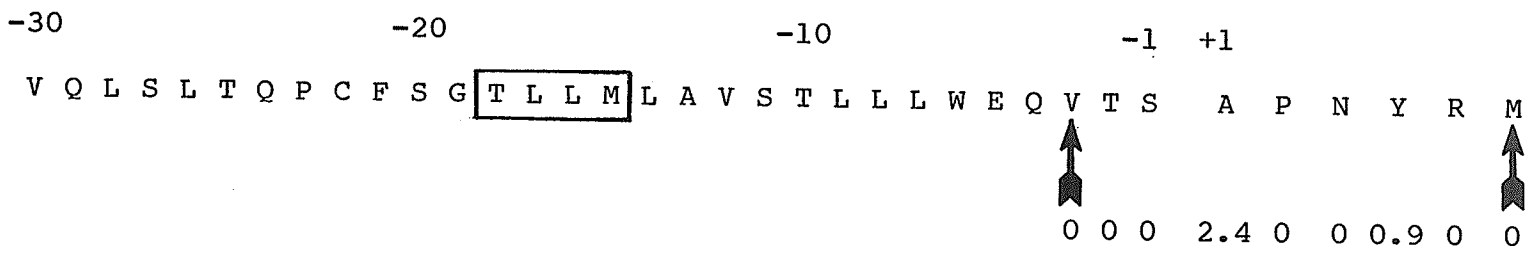


Table 13. Signal Peptide Lengths of Members of the PRL-GH Gene Family.

The number of amino acid residues in the signal peptides of rPL II and several other members of the PRL-GH gene family. The signal peptide of rPL II has not been completely characterized but is known to be at least 30 amino acids long. The signal peptide lengths of the other proteins were derived from the published mRNA sequences: hPL, Shine et al. (1977); hGH, Martial et al. (1979); bGH, Miller et al. (1980); mGH, Linzer and Talamantes (1985); rGH, Seeburg et al. (1977); mPRP, Linzer and Nathans (1985); rPLP-A, Peden, personal communication; mPLF-1, Linzer and Nathans (1984); mPLF-2, Linzer et al. (1985); rPRL, Cooke et al. (1980); mPRL, Linzer and Talamantes (1985); bPRL, Miller et al. (1981); hPRL, Cooke et al. (1981).

TABLE 13

Signal Peptide Lengths of Members of the PRL-GH Gene Family

	Number of Amino Acid Residues
hPL	26
hGH	26
bGH	26
mGH	26
rGH	26
mPRP	30
rPLP-A	31
rPL II	30+
mPLF-1	29
mPLF-2	29
rPRL	28
mPRL	29
bPRL	30
hPRL	28

of the other members of the PRL-GH gene family (Figure 13). However, the high homology could be due to the common function of the signal peptide rather than the relatedness of the family members.

Since the estimated size of 25 Kd *in vitro* is very close to the calculated size of 24,954 daltons, probably only a few residues from the amino terminus of rPL II are missing.

Glycosylation

There are no potential glycosylation signals, Asn-X-Ser/Thr (Bahl and Shah, 1977) within the mRNA sequence of rPL II. This correlates well with the decrease, not increase, in size of the *in vitro* rPL II mRNA translation product processed *in vitro* by dog pancreatic microsomes.

Both oPRL and hPRL contain the consensus glycosylation sequence, Asn-Leu-Ser, at positions 31-33. Lewis et al. (1984, 1985) have isolated glycosylated forms of PRL from ovine and human pituitaries. In RIA's for pituitary PRL, the glycosylated forms of ovine and human PRL are only one third as immunoreactive as the nonglycosylated forms (Lewis et al., 1984; 1985).

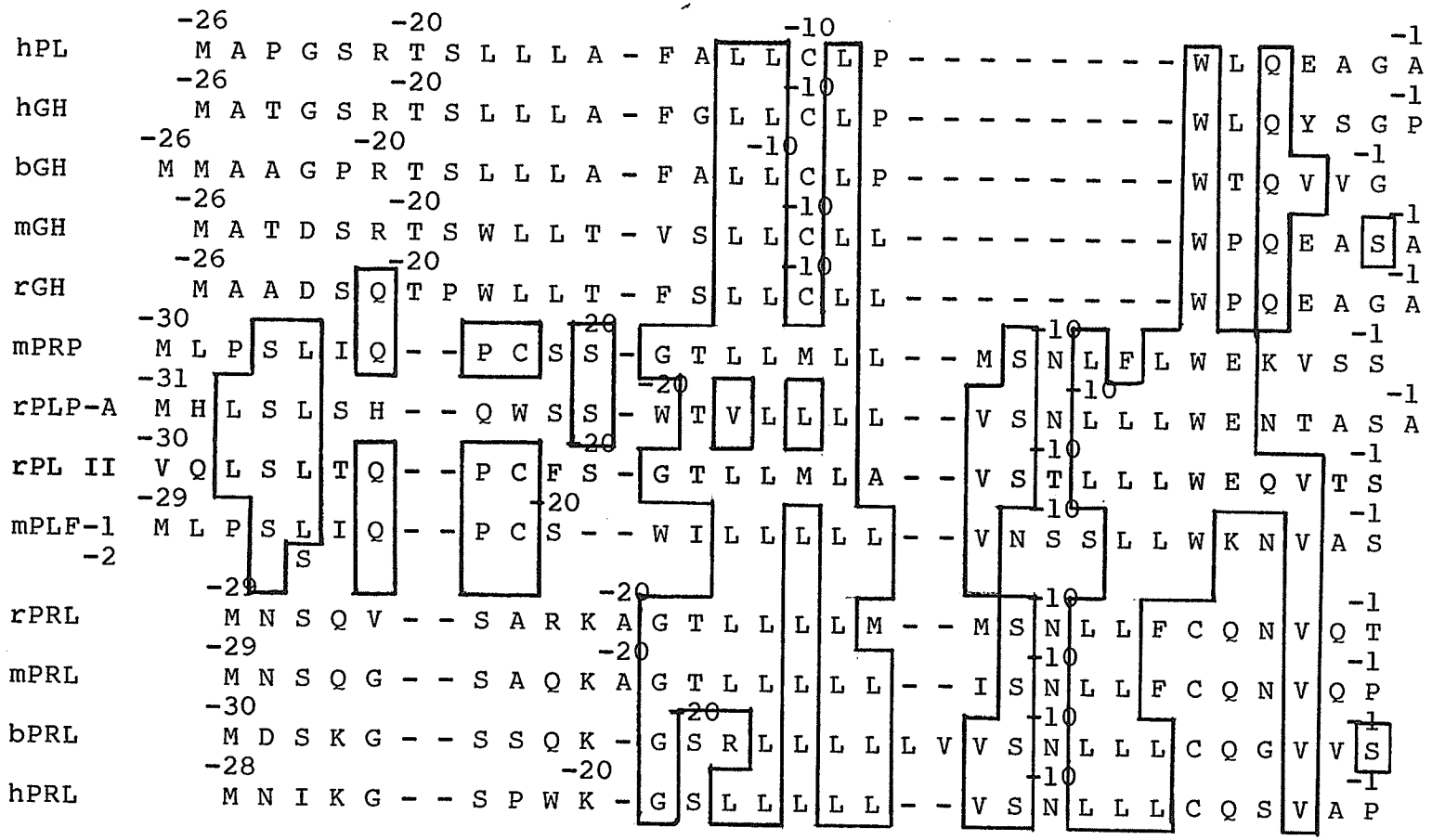
Several of the new members of the PRL-GH gene family also appear to exist as glycosylated forms. The mouse

Figure 13. Amino Acid Sequence Homology in the Signal Peptides of Members of the PRL-GH Gene Family.

The one-letter notation for amino acids, described in Figure 10, is used.

Residues homologous to rPL II are enclosed in the boxes. The signal peptide sequences are derived from the published sequences cited in Table 12. Alignment has been maximized by the introduction of arbitrary gaps.

Figure 13. Amino Acid Sequence Homology in the Signal Peptides of Members of the PRL-GH Gene Family.



clones, mPLF-1 and mPLF-2, contain three glycosylation signals at the amino end of the mature protein (Linzer and Nathans, 1984; Linzer et al., 1985). Mouse PRP also contains three potential glycosylation signals at the NH_2 end of the proposed mature peptide (Linzer and Nathans, 1985). The rat placental clone, rPLP-A contains two consensus glycosylation signals, Asn-Tyr-Thr, at positions 10-13 and 144-146.

The physiological significance of glycosylation is not known. Lewis et al. (1985) has proposed that the tertiary structure of a protein is altered by glycosylation. The structural alteration may expose or protect different portions of the molecule from proteolytic cleavage. The proteolytic cleavage may be necessary for correct post-translational processing.

Proteolytic Cleavage Sites

There is an Arg-Arg dipeptide at positions 167-168 in the deduced amino acid sequence of rPL II which may be a site of proteolytic cleavage.

Propolypeptides occur occasionally in the biosynthesis of polypeptide hormones. For example, insulin, gastrin, somatostatin, and glucagon are synthesized as prohormones which are subsequently cleaved at a site with basic

residues (Docherty and Steiner, 1982). The half-lives of the prohormones are generally longer. However, the physiological significance of this mechanism is unclear.

Proteolysis of hGH in the pituitary has been reported (Singh et al., 1974; Lewis et al., 1977). In a review, Mittra (1984) has suggested that proteolytic processing is essential for the growth-promoting and mitogenic actions of PRL and GH. However, this proposal remains to be proven.

The mouse proliferins, mPLF-1 and mPLF-2 contain 3 potential proteolytic sites at positions 120-122 (Lys-Lys-Lys), 145-146 (Lys-Lys) and 176-177 (Lys-Lys) (Linzer and Nathans, 1984; Linzer et al., 1985). Mouse PRP contains two dibasic peptides (Arg-Lys) at positions 35-36 and 84-85 (Linzer and Nathans, 1985). Rat PLP-A also contains three potential cleavage sites at positions 51-52 (Arg-Arg), 133-134 (Lys-Lys) and 174-175 (Lys-Lys).

C. Comparison of the Nucleotide and Amino Acid Sequences of the PRL-GH Gene Family Members to rPL II

Direct comparisons of the nucleotide and deduced amino acid sequences of rPL II with those of other members of the PRL-GH gene family are based on the alignments shown in Figure 14.

Figure 14. Alignment of rPL II with Other Members of the PRL-GH Gene Family.

To compare nucleotides, codons and amino acids among the PRL-GH gene family, the deduced amino acid sequences from the published mRNA sequences: hPL, Shine et al. (1977); hGH, Martial et al. (1979); bGH, Miller et al. (1980); mGH, Linzer and Talamantes (1985); rGH, Seeburg et al. (1977); mPRP, Linzer and Nathans (1985); rPLP-A, Peden, personal communication; mPLF-1, Linzer and Nathans (1984); mPLF-2, Linzer et al. (1985); rPRL, Cooke et al. (1980); mPRL, Linzer and Talamantes (1985); bPRL, Miller et al. (1981); hPRL, Cooke et al. (1981), have been aligned. A minimal number of gaps have been introduced arbitrarily to maximize the alignments. The one-letter notation for amino acids, described in Figure 10, is used.

FIGURE 14

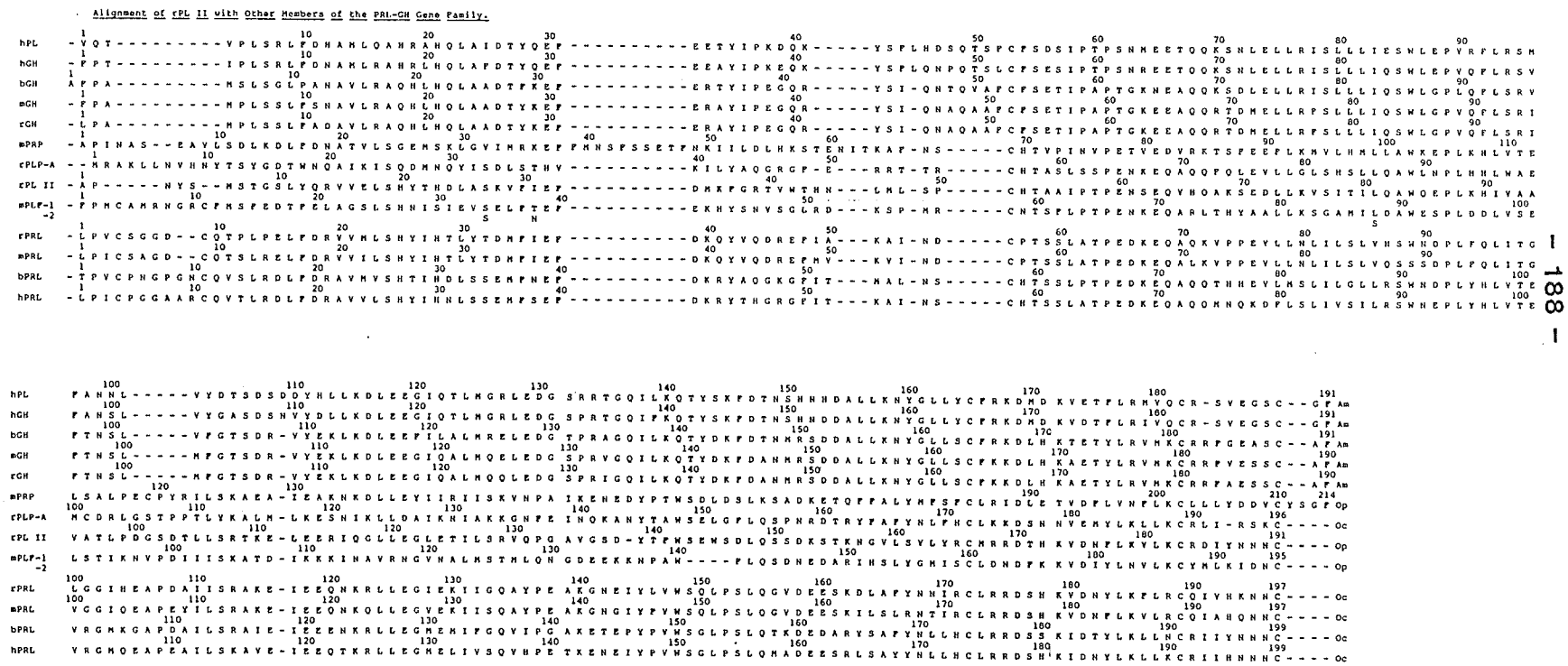

Alignment of rPL II with Other Members of the PRL-GH Gene Family.
hPL
hGH
bGH
mGH
rGH
mPRP
rPLP-A
rPL II
mPLF-1
-2
rPRL
mPRL
bPRL
hPRL

Table 14 shows the comparison of the nucleotide and deduced amino acid sequence of rPL II to other members of the PRL-GH gene family.

Amino Acid Sequence Homologies

Two unrelated amino acid sequences of the same length will display approximately 5% identity (Doolittle, 1981). All the members of the PRL-GH gene family are more than 5% homologous to rPL II at the amino acid level (Table 14).

Human PL and the GH's are 21-23% identical to rPL II at the amino acid level. The mouse clones, mPRP, mPLF-1, mPLF-2 and the rat clone, rPLP-A, are 28-31% identical to rPL II at the amino acid level. The PRL's are 37-40% homologous to rPL II. Therefore, rPL II appears to be most closely related to PRL's at the amino acid level.

Cooke et al. (1981) noted that hPL and hGH were 80% homologous at the amino acid level. Rat PL II is not as closely related to any of the family members. Interspecies comparisons of PRL's and GH's showed that PRL's as well as GH's are approximately 65% homologous among themselves at the amino acid level (Cooke et al., 1981). Rat PL II is not as closely related to any of the members of the family examined. However, the relationship of rPL II to GH's and

Table 14. Amino Acid and Nucleotide Comparisons of the PRL-GH Gene Family to rPL II.

The sequences are derived from the published sequences used in Figure 14. The alignments used to determine the values in the table are from Figure 14. Only the coding sequences of the mature peptides have been compared. Amino acids, codons and nucleotides corresponding to gaps, unmatched segments and the termination codons have been excluded for the calculations. Related amino acids are those described in Figure 11.

TABLE 14

Amino Acid and Nucleotide Comparisons of the PRL-GH Gene Family to rPL II

Comparison	Amino Acids				Nucleotides							
	Identical		Related		Identical Bases		Identical Codons		Codons Differing By One Base			
									Silent		Expressed	
rPL II vs.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
hPL	23	41/180	21	29/139	37	202/540	7	13/180	58	26/45	42	19/45
hGH	23	42/180	21	29/138	38	204/540	8	15/180	55	26/47	45	21/47
bGH	22	40/179	19	26/139	39	208/537	10	18/179	45	22/49	35	17/49
mGH	21	38/179	19	27/141	34	184/537	8	14/179	50	23/46	50	23/46
rGH	21	38/179	19	27/141	37	198/537	7	13/179	50	24/48	50	24/48
mPRP	31	59/190	18	24/131	52	297/570	20	38/190	31	17/54	69	37/54
rPLP-A	28	52/188	19	26/136	51	287/564	17	32/188	36	21/56	63	35/56
mPLF-1	29	54/187	18	24/133	50	283/561	18	34/187	34	19/56	66	37/56
mPLF-2	28	53/187	18	24/134	50	282/561	17	32/187	34	20/58	66	38/58
rPRL	37	69/189	25	30/120	56	317/567	24	45/189	39	23/59	61	36/59
mPRL	40	75/189	24	27/114	57	321/567	26	49/189	42	22/53	58	31/53
bPRL	40	76/189	23	26/113	56	318/567	24	45/189	49	29/59	51	30/59
hPRL	40	76/189	25	28/113	59	334/567	27	50/189	40	26/65	60	41/65

hPL, in terms of amino acid homology, is analogous to the homology seen between the PRL's and GH's (~25%).

Figure 15 shows a direct comparison of the predicted amino acid sequences of the mRNA's coding for rPL II and other members of the family. The members of the family and rPL II display several highly conserved regions.

Nucleotide Sequence Comparisons

Random nucleotide sequences display approximately 25% identity (Doolittle, 1981). In all the comparisons at the nucleotide level, shown in Table 14, the homology to rPL II exceeds 25%. Also, like othr members of the PRL-GH gene family (Cooke et al., 1981), the nucleotide sequence homology is greater than the amino acid homology in all cases.

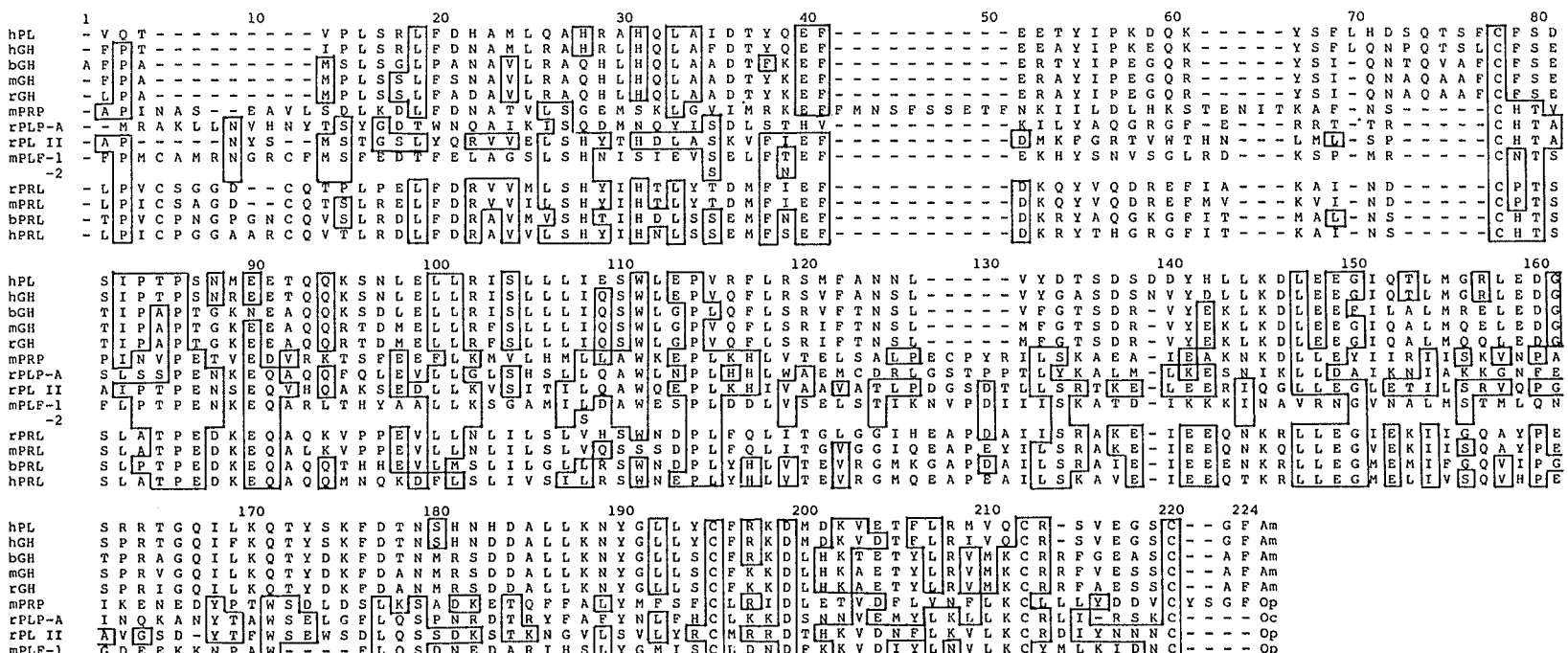
The nucleotide sequence homologies to rPL II appear to follow the same pattern as the amino acid sequence homologies. The highest identity (~57%) is seen between rPL II and the PRL's although it is not as high as the identity among PRL's (75%) (Cooke et al., 1981). A lower homology (~51%) is seen between rPL II and mPRP, mPLF-1, mPLF-2 and rPLP-A. Only approximately 37% identity is seen between the aligned nucleotide sequences of rPL II and GH's/hPL. This is similar to the homology seen between

Figure 15. Comparison of the Predicted Amino Acid Sequences of the mRNA's Coding for rPL II and Other Members of the PRL-GH Gene Family.

The alignment and sequences are taken from Figure 14. The boxed areas indicate amino acid residues identical to rPL II.

FIGURE 15

Comparison of the Predicted Amino Acid Sequences of the mRNA's Coding for rPL II and Other Members of the PRL-GH Gene Family.



PRL's and GH's (~40%) in general (Cooke et al., 1981).

D. Molecular Evolution

For the evolutionary comparisons of rPL II with other members of the PRL-GH gene family, it has been assumed that rPL II arose from a common ancestral gene, possibly by gene duplication.

Codon Comparisons

Codon by codon comparisons facilitate the identification of two additional types of nucleotide differences. Codons that differ by a single base may result in amino acid replacement (expressed substitution) or a synonymous codon (silent substitution).

As seen in Table 14, the percentage of silent substitutions is greater than the 25% that is seen when comparing random sequences (Jukes and King, 1979). Since this is considered an indication of evolution of genes from a single, common ancestral gene (Cooke et al., 1981), the number of silent substitutions further supports the hypothesis that rPL II arose from the same ancestor as PRL's and GH's.

Cooke et al. (1981) concluded that, in the case of hPL and hGH, positive selective influences caused a rapid

fixation of expressed substitutions in the recently diverged genes since the two sequences displayed the highest nucleotide sequence homology in the family (92%) and had the lowest ratio of silent to expressed single base substitutions, 0.45 (31%/69%).

The percentage of nucleotide sequence homology and ratios of silent to expressed single base substitutions of the PRL-GH gene family members compared to rPL II is shown in Table **15.** The lowest ratio is seen between rPL II and mPRP, rPLP-A and mPLF's. Therefore, the divergence of the nucleotide sequences of these proteins may be slowed by positive selective influences.

Evolutionary Relationship of rPL II to Other Members of the PRL-GH Gene Family

Dayhoff (1976) has developed methods for determination of the relative lengths of the branches of phylogenetic trees. The amount of evolutionary change can be measured in units of accepted point mutations (PAM's). The observed number of differences per 100 residues or bases is correlated to the number of differences that must have occurred. The method does not assign evolutionary relationships in terms of time, but rather in terms of relative evolutionary distance.

TABLE 15

Nucleotide Sequence Homology and
Ratio of Silent to Expressed Single Base Substitutions

	Ratio of Silent to Expressed Single Base Substitutions		Identical Bases (%)
rPL II			
vs.			
hPL	58%/42%	1.38	37
hGH	55%/45%	1.22	38
bGH	45%/35%	1.29	39
mGH	50%/50%	1.00	34
rGH	50%/50%	1.00	37
mPRP	31%/69%	0.45	52
rPLR-A	38%/63%	0.60	51
mPLF-1	34%/66%	0.52	50
mPLF-2	34%/66%	0.52	50
rPRL	39%/61%	0.64	56
mPRL	42%/58%	0.72	57
bPRL	49%/51%	0.96	56
hPRL	40%/60%	0.67	59

The data are derived from Table 14.

The evolutionary distances in PAM's between rPL II and other members of the PRL-GH gene family are shown in Tables 16 and 17. Using nucleotide (Table 16) and amino acid (Table 17) sequence divergence, the same pattern appears. Rat PL II is most closely related to the PRL's, followed by mPRP, rPLP-A, mPLF's and then, the GH's.

Another method for quantifying differences among related peptides is to compare their evolutionary divergences by using a parameter called the unit evolutionary period (UEP) which is defined as the length of time, in millions of years, for a 1% amino acid sequence difference to arise in two related proteins (Wilson et al., 1977). Wilson et al. (1977) calculated a UEP of 5.0 for PRL and 4.0 for GH. Cooke et al. (1981) and Miller et al. (1981) used the value 4.5 for comparisons between PRL-GH gene family members. This value will be used for looking at the evolutionary divergence of rPL II from the other related proteins.

Miller et al. (1981) found that amino acid sequence comparisons were just as accurate as nucleotide sequence comparisons for calculating the evolutionary divergence of PRL's and GH's using the UEP parameter. Therefore, only amino acid sequence comparisons have been used for looking

TABLE 16

Evolutionary Distance (PAM's) between rPL II and Other Members of the PRL-GH Gene Family derived from Nucleotide Sequence Divergence

	Observed Differences per 100 Bases	Evolutionary Distance in PAM's
hPL	63	136
hGH	62	131
bGH	61	125
mGH	66	158
rGH	63	136
mPRP	48	76
rPLP-A	49	79
mPLF-1	50	82
mPLF-2	50	82
rPRL	44	66
mPRL	43	63
bPRL	44	66
hPRL	41	59

The nucleotide sequence divergence (observed differences) was calculated from the nucleotide identities in Table 14. The PAM values were derived from the nucleotide PAM scale in Dayhoff (1976). Dayhoff's model assigns equal probability to all nucleotide changes.

TABLE 17

Evolutionary Distance (PAM's) between rPL II and Other Members of the PRL-GH Gene Family derived from Amino Acid Sequence Divergence

	Observed Differences per 100 Residues	Evolutionary Distance in PAM's
hPL	43	65
hGH	43	65
bGH	44	67
mGH	44	67
rGH	40	58
mPRP	36	50
rPLP-A	38	54
mPLF-1	38	54
mPLF-2	38	54
rPRL	33	44
mPRL	32	42
bPRL	32	42
hPRL	32	42

The amino acid sequence divergence (observed differences) is calculated from the amino acid identities in Table 14. Gaps and unaligned sequences were not included. Only the coding region of the mature peptides was used for the calculations. The PAM values are from the amino acid PAM scale in Dayhoff (1976).

at the evolutionary relationship in UEP's of rPL II with the other gene family members.

The time of evolutionary divergence of rPL II from the related PRL-GH family members is shown in Table 18. The comparisons imply that rPL II diverged from PRL's only 275 MYA whereas it diverged from GH's and hPL approximately 350 MYA. Rat PL II appears to have diverged from mPRP, mPLF and rPLP-A approximately 320 MYA. Therefore, PL II is more closely related to rPRL than rGH evolutionarily.

The time of rPL II divergence from GH (350 MYA) approximates the time calculated by Cooke et al. (1981) for the time of PRL and GH divergence (392 MYA). This also correlates well with the results of Acher (1976) who estimated that PRL and GH diverged 400 MYA near the time of fish and tetrapod divergence.

Rat PL II appears to have diverged from PRL and the other PRL-like molecules, mPLF, mPRP and rPLP-A, prior to mammalian radiation, 85-100 MYA (Romero-Herrera et al., 1973; McKenna, 1969) and the origin of placental mammals, approximately 100 MYA (Dickerson and Geis, 1970).

Unfortunately, this method does not distinguish between direct or indirect divergence. Rat PL II may have diverged from rPLP-A following the divergence from rPRL.

TABLE 18

Evolutionary Divergence of rPL II from PRL-GH Gene Family Members

	Divergence (%)	Time of Divergence (MYA)
rPL II vs.		
hPL	77	347
hGH	77	347
bGH	78	351
mGH	79	356
rGH	79	356
mPRP	69	311
rPLP-A	72	324
mPLF-1	71	320
mPLF-2	72	324
rPRL	63	284
mPRL	60	270
bPRL	60	270
hPRL	60	270

The percent divergence is based on the comparisons of the amino acid sequences shown in Figure 15. The time of divergence, millions of years ago (MYA), was calculated using a UEP value of 4.5.

However, a rough estimate of the evolutionary relationships has been obtained.

3' Untranslated Region

Human GH and hPL have diverged only 6.4% in the 3'-untranslated region of the mRNA whereas hPRL and hGH have diverged 87.5% (Cooke et al., 1981). Comparisons of the 3' untranslated region of rPL II compared, without introducing gaps, to rGH and rPRL (Figure 9) shows that rPL II has diverged 72% from rGH and 62% from rPRL. The divergence is greater than that seen in the coding region: rPL II vs rPRL, 45%; rPL II vs rGH, 63%.

In the PRL-GH gene family, except in the case of hGH and hPL, the 3' untranslated areas have diverged more than the coding areas (Cooke et al., 1981) suggesting that there is less evolutionary pressure to conserve 3' untranslated sequences, as might be expected.

Evolutionary Implications

Although the mRNA and amino acid sequences of other subprimate PL's are required for a more thorough evolutionary analysis, several things are suggested by these comparisons. Human PL and rPL II have arisen by separate mechanisms at different times in mammalian

evolution. Rat PL II is not as closely related to any other known molecule as hPL is to hGH. Human PL is believed to have arisen after mammalian radiation (Cooke et al., 1981; Miller et al., 1981) whereas rPL II seems to have arisen before mammalian radiation. Miller et al. (1981) suggested that if hPL arose recently in humans, then a separate gene duplication to produce PL, must have taken place in each mammalian species. However, the human PL may be unique and it may be that PL's in most subprimate species are more primitive. What is known is that a placental hormone, resembling the pituitary hormones GH and PRL, has been generated at least twice in mammalian evolution, once in rats and once in humans. Rat PL II could have arisen from the duplication of the PRL gene as suggested by Hurley et al. (1977). Mouse PLF, mPRP and rPLP-A also must have arisen prior to mammalian radiation, possibly from duplications of the PRL gene.

The evolutionary comparisons raise many questions. For example, is a gene resembling rPL II present in all mammals, including humans? Is the rPL II gene on the same chromosome as rPRL? Is it closely linked to the PRL gene as well as genes for rPLP-A and the rat equivalents of mPLF and mPRP? Does the homology between rPL II and rPRL which

is seen in the coding region extend into the introns? Are all other subprimate PL's similar to rPL II?

E. Structural Comparisons to the rPL II Protein

Common structural features which the rPL II protein shares with other proteins further suggest the descent of rPL II and other PRL-GH gene family members from a common ancestral protein. In addition, there is evidence that the primary role of rPL II during pregnancy may be as a mammotropin/lactogen. Rat PL II, unlike hPL and oPL, has not been shown to possess any somatotropic activity. Therefore, the conservation or absence of residues and regions implicated in biological activity may aid in elucidating the biological role of rPL II and the validity of the implied residues. Human PL, hGH and the PRL's are known to be lactogenic. The mouse proteins, mPLF and mPRP and rPLP-A have not yet been shown to have lactogenic activity.

Surprisingly, rPL II, at the level of amino acid and nucleotide sequence, is not any more homologous to the lactogenic hGH than it is to the non-lactogenic rGH (see Table 14).

Most studies employed to determine essential residues have used chemical modification of residues that often

drastically disrupts the conformation of the molecules.

Three-dimensional structural determination is the best method for comparing proteins but very few proteins have been crystallized. Current methods for prediction of tertiary structure are not very accurate.

Rat PL II displays several other structural features characteristic of the PRL-GH gene family products. A diagramatic representation of several structural features of the PRL-GH gene family members and rPL II is shown in figure 16. Rat PL II appears to have common structural features with both the PRL-like and GH-like molecules. The members of the PRL-GH gene family share a similar molecular weight (~22 kd) as well as polypeptide chain length (~200 amino acids) with rPL II.

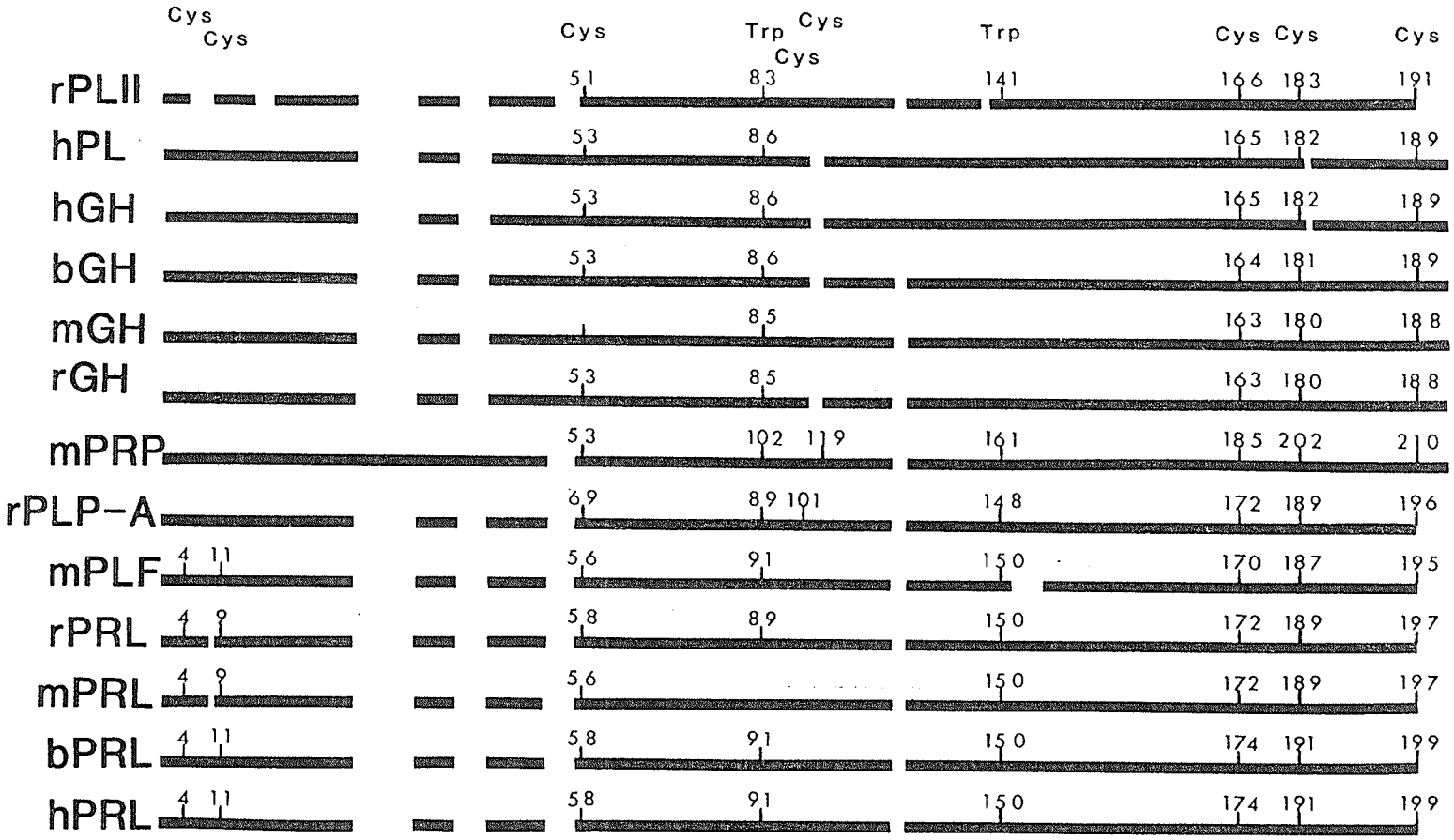
Like the PRL's, the rPL II polypeptide chain terminates at the last cysteine residue. The GH's, hPL and mPRP extend beyond the final cysteine.

Tryptophan and cysteine residues are among the least frequently occurring amino acids. Usually, only highly related proteins conserve tryptophan and cysteine residues (Doolittle, 1981). In addition, Dayhoff and Barker (1972) have concluded that conservation of these residues demonstrates evolutionary relationships among proteins.

Figure 16. Conserved Structure of the PRL-GH Gene Family Members.

The locations of cysteine and tryptophan residues are shown. The numbers refer to the amino acid positions in the mature proteins. Gaps and alignments are based on Figure 15.

Figure 16



Conserved Structure of the PRL-GH Gene Family Members

Tryptophan and cysteine residues they find are the least subject to replacement in the course of protein evolution.

Tryptophan Residue Conservation

Rat PL II, like the PRL's, mPLF, mPRP and rPLP-A, has two tryptophan residues in conserved positions. Kawauchi et al. (1973) suggested that the first tryptophan, seen in a similar position in oPRL, was essential for lactogenic activity. Selective modification of Trp-149, with o-nitrosulphenyl chloride, did not abolish lactogenic activity but modification of both Trp-90 and Trp-149 completely abolished biological activity. However, mPRL does not contain Trp-90 (Kohmoto et al., 1984) yet still acts as a lactogen. This tryptophan is also not found in hPL or hGH, which are both known to have lactogenic activity. Kawauchi et al. (1973) reported that the doubly modified oPRL underwent drastic changes in its quaternary structure as shown by circular dichroism spectra. Therefore, the drastic alteration in the conformation of the molecule may have caused the loss of biological activity. The second tryptophan residue, which according to Kawauchi et al. (1973) was not required for lactogenic activity, is conserved in the same molecules as the first tryptophan. It is also found in mPRL.

Cysteine Residue Conservation

Rat PL II contains 4 cysteine residues within the coding region of the mature protein spaced at positions equivalent to the 4 cysteines in GH's and hPL. The PRL's, in general, and mPLF have 6 cysteine residues. Rat PLP-A and mPRP contain 5 cysteines, 4 of which are in the same locations as the residues in the GH's, hPL and rPL II.

Although the complete primary structures of other subprimate PL's have not been determined, the amino acid compositions of ovine, rabbit and bovine PL have been reported. A comparison between rPL II and these other subprimate PL's is shown in Table 19.

The calculated molecular weights are approximately 22 kd except for bPL which is considerably larger (~31 kd). The isoelectric points range between 5.5 and 7.7. Rat PL II (pI 6.6) is within this range. The amino acid compositions appear to be similar although the numbers of half-cystine and tryptophan residues vary. Like the GH's and hPL, rPL II and bPL contain 4 half-cystine residues. However, oPL and rabbit PL contain 6 half-cystine residues like the PRL's, in general. Rat PL II contains 4 tryptophan residues whereas oPL and rabbit PL contain only two. The number of amino acid residues are approximately the same (191-198) except for the longer bPL (277).

Table 19. Comparison of Molecular Weight, Isoelectric Point and Amino Acid Composition of rPL II with Other Subprimate PL's.

The molecular weights as reported in the published data: oPL, Hurley et al. (1977); rabbit PL, Bolander and Fellows (1976b); bPL, Arima and Bremel (1983). The amino acid composition of bPL is shown for bPL-2, one of the three isoforms isolated by Arima and Bremel (1983). Bovine PL-2 was the most potent form biologically. Tryptophan analysis was not performed on bPL. Glx represents Gln/Glu and Asx represents Asn/Asp.

TABLE 19

Comparison of Molecular Weight, Isoelectric Point and Amino Acid Composition of rPL II with Other Subprimate PL's

	rPL II	oPL	Rabbit PL	bPL
Estimated Mol. Wt. (daltons)	21,693	21,418	22,100	31,100
pI	6.6	7.7	6.1	5.5
Amino Acid Composition				
Lys	11	14	15	19
His	7	4	6	8
Arg	10	10	8	16
Asx	19	19	21	29
Thr	14	10	11	18
Ser	18	15	15	19
Glx	20	24	22	36
Pro	7	10	12	16
Gly	8	15	15	15
Ala	10	13	15	21
½-Cys	4	6	6	4
Val	14	12	15	15
Met	4	5	3	5
Ile	8	10	4	10
Leu	22	13	13	26
Tyr	6	4	7	8
Phe	5	7	8	12
Trp	4	2	2	ND
Total Number of Residues	191	193	198	277

Rat PL II has been shown to be remarkably similar to the amino terminal of the late form of mPL. The homology at the amino-terminus of the mature peptides was shown in Figure 11. Like rPL II, mPL II does not contain the two NH_2-terminal half-cystine residues.

Secondary Structure

Disulfide Bonding

Since cysteine residues contain free sulfhydryl groups at the end of the side chain, cysteines combine to form covalent disulfide bonds which cross-link regions of the molecule.

The PRL's, in general, contain 6 half-cystine residues within the mature peptide. These residues interact to form 3 disulfide bonds (Li, 1972). The bonds are formed as an NH_2-terminal loop, a central disulfide bridge and a COOH-terminal loop. GH's and hPL lack the amino-terminal loop.

Treatment of rPL II with mercaptoethanol, to disrupt the disulfide bonds, caused a loss of more than 90% of the bioactivity of the protein (Robertson et al., 1982). This suggests that the disulfide bonds, or the molecular conformation of rPL II dictated by these bonds, are essential for the biological activity of the molecule.

Equine, salmon and Tilapia PRL's do not contain the amino-terminal disulfide bond. They contain only 4 half-cystine residues (Li and Chung, 1983; Doneen et al. 1979; Kawauchi et al., 1982). Equine PRL, however, is equipotent with oPRL in the pigeon crop-sac assay (Li and Chung, 1983). Salmon PRL is also equipotent to oPRL in the same assay (Kawauchi et al., 1982). Although Farmer et al. (1977) found that Tilapia PRL was not active in the pigeon crop-sac or mouse mammary gland bioassays, Houdebine et al. (1981) found that the Tilapia PRL was lactogenic in the rabbit. Therefore, the NH_2-terminal loop does not appear to be required for the hormonal function of lactogenic molecules.

In 1979, Doneen et al. concluded that 2 of the 3 disulfide bonds are not essential for the bioactivity of oPRL. Reduction of the amino-terminal and carboxyl-terminal disulfide linkages caused no loss in biological activity whereas the integrity of the central disulfide bond was essential for retention of biological activity. Therefore, it appears that only one disulfide bond, the central bond, is required for biological activity.

The 5 cysteine residues, in rPLP-A and mPRP, may permit these molecules to form alternate disulfide bonds and thus, possibly change their molecular conformation.

Thus, rPL II, mPL and bPL contain 2 disulfide bonds like the GH's whereas rabbit and ovine PL form contain 3 disulfide bonds like most of the PRL's.

Rat PL II also displays similarity to rat decidual luteotropin (Jayatilak et al., 1985). Rat decidual luteotropin has a molecular weight of 23.5 kd and contains disulfide linkages.

α-Helix

By secondary structure prediction (Garnier et al., 1978), 36% of the rPL II protein exists in an α-helical state. Using the same prediction method, mPLF-1 is 44% α-helix; rPLP-A is 40% α-helix and rPRL is 36% α-helix. Equine and ovine PRL form 50% and 65% α-helix, respectively, as determined by circular dichroism spectra (Li and Chung, 1983). Therefore, rPL II and rPRL share a similar content of α-helical structure.

Hydropathy

The hydropathy of the rPL II protein was shown in figure 7. The hydropathy plot may predict the molecular conformation of rPL II.

Two highly hydrophobic regions are seen in the protein. The first occurs at the amino-terminal and corres-

ponds with the predicted signal peptide seen in all family members. The second region occurs between residues 73 and 81 of the mature protein. This region is highly conserved in all members of the PRL-GH gene family (Figure 17).

Hydrophilic regions predict exposed, possibly antigenic domains. There are three regions of hydrophilicity within the mature rPL II protein at amino acid positions 58-71, 139-157 and 166-175. These regions are highly conserved among the PRL's, mPLF, mPRP and rPLP-A (Figure 17). When oPRL was cleaved with fibrinolysin between Met-53 and Ala-54, the molecule lost all immunological and biological activity (Birk and Li, 1978). The region 58-71 may be the antigenic recognition site of the molecules and the high homology may explain the immunological cross-reactivity seen between the proteins.

Conservation of Residues Implicated in Biological Activity

Li and Frankel-Conrat (1947) suggested that carboxyl groups are essential for lactogenic activity since esterification of the carboxyl groups in oPRL caused a decrease in the lactogenic potency of the hormone, in squabs, that was proportional to the number of modified carboxyl groups. However, the authors also suggested that the decrease in bioactivity could be due to changes in the

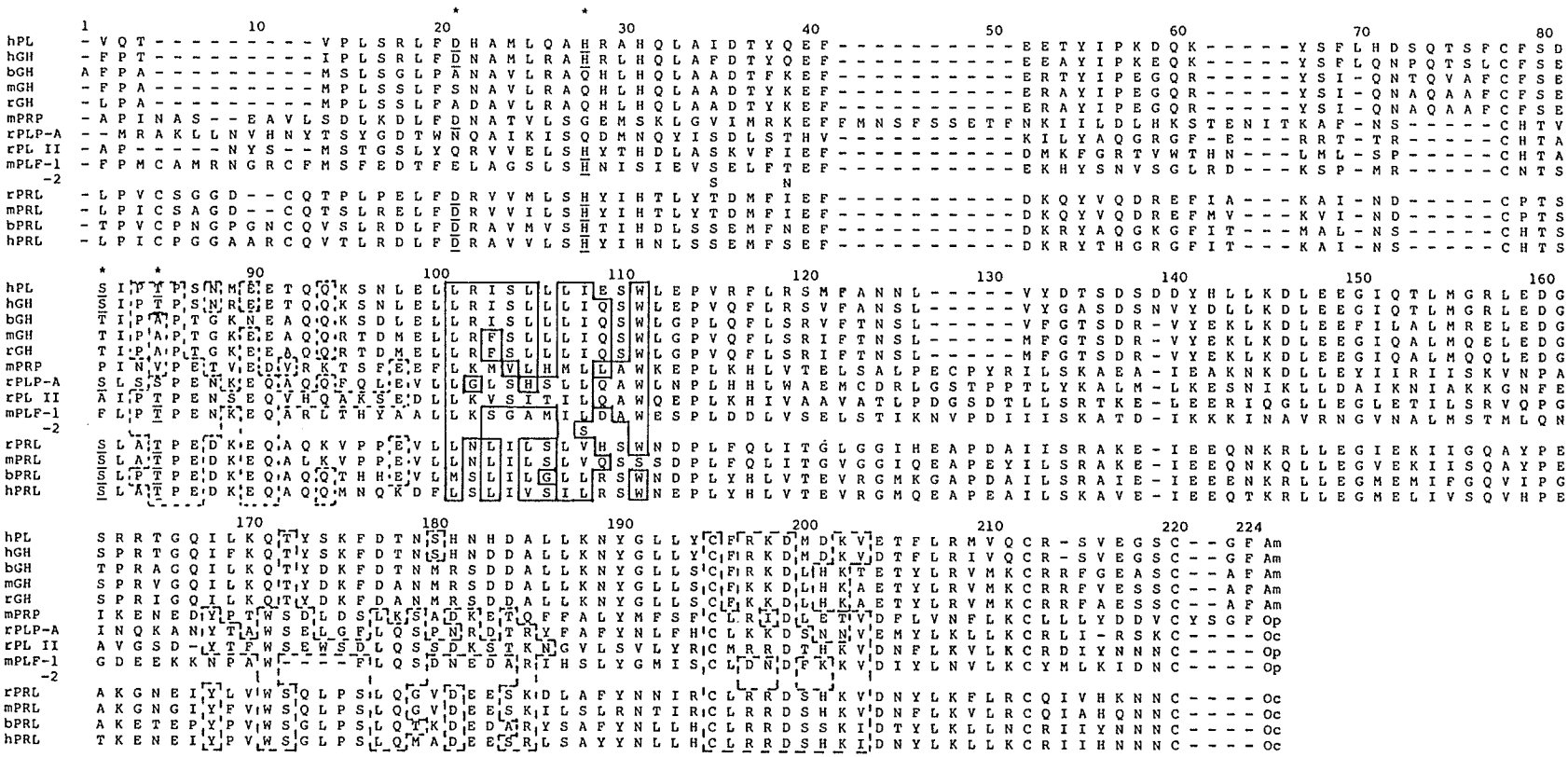
Figure 17. Hydrophobic and Hydrophilic Regions, and the Residues Implicated in Lactogenic Activity Shared by rPL II and the PRL-GH Gene Family Members.

Based on the alignment shown in Figure 14, the amino acid sequences are compared. The common hydrophobic region is denoted by the closed boxed area. The hydrophilic regions are shown by the open boxed areas. Both identical and related amino acids are enclosed in the boxes.

The residues implicated in lactogenic activity by Kohmoto et al. (1984) are denoted by asterisks. Amino acids identical to the residues described by Kohmoto et al. (1984) are underlined.

FIGURE 17

Hydrophobic and Hydrophillic Regions and the Residues Implicated in Lactogenic Activity Shared by rPL II and the PRL-GH Gene Family Members.



physical structure of the molecule such as disruption of hydrogen or ionic bonds or a change in the net molecular charge.

Most of the carboxyl residues in rPL II are highly conserved in PRL's and not as highly conserved in GH's.

Several investigators have suggested that residues found in the amino-terminal of lactogenic hormones are involved in the binding of the hormones to the lactogenic receptor. Anderson and Ebner (1979) found that chemical modification of the histidine residues in bPRL with ethoxyformic anhydride caused changes in the ability of the hormone to bind to rabbit mammary gland lactogenic receptors in the RRA-PRL. All activity was lost when all the histidine residues were modified. However, when only 5 of the 7 histidines were modified, the hormone still bound to the receptors. The activity in the assay was restored when the ethoxyformyl groups were removed. The authors then compared the amino acid sequence of bPRL and hGH and found that His-27 and His-30 of bPRL were the only two histidines also present in hGH. Therefore, they concluded that those two histidine residues are located in the lactogenic receptor binding domain of bPRL and are essential to lactogenic activity. These two histidines are found in all the PRL's, and in rPL II, hGH and hPL. There

are two histidines in bovine, mouse and rat GH but the first histidine is displaced by one position. Mouse PRP, mPLF and rPLP-A do not contain the two histidines.

Kawauchi et al. (1977) concluded that Tyr-28, but not Tyr-44 or Tyr-147 of oPRL, was essential for the lactogenic activity of the hormone. When Tyr-28 of oPRL was selectively nitrated, the modified hormone did not bind to lactogenic receptors. Nitration of the other tyrosines did not affect the binding activity. Tyr-28 is found next to the highly conserved His-27 in oPRL. The tyrosine at this position is found in rPL II and rat, mouse and human PRL's. However, it is not conserved in hGH, hPL or bPRL which are known lactogens.

Kohmoto et al. (1984), by comparison of the mouse, rat, porcine, ovine and human PRL, hPL and hGH amino acid sequences concluded that Asp-21, His-28, Ser-82 and Thr-85 were essential to lactogenic activity. They based this conclusion on the observation that these residues were found in all the lactogenic hormones but were not present in non-lactogenic subprimate GH's. Kohmoto et al. (1984) suggested that these residues are involved in lactogenic receptor binding. All 4 residues reside within the "active core" of hGH (1-134) (Reagan et al., 1978). The conservation of these residues in rPL II and the other PRL-

GH gene family members is shown in Figure 17. Asp-21 is replaced by glutamine in rPL II. His-28 is conserved in rPL II. Ser-82 is replaced by alanine and Thr-85 is replaced by the related amino acid Ser in rPL II. Therefore, Asp-21 and Ser-82 are probably not essential to lactogenic activity.

Kohmoto et al. (1984) further suggested that the other residues common to the family members are important for maintenance of hormonal structure.

Houghten and Li (1976) suggested that the three methionine residues found at positions 36, 81 and 132 were essential for the lactogenic activity of oPRL. Modification of four of the seven methionines, by alkylation, with iodoacetic acid and by oxidation with hydrogen peroxide caused little change in biological potency whereas modification of the remaining three, Met-36, Met-81 and Met-132, caused a dramatic decrease in activity. However, circular dichroism spectra indicated that the chemical modification of all the methionines, may have exposed buried tryptophan residues and changed the conformation of the molecule.

Rat PL II does not share any of these methionine residues in homologous positions. Therefore, they cannot be essential for lactogenic activity in rPL II.

Although a growth-promoting role has been suggested for PL's during pregnancy, oPL is the only subprimate PL which has been shown to have somatotropic activity. Rat PL II has not yet been shown to exert somatotropic effects. Chene et al. (1984) concluded that the lysine residues in oPL were important for the somatotropic activity of the hormone, possibly for the interaction of the molecule with the somatotropic receptors. Modification of the 14 lysine residues by methylation, ethylation, guanidation or acetamidination caused a slight decrease in the RRA-PRL activity of oPL whereas the RRA-GH activity was dramatically decreased. Although the primary structure of oPL is not known, hGH, a known somatotropin, contains 9 lysine residues. These residues are highly conserved among the GH's and hPL. In rPL II, only 1 of the 11 lysine residues is in a similar position to those in the GH's. One might predict from this that rPL II does not act as a somatotropin, in contrast to oPL and GH's.

The 20 K form of hGH does not possess the diabetogenic actions of 22 K hGH (Miller and Eberhardt, 1983). The 20 K variant is missing amino acid residues 32-46. The alignment of rPL II with hGH shows that rPL II is not homologous to this region of hGH (Figure 18) and may further suggest rPL II does not possess GH-like metabolic activity.

Figure 18. Comparison of hGH to rPL II-Region Implicated in Diabetogenic Activity.

Alignment of hGH, residues 32-46, with rPL II, residues 34-49, based on the alignment shown in Figure 14. None of the residues are conserved between the two hormones.

Figure 18.

Comparison of hGH and rPL II - Region Implicated in Diabetogenic Activity.

		32																46		
hGH	.	.	.	E	E	A	Y	I	P	K	E	Q	K	-	-	Y	S	F	L	Q	.	.	.
rPL II	.	.	.	D	M	K	F	G	R	T	V	W	T	H	N	L	M	L	-	S	.	.	.
		34																49		

VI. GENERAL CONCLUSIONS

The primary structure of rPL II has been determined. This is only the second PL, other than hPL, and the first subprimate PL to be characterized structurally. Thus, the rat is the second species, other than the human, in which the PRL, GH and PL triad has been described.

As suggested by earlier studies, rPL II shares many features with PRL's. However, rPL II also resembles GH's and has several unique characteristics.

Following the isolation and characterization of hPL, assumptions were made that a similar hormone would exist in other species. However, as shown by the characterization of rPL II, the PL in rats bears little resemblance to hPL. The PL in primates appears to be a unique hormone which has arisen recently from the GH gene exclusively in the primates. Comparisons of the primary structure of rPL II to other hormones, indicate that rPL II is closely related to PRL but not as closely related as hPL is to GH. Rat PL II, while probably arising from the same ancestral gene as PRL's and GH's, appears to have evolved from PRL prior to mammalian radiation and homologues may exist in many other mammalian species. Characterization of the rPL II gene

structure and organization may clarify the evolutionary relationship of the PRL-GH gene family members.

Although only limited information is available on other subprimate PL's, it appears that PL's vary functionally and structurally. "PL's," placental lactogenic hormones, may be species specific hormones that mistakenly share a common name. The GH's and PRL's are known to vary structurally and functionally between species (Nicoll, 1980). More information about other subprimate PL's is needed to clarify the relationship of rPL II to other subprimate PL's.

The placentae of pregnant rodents synthesize several PRL-like proteins in a specific temporal manner. One of these proteins is rPL II. Rat PL II appears to act primarily as a mammotropin/lactogen. The PRL's are known to act as multi-functional hormones(Nicoll, 1980) and it may be that the spectrum of PRL-like proteins in the rat take over specific functions of PRL during pregnancy which affect fetal development and maternal physiology. The physiological role of rPL II is unknown but elucidation of its role may lead to a better understanding of the actions of PRL and the developmental process.

VII. FUTURE STUDIES

Several future studies on rPL II will be possible because of the elucidation of the primary structure of rPL II.

The cDNA sequence will be useful as a guide for accurately mapping the rPL II gene in terms of flanking sequences and exon/intron organization.

Specific oligonucleotides, useful as primers and hybridization probes, can now be constructed using the rPL II nucleotide sequence.

The length of the signal peptide, structure of the potentially regulatory 5′ untranslated region and the 5′ boundary of Exon I of the rPL II gene can be determined with a full-length cDNA clone to rPL II mRNA. A full-length clone can be isolated by priming the 5′ end of the Day 18 placental mRNA with an oligonucleotide corresponding to the most 5′ portion of the rPL II cDNA (Smith, 1980). The sequence of the rPL II mRNA could also be determined by performing nucleotide sequence analysis on a 5′ rPL II genomic clone.

The cDNA subclones to rPL II mRNA generated for nucleotide sequence analysis in M13 vectors can be used as single-stranded strand specific probes (Dutton and Chovnick, 1984) for *in situ* hybridization studies (Gee and

Roberts, 1983). While immunocytochemical localization techniques do not differentiate between newly synthesized, receptor-bound or stored proteins, in situ hybridization localizes proteins to the cells involved in mRNA synthesis. Regulation of rPL II gene expression by various factors could be investigated in maternal, fetal and placental tissues throughout gestation.

Since rPL II is difficult to isolate from rat placentae, two approaches are possible for producing the rPL II protein. RPL II cDNA clone constructs containing the complete coding region for the mature rPL II protein could be ligated into expression vectors containing the appropriate reguatory elements. Bacterial cells, transformed with the expression vector, would produce the rPL II protein in vitro. The protein could be isolated from the bacterial cultures.

The importance of specific residues to the bioactivity of rPL II could be investigated. Although chemical modification studies have provided some insight into the amino acid residues necessary for biological activity, these methods lack specificity and often alter the three-dimensional structure. Site-directed mutagenesis techniques allow the investigator to modify specific bases in amino acid codons and produce mutant proteins altered in

a precise, known location. Therefore, specific residues in the coding region of rPL II coding region could be altered. The mutants protein produced in vitro by bacterial cells could be tested for bioactivity in assays. For example, the role of a specific residue in lactogenic activity could be tested in the Nb_2 bioassay or the RRA-PRL.

The protein sequence provided by the DNA sequence of rPL II could be used to synthesize peptides. Peptides corresponding to potential active domains could be tested for biological functions. Specific hydrophilic regions of rPL II could be synthesized and used to produce monoclonal antibodies to rPL II.

Recently, in our laboratory, by Western blotting of two-dimensional polyacrylamide gels of conditioned medium from Day 18 placental cell cultures, it was discovered that the rabbit polyclonal antibodies to purified rPL II cross-reacted with several other placental proteins within the 20-25 kd range with varying isoelectric points (M. Robertson, personal communication). A mouse monoclonal antibody to hPRL also cross-reacted with several of the same proteins, although with a lower affinity.

Considering the high homology between rPL II and several other PRL-GH gene family members in the predicted antigenic domains, this is not surprising. However, these

results show that a specific monoclonal antibody to rPL II is needed.

Specific monoclonal antibodies to rPL II which differentiate between rPL II and related proteins, would be useful for many investigations. Immunoaffinity columns could be used for protein purification. A radioimmunoassay for rPL II could be developed to study the secretion of rPL II. Immunolocalization studies could be done to determine which cells contain rPL II.

Since it is not known whether rPL II is required for normal pregnancy and fetal development in the rat, specific monoclonal antibodies to rPL II could be injected into pregnant rats and the physiological effects of the selective ablation of rPL II observed.

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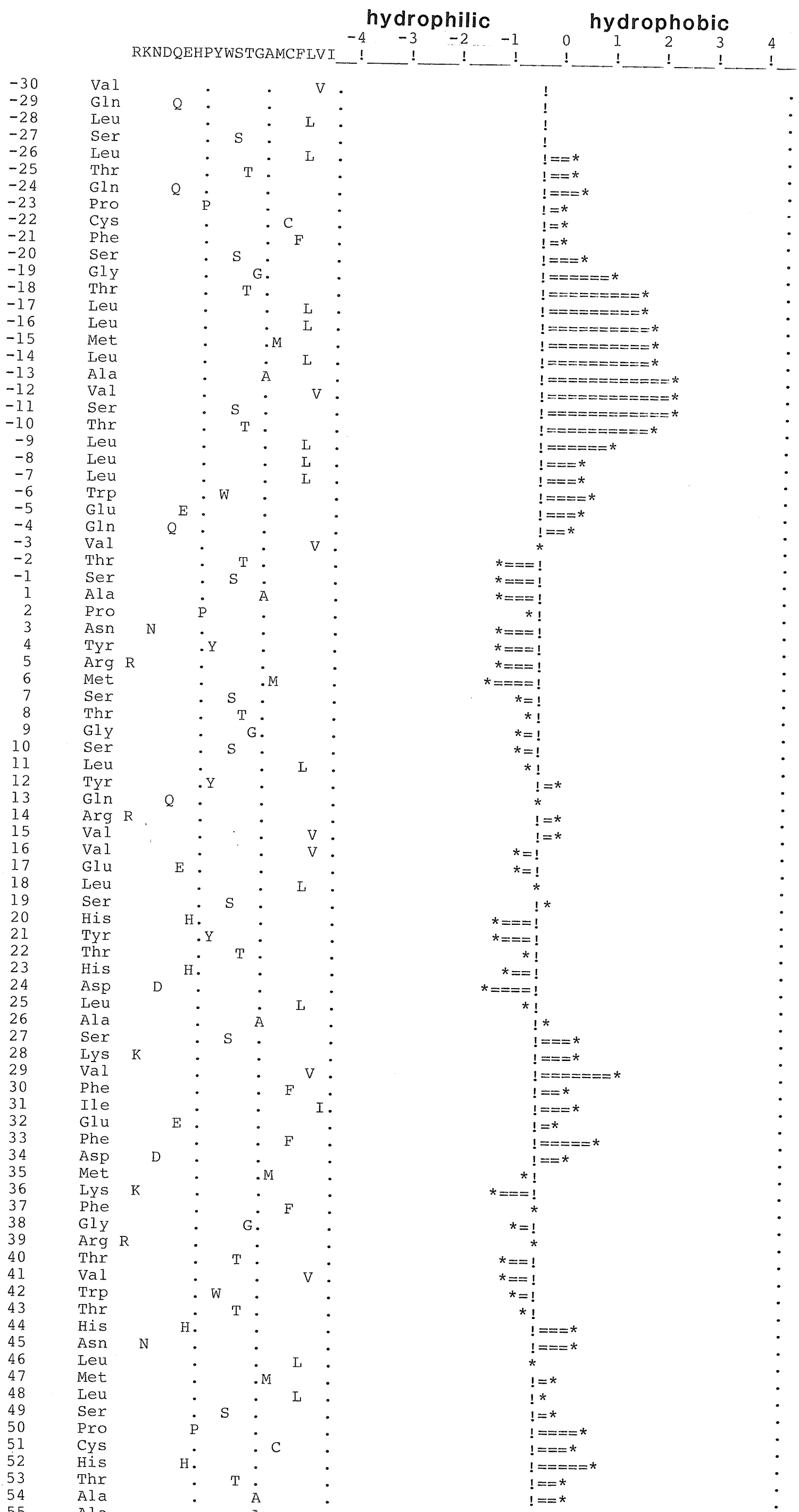
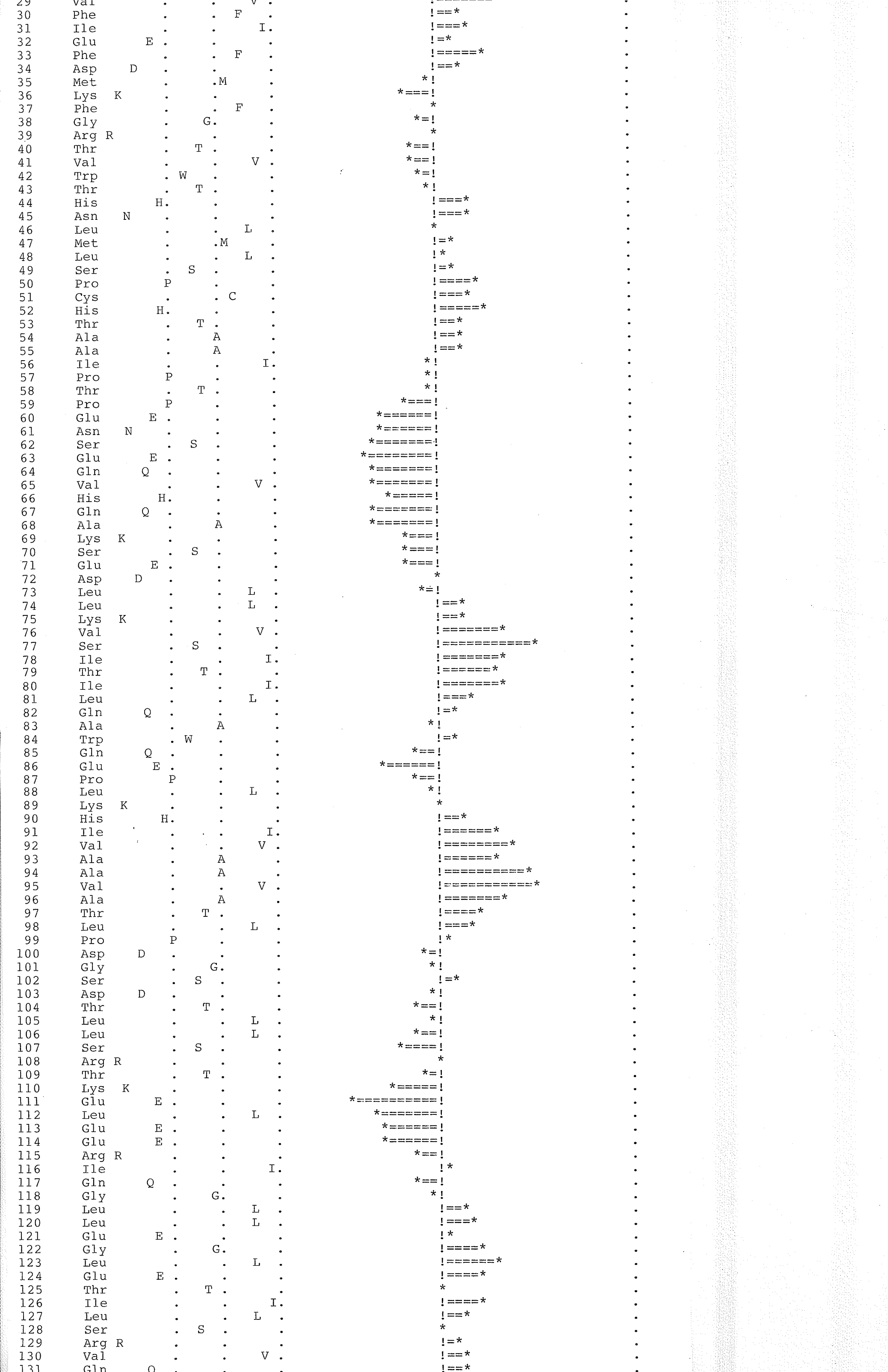
FIGURE 7

P U S T E L L S E Q U E N C E A N A L Y S I S P R O G R A M S
International Biotechnologies, Inc.

AMINO ACID PLOTTING PROGRAM Version 4.1

HYDROPATHY PLOT OF **rPLII**
Segment Length = 9





```
  91   Ile              .         .           I.                !======*               .
  92   Val              .         .          V .                !========*             .
  93   Ala              .         A            .                !======*               .
  94   Ala              .         A            .                !==========*           .
  95   Val              .         .          V .                !===========*          .
  96   Ala              .         A            .                !=======*              .
  97   Thr              .      T  .            .                !====*                 .
  98   Leu              .         .        L   .                !===*                  .
  99   Pro              P         .            .                !*                     .
 100   Asp       D      .         .            .              *=!                      .
 101   Gly              .        G.            .               *!                      .
 102   Ser              .    S    .            .                !=*                    .
 103   Asp       D      .         .            .               *!                      .
 104   Thr              .      T  .            .             *==!                      .
 105   Leu              .         .        L   .               *!                      .
 106   Leu              .         .        L   .             *==!                      .
 107   Ser              .    S    .            .           *====!                      .
 108   Arg R            .         .            .                *                      .
 109   Thr              .      T  .            .              *=!                      .
 110   Lys   K          .         .            .          *=====!                      .
 111   Glu            E .         .            .     *==========!                      .
 112   Leu              .         .        L   .        *=======!                      .
 113   Glu            E .         .            .         *======!                      .
 114   Glu            E .         .            .         *======!                      .
 115   Arg R            .         .            .             *==!                      .
 116   Ile              .         .           I.                !*                     .
 117   Gln          Q   .         .            .             *==!                      .
 118   Gly              .        G.            .               *!                      .
 119   Leu              .         .        L   .                !==*                   .
 120   Leu              .         .        L   .                !===*                  .
 121   Glu            E .         .            .                !*                     .
 122   Gly              .        G.            .                !====*                 .
 123   Leu              .         .        L   .                !======*               .
 124   Glu            E .         .            .                !====*                 .
 125   Thr              .      T  .            .                *                      .
 126   Ile              .         .           I.                !====*                 .
 127   Leu              .         .        L   .                !==*                   .
 128   Ser              .    S    .            .                *                      .
 129   Arg R            .         .            .                !=*                    .
 130   Val              .         .          V .                !==*                   .
 131   Gln          Q   .         .            .                !==*                   .
 132   Pro              P         .            .                *                      .
 133   Gly              .        G.            .                *                      .
 134   Ala              .         A            .                !*                     .
 135   Val              .         .          V .              *=!                      .
 136   Gly              .        G.            .                *                      .
 137   Ser              .    S    .            .                !=*                    .
 138   Asp       D      .         .            .                !=*                    .
 139   Tyr              .Y        .            .                *                      .
 140   Thr              .      T  .            .            *===!                      .
 141   Phe              .         .     F      .            *===!                      .
 142   Trp              .  W      .            .            *===!                      .
 143   Ser              .    S    .            .            *===!                      .
 144   Glu            E .         .            .               *!                      .
 145   Trp              .  W      .            .             *==!                      .
 146   Ser              .    S    .            .           *====!                      .
 147   Asp       D      .         .            .           *====!                      .
 148   Leu              .         .        L   .          *=====!                      .
 149   Gln          Q   .         .            .         *======!                      .
 150   Ser              .    S    .            .         *======!                      .
 151   Ser              .    S    .            .         *======!                      .
 152   Asp       D      .         .            .         *======!                      .
 153   Lys   K          .         .            .     *==========!                      .
 154   Ser              .    S    .            .       *========!                      .
 155   Thr              .      T  .            .          *=====!                      .
 156   Lys   K          .         .            .            *===!                      .
 157   Asn    N         .         .            .              *=!                      .
 158   Gly              .        G.            .                !=*                    .
 159   Val              .         .          V .                !====*                 .
 160   Leu              .         .        L   .                !===*                  .
 161   Ser              .    S    .            .                !===*                  .
 162   Val              .         .          V .                !======*               .
 163   Leu              .         .        L   .                !========*             .
 164   Tyr              .Y        .            .                !===*                  .
 165   Arg R            .         .            .               *!                      .
 166   Cys              .         .  C         .              *=!                      .
 167   Met              .         .M           .           *====!                      .
 168   Arg R            .         .            .       *========!                      .
 169   Arg R            .         .            .      *=========!                      .
 170   Asp       D      .         .            .           *====!                      .
 171   Thr              .      T  .            .       *========!                      .
 172   His             H.         .            .    *===========!                      .
 173   Lys   K          .         .            .        *=======!                      .
 174   Val              .         .          V .             *==!                      .
 175   Asp       D      .         .            .             *==!                      .
 176   Asn    N         .         .            .               *!                      .
 177   Phe              .         .     F      .                !==*                   .
 178   Leu              .         .        L   .                !==*                   .
 179   Lys   K          .         .            .                !=*                    .
 180   Val              .         .          V .                !=*                    .
 181   Leu              .         .        L   .                !=*                    .
 182   Lys   K          .         .            .                !==*                   .
 183   Cys              .         .  C         .                *                      .
 184   Arg R            .         .            .                *                      .
 185   Asp       D      .         .            .            *===!                      .
 186   Ile              .         .           I.        *=======!                      .
 187   Tyr              .Y        .            .           *====!                      .
 188   Asn    N         .         .            .                !                      .
 189   Asn    N         .         .            .                !                      .
 190   Asn    N         .         .            .                !                      .
 191   Cys              .         .  C         .                !                      .
```