Stephanie Wilkins Barnes. **IDENTIFICATION, CHARACTERIZATION, AND EXPRESSION OF HAMSTER PROLIFERIN-RELATED PROTEIN.** (Under the direction of Dr. Randall H. Renegar) Department of Biology, August, 1994.

ABSTRACT

In the hamster, serum total lactogenic activity increases during the latter half of gestation (Days 8-16). On Days 10 and 12 a substantial amount of lactogenic activity cannot be attributed to prolactin and hamster placental lactogen-II (haPL-II); therefore, the presence of a molecule similar to placental lactogen-I (PL-I) as found in the rat and mouse has been hypothesized for the hamster. The objectives for this study were to obtain the nucleotide sequence of this placental molecule, determine the levels of expression throughout gestation, and establish which cells synthesize and secrete this molecule. Preliminary Northern analysis indicated that a mouse PL-I cDNA did not hybridize to total hamster placental RNA. Susequently, a 21 bp oligonucleotide derived from a region of nucleotide homology for mPL-I and rPL-I was used in 3' RACE methodology. A 444 bp cDNA fragment that had nucleotide sequence similarity with members of the prolactin-growth hormone gene family was generated. This cDNA fragment was utilized to screen a Day 16 placental bacteriophage cDNA library, and a clone containing the entire coding region was identified and sequenced. The molecule had 77% nucleotide sequence homology with mouse proliferin-related protein (mPRP) and somewhat less homology (~60%) with hamster, rat and mouse prolactin or placental lactogens. As a result, this molecule will be tentatively referred to as hamster proliferin-related protein (haPRP). The

deduced amino acid sequence of this molecule contained a 15 residue signal sequence and a 219 residue peptide with a calculated molecular weight of 25,477 daltons. The peptide shared 58% amino acid sequence identity with mPRP.

Temporal expression of haPRP mRNA during the latter half of gestation was evaluated by Northern and slot blot analysis using the 444 bp cDNA fragment as a hybridization probe. A 1-kb transcript was first detected on Day 9. Expression increased between Days 9 and 10, remained high on Day 11, and decreased on Day 12. Low levels of expression were detected on Day 14, but the 1-kb transcript was not detected on Day 15. Interestingly, a larger (~1.2 kb) transcript was detected on Day 15. Immunocytochemistry, as well as preliminary *in situ* hybridization studies, indicate that cytotrophoblast cells of the trophospongium are responsible for production of haPRP.

The nucleotide sequence and site of synthesis of the identified molecule suggest that it may be the hamster homologue of mouse proliferin-related protein. Although the temporal expression of this molecule parallels previously detected serum lactogenic activity in pregnant hamsters, further study is required to determine if it has lactogenic activity.

IDENTIFICATION, CHARACTERIZATION AND EXPRESSION OF HAMSTER PROLIFERIN-RELATED PROTEIN

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IDENTIFICATION, CHARACTERIZATION, AND EXPRESSION OF HAMSTER PROLIFERIN-RELATED PROTEIN

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DEDICATION

To my mom for instilling within me the desire to setforth goals and the ability to achieve them.

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INTRODUCTION

In vivaparous species, proper growth and maturation of the fetus within the uterus is accomplished largely by processes under the control of hormones produced by the anterior pituitary, ovary, placenta, and uterus (reviewed by Soares et al., 1991). The placenta is the source of steroid, protein, and polypeptide hormones (reviewed by Talamantes and Ogren, 1988) and several of these placental polypeptides belong to the prolactin (PRL) - growth hormone (GH) gene family; both lactogenic and non-lactogenic proteins have been identified.

Pregnancy-specific proteins which demonstrate lactogenic activity in bioassays are termed placental lactogens (PLs) (Ehrhardt, 1936; Talamantes and Ogren, 1988; Southard and Talamantes, 1991). Placental lactogens are structurally similar to the pituitary hormones, PRL and GH, and likewise have been shown to regulate processes such as mammary gland differentiation, intermediary metabolism, steroidogenesis, and somatic growth (Ogren and Talamantes, 1988). In addition, placental lactogens are characterized as potent haemopoietic hormones (Berczi and Nagy, 1991).

In rodents, two distinct forms of PLs have been described: PL-I and PL-II. Placental production of PL-II begins at midpregnancy in the hamster, rat, and mouse in association with formation of the chorioallantoic placenta and continues to parturition. In the hamster, low concentrations, 10.6 ng/ml, are first detected on Day 10 of pregnancy, but serum values reach 12.3 μg/ml by the day of parturition, Day 16 (Southard and

Talamantes, 1987). Placental lactogen-I is the "midpregnancy" lactogen with production of this hormone occurring during a few days around midpregnancy. Mouse and rat PL-I have been isolated and characterized, and serum levels have been determined using homologous radioimmunoassays (RIAs) (Colosi et al., 1987a; Robertson and Friesen, 1981). In contrast, relative serum levels of a putative hamster (ha) PL-I have been determined indirectly using the difference between radioreceptor estimates of lactogenic activity and radioimmunoassayable PRL and haPL-II concentrations. Using this crude estimate of the suspected molecule, the serum profile is characterized by a large peak slightly after midpregnancy (approximately Day 10) with hormone concentrations falling to low levels by Day 12 (Soares and Talamantes, 1982; Southard et al., 1986; Southard et al., 1987).

In addition to PLs, other molecules that have amino acid sequence homology with pituitary PRL are produced by rat and mouse placentas (Ogren and Talamantes, 1988). These PRL-like proteins appear to bind weakly or not at all to lactogenic receptors and have little or no lactogenic activity. Non-lactogenic PRL-like proteins include mouse proliferin (mPLF: Linzer and Nathans, 1984), mouse proliferin-related protein (mPRP: Linzer and Nathans, 1985), rat placental lactogen-I variant (Deb et al., 1991a) as well as rat prolactin-like proteins (PLP) A (Campbell et al., 1989), B (Ogilvie et al., 1990), and C (Deb et al., 1991b, c).

The objectives of this research were to 1) identify and characterize the putative haPL-I, 2) determine the relative levels of gene expression of this molecule throughout

gestation, which may provide information on its role during pregnancy, and 3) determine the placental cell that produces this molecule. Sequence data will allow comparison of nucleotide and amino acid structures of haPL-I and other PLs. Ultimately, sequence data will allow production of antibodies for RIA development and nucleic acid probes for *in situ* hybridization histochemistry. These reagents together with those available for other hamster placental hormones (e.g., PL-II and relaxin) will facilitate the study of placental hormone synthesis, secretion, and function.

REVIEW OF LITERATURE

Placenta

Embryonic development in mammals requires regulatory factors from maternal, extraembryonic, and embryonic tissues. The placenta, a specialized extraembryonic tissue, develops in concert with the embryo and provides the embryo with access to maternal resources. This disposable organ is a source of growth factors and cytokines, as well as a diverse group of hormones. Several essential functions performed by the placenta include: 1) regulation of nutrient and waste transport, 2) prevention of rejection of the genetically disparate embryo, and 3) synthesis and secretion of steroid and polypeptide hormones that participate in transduction of fetal and maternal signals.

Development

The placenta arises from the implanting blastocyst. In the hamster, the blastocyst consists of an outer single-cell layer termed the trophectoderm which surrounds the inner cell mass (future embryo) and the blastocoele. At Day 4, implantation is initiated when the blastocyst attaches to the epithelium of an implantation groove which is formed by folds of the antimesometrial uterine endometrium. Trophectoderm cells directly involved in the initial physical contact are located at the abembryonic pole of the blastocyst and differentiate into primary giant trophoblast cells (GTC-1). Also at this phase, cells of the inner cell mass differentiate into ectoderm and endoderm. Ultimately, cells of inner cell

mass lineage give rise to the embryo, amnion, and allantois while cells of trophectoderm lineage form extraembryonic structures that establish and maintain physical and chemical contact with maternal tissues and vasculature (Stevens, 1975).

By Day 5, trophectoderm cells at the embryonic pole of the conceptus proliferate and migrate mesometrially forming the träger. Trophectoderm cells at the interface between the uterus and träger enlarge and differentiate to form secondary giant trophoblast cells (GTC-2). In the inner cell mass, cells of the endoderm and ectoderm proliferate and extend downward into the blastocoele with the endoderm on the outside of the ectoderm to form the egg cylinder. Subsequently a primitive amniotic cavity appears in the ectoderm and this layer further differentiates into embryonic (lower) and extraembryonic (upper) ectoderms.

By Day 7, on either side of the egg cylinder between endoderm and ectoderm, mesodermal cells arise and proliferate at the junction between the embryonic and extraembryonic ectoderms. This mesoderm pushes inward carrying ectoderm with it until the two folds meet and fuse at the center of the primitive amniotic cavity. As a result of this fusion event three cavities are formed: ectoplacental (upper/mesometrial), exocoelomic (middle), and definitive amniotic (lower/antimesometrial). Shortly thereafter, allantoic mesoderm arises from the caudal end of the embryo and migrates mesometrially across the exocoloem to contact the floor of the ectoplacental cavity. During the 8th day, this allantois pushes the ectoplacental ectoderm (floor) into contact with the träger, thus forming the ectoplacental cone. At Day 9 the ectoplacental cone becomes vascularized by

allantoic arteries to mark formation of the chorioallantoic placenta which contains two functionally and structurally distinct zones. The region in direct contact with the allantois is the labyrinth which is composed of fetal capillaries arising from the allantois separated from maternal blood by a thin layer of trophoblast. The labyrinth is the only region where maternal and fetal vasculature are in close proximity, and the extent of the labyrinth is defined by the degree of fetal vascular penetration into the ectoplacental cone. Adjacent to the labyrinth is the trophospongium composed of cytotrophoblast and secondary giant trophoblast cells (from the ectoplacental cone) surrounded by vascular spaces containing maternal venous blood. This is the major endocrine portion of the placenta. Fetal blood flows within large vessels to the labyrinth/trophospongium interface and then back through the labyrinth in fetal capillaries to enter veins at the fetal surface of the placenta. Maternal blood traverses the trophospongium and labyrinth in placental arteries to reach the fetal surface of the placenta where it enters channels in the labyrinth between the trophoblast cells surrounding fetal arteries. Maternal blood then enters the vascular spaces of the trophospongium to ultimately enter uterine veins.

As gestation proceeds, these placental zones continue specialization and refinement. The cytotrophoblasts of the trophospongium proliferate and differentiate into additional GTC-2 while the labyrinthine trophoblast cells tend to fuse forming a syncytium. Maternal and fetal vasculature increase throughout the remainder of gestation. The specific arrangement of fetal vessels surrounded by freely flowing maternal blood within the labyrinth zone permits exchange of nutrients, gases, wastes and hormones

between the fetal and maternal compartments (Ward, 1948; Orsini, 1954; Butler, 1970). Likewise, the vascular arrangement of the trophospongium directs its secretory products to the maternal compartment.

Giant Trophoblast Cells

Sixty percent of the hamster placenta is of trophectoderm origin (Rossant, 1986). At the initiation of implantation, trophectoderm cells at the abembryonic pole of the blastocyst differentiate into primary giant trophoblast cells (GTC-1). Secondary giant trophoblast cells (GTC-2) arise from cells of the träger around the fifth day of gestation. These giant cells exhibit several unique characteristics which contribute to successful implantation and homeostasis throughout pregnancy.

All giant cells possess certain common characteristics including the ability to migrate, the tendency to form a syncytium, and the occurrence of nuclear division without subsequent cytoplasmic division which is termed endoreduplication. (Orsini, 1954). This lack of mitotic activity may explain the increased cell size and amplified genome of these cells (Orsini, 1954). In the mouse, endoreduplication is a general feature of extraembryonic development yet the regulatory mechanism(s) and the significance of this process remain to be elucidated (Ilgren, 1980; Faria et al., 1990).

Giant cells are believed to participate in several processes requisite for successful implantation and conceptus development. These include: 1) penetration of uterine tissues and the maternal vasculature, 2) prevention of immunological rejection of the developing

fetus, and 3) production of steroid and peptide hormones. Several aspects of these functions overlap. Migration of giant cells into surrounding tissue is accomplished by extension of cytoplasmic processes and phagocytosis. Previous studies have also suggested giant trophoblast cells secrete proteolytic and cytolytic factors (Runner, 1947; Fawcett et al., 1947; Blandau, 1949; Grobstein, 1950; Fawcett, 1950; Orsini, 1954). These features may permit an active role in penetration of the uterus, enlargement of the decidual cavity, and degeneration of maternal tissue (Orsini, 1954). The migratory abilities of giant cells involves rapid migration into endometrium and rapid invasion into maternal vessels. Enders and Welsh (1993) suggested the rapid pace of this migration/invasion is necessary for efficient gas and nutrient exchange as well as prevention of an immune response to these placental cells. By residing within maternal vessels where effector cells of the immune system are less active, these trophoblast cells may exist without stimulating an immune response.

Trophoblast cells, with the potential to express both maternal and paternal antigens directly, interact with the uterus yet immunological rejection of the conceptus is rare. Giant cells in particular are in a position to stimulate an immune response with the very earliest invasion of the antimesometrial uterus by GTC-1 followed by GTC-2 invasion of the decidua basalis. Several observations have been reported to explain this phenomenon. Trophoblast cells from most species show reduced and/or modified histocompatibility factors; thus, antigenicity seems to be suppressed at the maternal-placental interface (Enders and Welsh, 1993). In the mouse, preferential inactivation of the paternally

derived X chromosome has been demonstrated in cells of extraembryonic trophectoderm and primary endoderm lineages (Takagi and Sasaki, 1975; Krumlauf et al., 1986). Absence of protein products encoded within these paternal genes could be a regulatory mechanism for preventing an immune response. Both of these examples demonstrate non-stimulation of an immune response. The production of an inhibitory factor could also prevent immunological rejection. Numerous factors produced and isolated from the maternal-placental interface have been associated with immunosuppression. These include human chorionic gonadotropin and human placental lactogen which have immunosuppressor activity in some *in vitro* assays (Beer and Billingham, 1978). Both of these polypeptide hormones are synthesized and secreted by trophoblast cells (Maruo et al., 1992).

Renegar et al. (1990) reported cytoplasmic features of giant trophoblast cells in the hamster placenta. These cells possess an extensive Golgi apparatus, an abundant endoplasmic reticulum, and cytoplasmic organelles organized in a manner which is characteristic of cells actively involved in protein synthesis. Accordingly, several polypeptides have been reported to by synthesized by GTC from various species. These include hamster relaxin (Renegar et al., 1990), placental lactogens (haPL-II: Jones and Renegar, 1994; mPL-I: Faria et al., 1991; mPL-II: Lee et al., 1988; rPL-I: Robertson et al., 1990; rPL-II: Duckworth et al., 1990; rPL-Iv: Robertson et al., 1991), mouse proliferin (Lee et al., 1988), and rat prolactin-like proteins-A (Duckworth et al., 1990) and C (Deb et al., 1991b,c). Interestingly, several placental polypeptides are synthesized by the same GTC: PL-II and relaxin in the hamster placenta (Renegar et al., 1990) and PL-I,

PL-II, and PLF in the mouse placenta (Yamaguchi et al., 1992; Yamaguchi et al., accepted for publication).

PRL-GH Gene Family

In 1905 Halban suggested that the placenta is an endocrine organ. Since then, the placenta of several species has proven to be the source of steroid and polypeptide hormones (reviewed by Ogren and Talamantes, 1994). The types and quantities of hormones produced by the placenta is highly species dependent. Several of these placental polypeptide hormones are structurally and functionally similar to the pituitary hormones prolactin (PRL) and growth hormone (GH) and, therefore, are considered members of the PRL-GH gene family. Members of this gene family appear to have emerged from a common ancestral gene which diverged to give rise to separate PRL and GH lineages (Nicoll et al., 1986).

Placental lactogens (PLs) are the best characterized placental members of the PRL-GH gene family. Initially PLs were defined as placental substances which demonstrated PRL-like (lactogenic) activity in bioassays and radioreceptor assays (RRA). Recently, additional placental polypeptides have been classified as PLs based on biochemical characterizations (Ogren and Talamantes, 1988). Placental lactogens have been characterized in human (Josimovich and MacLaren, 1962; Cohen et al., 1964; Friesen, 1965; Hunt et al., 1981), rhesus monkey (Shome and Friesen, 1971; Vinik et al., 1973), baboon (Josimovich et al., 1973), mouse (mPL-I: Colosi et al., 1987a, b; mPL-II: Colosi

et al., 1982; Jackson et al., 1986), rat (rPL-I: Robertson and Friesen, 1981; rPL-II: Robertson and Friesen, 1975; Duckworth et al., 1986a), hamster (haPL-II: Southard et al., 1986; Southard and Talamantes, 1987), sheep (Hurley et al., 1975; Martal and Djiane, 1975; Chan et al., 1976) and cow (Murthy et al., 1982; Eakle et al., 1982; Arima and Bremel, 1983). Based upon amino acid sequence data, Nicoll et al. (1986) suggested that primate PLs belong to the GH lineage while rodent PLs belong to the PRL lineage. Rodent placentas produce more than one distinct PL during gestation (Southard and Talamantes, 1991).

In addition to PLs, other placental polypeptides have been localized within fetal and maternal compartments of many rodent placentas (Ogren and Talamantes, 1988). Although several of these pregnancy specific polypeptides also share amino acid similarities with pituitary PRL, they bind weakly or not at all to PRL receptors and have little or no lactogenic activity. Collectively this group of placental polypeptides is referred to as non-lactogenic PRL-like proteins and are members of the PRL-GH gene family as well (Southard and Talamantes, 1991). Non-lactogenic PRL-like proteins include mouse proliferin (mPLF), mouse proliferin-related protein (mPRP), rat placental lactogen-I variant (rPL-Iv), and rat prolactin-like proteins (rPLPs) A, B, and C. This PRL-GH gene family is continuously expanding since characterization/purification of each polypeptide has revealed the existence of yet another mRNA/protein. Proliferin was identified as a 1kb mRNA species isolated from growth factor/serum-stimulated mouse culture cells (Linzer and Nathans, 1984). During isolation of PLF cDNA from a placental cDNA plasmid

library, PRP cDNA was detected as a clone that hybridized weakly to a PLF cDNA probe (Linzer and Nathans, 1985). Rat PLP-A and PLP-B cDNAs were identified during characterization of rPL-II cDNA (Duckworth et al., 1986b; Duckworth et al., 1988), while PLP-C was observed as a contaminating protein during purification of PLP-A (Deb et al., 1991b). A second contaminating protein detected during purification of PLP-A is now referred to as rPL-Iv (Deb and Soares, 1990; Deb et al., 1991a). Rat PL-Iv was also detected as a 1kb mRNA species from late gestation placentas which hybridized with the rPL-I cDNA probe (Robertson et al., 1990).

Biochemical Characterization

Two structural forms of PL have been described: 1) PLs which are similar in structure to the PRL and GH of the same species, and 2) PLs which differ somewhat from those of PRL and GH. The first group of PLs consists of single chain polypeptides with molecular weights of 20,000 to 25,000 daltons. This group is also characterized by two or three intrachain disulfide bonds in positions analogous to those of GH and PRL (Ogren and Talamantes, 1988; Talamantes and Ogren, 1988). Examples of this group include hamster PL-II (Southard et al., 1986; Southard and Talamantes, 1987), as well as human PL (Josimovich and MacLaren, 1962; Cohen et al., 1964; Friesen, 1965; Bewley et al., 1972; Cooke et al., 1981; Hunt et al., 1981), mouse PL-II (Colosi et al., 1982; Jackson et al., 1986), and rat PL-II (Robertson and Friesen, 1981; Duckworth et al., 1986a,b). The second group of PLs is also composed of single chain polypeptides with molecular weights

of 30,000 to 34,000 daltons. These are found primarily in rodent species and include mouse PL-I (Colosi et al., 1987a, b) and rat PL-I (Robertson et al., 1982). These hormones have been characterized extensively, and unlike PL-IIs, PL-Is contain one or more potential N-linked glycosylation sites. A molecule of similar molecular weight (35,000-37,000 daltons) has been detected in gel filtration chromatography fractions from pregnant hamster serum using a PRL radioreceptor assay; however, the structure of this molecule has not been determined (Southard et al., 1987).

Biochemical information concerning non-lactogenic PRL-like proteins has been determined from cDNA nucleotide and amino acid sequences and from analysis of recombinant proteins. These molecules have molecular weights ranging from 24,000 to 44,000. Molecular weight variation occurs within a single protein species due to secretion of the protein as a mixture of glycosylated forms (reviewed by Southard and Talamantes, 1991). Comparison of the amino acid sequences for haPL-II, mPLs, rPLs, mPLF, mPRP, rPLP-A, rPLP-B and rPLP-C revealed that signal peptides range from 29-31 amino acid residues while the secreted peptides range from 192-214 amino acid residues. Variation does exist between the primary structures of these proteins; the three PL-IIs have 69% to 71% sequence identity just as mPL-I and rPL-I share 71% sequence identity. In contrast, the non-lactogenic PRL-like proteins have 22% to 32% sequence identity with each other and the PLs (Southard and Talamantes, 1991). There is also considerable variation in the number of cysteine residues and glycosylation sites among peptide sequences with significant differences between pituitary PRL and the placental PRL-like proteins. Prolactins from numerous vertebrate species contain four or six cysteines in conserved positions but rodent placental PRL-like proteins contain four, five, six and nine cysteine residues which can be found in a variety of positions (Southard and Talamantes, 1991).

The majority of mammalian PRLs exist as non-glycosylated proteins yet seven rodent placental PRL-like proteins contain one or more potential N-linked glycosylation sites (Southard and Talamantes, 1991). Further analysis is necessary to establish the presence and degree of glycosylation of these placental PRL-like proteins.

Gestational Profiles

Placental PRL-like polypeptide secretion varies among species. Maternal serum concentrations for PL have been assessed in a number of mammals. Human PL (hPL) appears in circulation at ~6 weeks with levels increasing linearly until ~ 30 weeks; afterwhich, hPL levels plateau and are maintained until parturition (Grumbach et al., 1968). Monkey PL (mPL) levels are low on Day 42 but increase significantly by Day 157 (Walsh et al., 1977). In cows serum PL levels are low during the first two trimesters yet rise rapidly between Days 160 and 200 (Bolander et al., 1976). Ovine PL (oPL) is first detected in serum between Days 41-50 and then concentrations increase linearly to peak between ~ Days 121-141. Subsequently, oPL levels decline as parturition approaches (Handwerger et al., 1977).

In rodents, the gestational serum profiles for both placental lactogens and non-lactogenic PRL-like proteins have been determined. Lactogenic activity in placental tissue and blood from pregnant hamsters was first reported by Kelly et al. in 1976. Relative serum levels of a putative haPL-I molecule have been determined indirectly using the

difference between radioreceptor estimates of lactogenic activity and radioimmunoassayable PRL plus haPL-II concentrations. Using this crude estimate of the suspected molecule, the hamster serum profile is characterized by a large peak slightly after midpregnancy (approximately Day 10) with hormone concentrations falling to low levels by Day 12 (Soares and Talamantes, 1982; Southard et al., 1986; Southard et al., 1987). Serum concentrations of haPL-II have been determined using a specific homologous radioimmunoassay. Low concentrations, 10.6 ng/ml, are first detected on Day 10 of pregnancy, but serum values reach 12.3 μg/ml by the day of parturition (Southard and Talamantes, 1987).

Placentas of the mouse and rat synthesize and secrete two distinct PLs in addition to several non-lactogenic PRL-like proteins. Analysis of pregnant mouse serum indicates the presence of PL-I on Day 7 with maximal concentrations occurring on Day 11 and then quickly diminishing (Ogren et al., 1989). Mouse PL-II appears around midgestation (Day 10) and increases to term (Soares et al., 1982). Proliferin serum levels are highest at Day 11 and then decline slowly to term (Linzer et al., 1985). Proliferin-related protein is detectable between Days 10 and 18 with elevated concentrations occurring between Days 12 and 14 (Colosi et al., 1988).

Rat placental lactogen-I is present on Day 7 but aburptly declines by Day 12 (Faria et al., 1990). During the decline of PL-I, PL-II serum levels rise on Day 11 and continue increasing to term (Faria et al., 1990). PL-Iv (Deb et al., 1991a) and PLPs A (Deb et al.,

1989), B (Roby and Soares, 1991), and C (Deb et al., 1991b) are detectable at midpregnancy and increase during the second half of pregnancy.

In general, rodent PLs with maximal serum concentrations during midpregnancy are referred to as "PL-Is" while PLs with maximal serum concentrations during the second half of pregnancy are referred to as "PL-IIs". Non-lactogenic PRL-like placental proteins demonstrate tremendous variation in their secretion patterns.

Synthesis

Placental PRL-like polypeptides have distinct cellular and temporal patterns of expression. Cell types for PLs have been determined for the human, sheep, hamster, mouse and rat. In all cases, PL-producing cells have originated in the conceptus. After week 6, the major source of hPL mRNA has been localized to syncytiotrophoblasts (McWilliams and Boime, 1980). Ovine PL has been localized to the binucleate cells of the placenta (Wooding, 1981).

Unfortunately there is considerable inconsistency in the terminology pertaining to the chorioallantoic placenta of the hamster, mouse, and rat. The following terms are equivalent: trophospongium = basal zone = junctional zone and cytotrophoblasts = spongiotrophoblasts. In the hamster placenta, PL-II mRNA has been localized to GTC of both the labyrinth and trophospongium. Messenger RNA levels are detected at Day 10 with levels increasing by Day 12 and continuing until parturition (Jones and Renegar,

1994). Renegar et al. (1990) localized both haPL-II and relaxin proteins to the same GTC of the hamster placenta. Localization of the proposed haPL-I remains to be determined.

Mouse placental PRL-like mRNAs, with one exception, have been localized to GTC of the placenta. Placental lactogen-I mRNA is detected between Days 8 and 12 with maximum levels occurring at Day 10 (Colosi et al., 1987b; Faria et al., 1991) while PL-II mRNA levels are initially detected at Day 10, maximize at Day 12, and are maintained to term (Jackson et al., 1986; Lee et al., 1988). Proliferin mRNA increases sharply between Days 8 and 10 with a gradual decrease in levels to Day 18 (Linzer et al., 1985). Lee et al. (1988) demonstrated individual giant cells of the mouse placental contained both PL-II and PLF proteins. Unlike other placental PRL-like mRNAs, PRP mRNA has been localized to cells of the basal zone of the mouse placenta; cytotrophoblast cells are proposed to be the specific cell population, but further analysis is necessary (Colosi et al, 1988). Proliferin-related protein mRNA levels peak at Day 12 and decrease gradually to term (Linzer and Nathans, 1985).

Rat PL-I mRNA appears in GTC at Day 10 and reaches elevated levels between Days 11 and 12 (Robertson et al., 1990). At Day 12, rPL-II mRNA is detected in GTC-1 and GTC-2. Later in pregnancy rPL-II mRNA continues in GTC of the basal zone but also appears in cells of the labyrinth (Duckworth et al., 1990). Messenger RNA for non-lactogenic PRL-like proteins of the rat are detected by Day 14 and remain elevated until parturition. Prolactin-like protein-A (Duckworth et al., 1990), PLP-C (Deb et al., 1991b), and PL-Iv (Robertson et al., 1991) mRNAs have been localized to GTC as well as a few

cytotrophoblast cells of the basal zone. Prolactin-like protein-B mRNA is unique in its localization only in cytotrophoblast cells of the rat placenta (Duckworth et al., 1990).

Trophoblast cells are the major endocrine cells of the rodent placenta. Often a single trophoblast cell is responsible for the production of multiple PRL family members. The factors involved in regulating expression of these hormones remain to be determined.

Biological Activities

Each member of the PRL-GH gene family is a major secretory product during pregnancy, thus implying functional significance. Physiological functions for PLs are not entirely understood and even less is known about the non-lactogenic PRL-like proteins. Due to the structural similarities shared between PLs and pituitary PRL and GH, functions proposed for PLs include regulation ovarian steroidogenesis, preparation of mammary glands for postpartum lactation, and stimulation of fetal growth (Talamantes et al., 1980). Initially, PLs were believed to be the agents, secreted by the conceptus, which stimulated progesterone production by the ovaries during the latter half of gestation. However, further investigation demonstrated several PLs (e.g., hPL and mPL-I) were unable to stimulate progesterone secretion *in vitro* and, therefore, may not be luteotrophic factors (reviewed by Ogren and Talamantes, 1988).

Preparation of the mammary gland for lactation involves ductal and lobuloalveolar growth and acquisition of the ability to synthesize specific milk constituents, all which are totally or partially under the control of PRL (Ogren and Talamantes, 1988). The ability of

PRL to stimulate cellular proliferation has been exploited to develop a sensitive and specific assay for lactogenic activity (Tanaka et al., 1980). Prolactin from several species stimulates rat Nb2 lymphoma cells to proliferate while other hormones and growth factors are without effect. Consistent with a role for PLs in mammary gland growth, several of these hormones also stimulate proliferation of Nb2 cells (Tanaka et al., 1980; Robertson et al., 1994). The ability of PLs to control synthesis and secretion of specific milk constituents has been demonstrated *in vitro*. Human PL (Ways et al., 1979), haPL-II (Southard et al., 1986), mPL-I (Colosi et al., 1987a), and mPL-II (Thordarson et al., 1986) stimulate synthesis and/or secretion of α-lactalbumin by mouse mammary epithelial cells.

Direct participation of PLs in the control of fetal growth is likely since specific PL receptors have been detected in numerous human fetal tissues, including liver, kidney, adrenal glands, and brain (Hill et al., 1988). Indirect stimulation may also exist; during prolonged periods of low nutrient availability, hPL is believed to reserve glucose from maternal circulation for utilization by the fetus (Kim and Felig, 1971). Finally, there is some evidence that PLs have immunosuppressive properties (Beer and Billingham, 1978) and stimulate haemopoiesis (Berczi and Nagy, 1991).

Functional characterization of non-lactogenic PRL-like proteins has been hindered by the inability to obtain highly purified proteins. Recently, Linzer et al. (personal communication, 1994) demonstrated that PLF possesses angiogenic properties while PRP possesses anti-angiogenic properties. Linzer and Nathans (1983) reported the expression

of several molecules following serum-stimulated growth of BALB/c 3T3 cells, and one of these molecules was subsequently determined to be mPLF (Linzer and Nathans, 1984). These authors hypothesized that mPLF may be involved in proliferation of maternal and/or fetal tissues.

Even though the precise functions for placental PRL-like proteins remain to be determined, each member of the PRL-GH gene family is believed to contribute to endocrine homeostasis throughout gestation which would aid in the maintainance of pregnancy and the promotion of fetal growth and well being (Ogren and Talamantes, 1994). Accordingly, placental PRL-like proteins synthesized immediately post-implantation may serve as systemic or local signals that indicate successful implantation of the embryo or act in a paracrine manner to support growth and differentiation of uterine decidual tissue. Placental PRL-like proteins are synthesized and secreted by trophoblast cells and, therefore, may function as autocrine or paracrine regulators of trophoblast cell growth and differentiation.

MATERIALS AND METHODS

Animals

Female Golden (Syrian) hamsters (Charles River; Kingston, NY) were maintained in the Department of Comparative Medicine on a 14L:10D schedule. Between the hours of 1900 and 2100 females were checked for estrus as determined by the expression of lordosis in the presence of male hamsters. Animals in estrus were housed overnight with a male, and the following day was designated Day 1 of gestation. All maintenance and handling was in accordance with University guidelines. On Days 8, 9, 10, 11, 12, and 14 of gestation between the hours of 1000 and 1200 and on Day 15 between the hours of 2000 and 2100, animals were anesthetized with methoxyflurane (Pitman-Moore; Mundelein, IL) and sacrificed by cervical dislocation. Placentae and liver were frozen in liquid nitrogen immediately upon removal and stored at -70°C or fixed by immersion and processed for histology.

Treatment of RNA Solutions and Equipment

All equipment and solutions were handled with gloves to minimize ribonuclease contamination. Purchased solutions were assumed to be RNase-free and did not receive additional treatment. All glassware was baked at 200°C for approximately 12 hours. Plasticware and other non-bakable materials were soaked in active 0.1 % DEPC water overnight followed by autoclaving. Pipette tips were also autoclaved. Stock solutions

made in the laboratory contained inactivated 0.1% DEPC water and were placed in RNase-free glassware/plasticware.

RNA Extractions

(Appendices A and B)

Total RNA was isolated from placentae and liver of the various gestational stages by a modification of the procedure of Chomczynski and Sacchi (1987). Total RNA was quantitated by spectrophotometric methods. RNA quality was determined by ultraviolet light visualization of ethidium bromide-stained agarose formaldehyde gels.

Treatment and Handling of DNA Solutions

Purchased solutions were assumed to be sterile. Stock solutions made in the laboratory as well as all pipette tips, glassware, and plasticware were autoclaved for 20 minutes to inactivate deoxyribonucleases and eliminate microbial contamination. Working solutions were stored in sterile glassware/plasticware.

3' Rapid Amplification of cDNA Ends (RACE)

(Appendices C and D)

Custom synthesized oligonucleotide primers based on consensus regions for mouse PL-I and rat PL-I were utilized in 3' RACE methodology as described in the 3' RACE system (Gibco BRL; Gaithersburg, MD). This methodology takes advantage of the 3'

poly-A tail present on most mRNAs. In the first step an oligo(dT) primer anneals to the poly-A tail region and the enzyme reverse transcriptase (Superscript; Gibco BRL; Gaithersburg, MD) is added to generate cDNAs from all mRNAs present. Specificity for the mRNA of interest is accomplished by adding the gene specific oligonucleotide primer (e.g., PL-I) along with the cDNA and the enzyme Taq polymerase (Taq DNA Polymerase; Stratagene Cloning Systems; La Jolla, CA). Repeated cycles (n=35) of annealing, elongation, and denaturation were accomplished by varying the temperatures of the reaction (polymerase chain reaction; PCR). An aliquot from each PCR reaction and size markers were simultaneously electrophoresed in a 1.5% agarose gel. PCR products corresponding to the predicted size were cut and eluted from the gel using glass fiber spin columns. The cDNA was concentrated by ethanol precipitation.

Oligonucleotide Primers

Four sequences (A, B, C, D) from regions of homology shared by mPL-I and rPL-I cDNA sequences were used as oligonucleotide primers in 3' RACE methodology. The primer (sense) sequences and their locations as they appeared within the mPL-I cDNA sequence were as follows:

A GATGTATACCGTGAATTTGAT spanning nucleotides 223-243

B ACTGCTTCCATCCATACTCC spanning nucleotides 298-317

C TGGAAAGAACCTCTGAAACA spanning nucleotides 391-411

D GAAAATTTTGACTACCCTGCCTGG spanning nucleotides 557-580

Ligation and Transformation

Ligation of PCR products into plasmid vector (pCR II) was accomplished according to the instructions provided in the TA Cloning Kit (Invitrogen Corporation; San Diego, CA). During the polymerase chain reaction, Taq DNA Polymerase generated deoxyadenosine overhangs at the 3' ends of all duplex cDNA. These overhangs were then utilized by DNA ligase to ligate the purified cDNA into the polylinker of the plasmid vector (pCR II): this vector was provided in the TA Cloning Kit. In addition to this polylinker, pCR II has been engineered to contain scorable and selectible markers. The scorable marker is a lac Z gene located within the polylinker while the selectible markers are ampicillin and kanamycin resistance genes. After ligation, the plasmid with insert was used to transform INV α F' E. coli cells. This transformation was accomplished by using the heat shock procedure described in the TA Cloning Kit. An aliquot of each transformation was spread on the surface of LB agar containing ampicillin and X-gal. E. coli cells transformed with plasmids lacking cDNA inserts were identified as ampicillin-resistant blue colonies while positive transformants were identified as ampicillin-resistant white colonies. When bacterial cells are transformed with a plasmid containing an intact lac Z gene, β-galactosidase is produced and lactose is cleaved into its corresponding monosaccharides. If a chromagenic substrate such as X-gal is present, the catalytic activities of β-galactosidase are detected by the presence of blue colored colonies; therefore, positive transformants are white because the cDNA insert has disrupted the lac

Z gene, β-galactosidase is not produced, and X-gal is not catalyzed. Several white colonies were selected and amplified by inoculation of Luria broth containing ampicillin. Isolation of the amplified plasmid with insert was accomplished using an alkaline lysis method (Appendices E and F). The size of the cDNA inserts were verified by restriction endonuclease (ECO RI) digestion of the plasmid with insert was followed by electrophoresis in a 1.2 % agarose gel. Size markers were electrophoresed in this gel as well.

Sequencing of the cDNA Insert

(Appendices G and H)

Once purified, the cDNA insert within the plasmid was sequenced by the chain termination procedure using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical; Cleveland, OH). Denaturation of this double stranded vector construct was followed by annealing of synthetic oligonucleotide primers. Initially, the primers were complimentary to vector sequences located adjacent to the cDNA insert; however, as sequence data was obtained, custom synthesized gene specific primers were utilized in a primer walking application. The 3' end of each primer was extended by the addition of deoxynucleoside triphosphates including radioactively labeled [^{35}-S]-dATP. Termination of this elongation process was accomplished by incorporation of a specific dideoxynucleoside triphosphate. Each sequencing reaction was analyzed by

electrophoresis in a 6% polyacrylamide DNA sequencing gel. These methods were used to obtain the complete sequence for both the sense and antisense cDNA strands.

Computer Analysis of Nucleotide Sequences

The Sequence Analysis Software Package by Genetics Computer, Inc. was utilized for comparison of the mouse PL-I and rat PL-I cDNA sequences at the nucleotide sequence level prior to selecting 3' RACE primers. This program was also used to assemble sequencing fragments into a consensus nucleotide sequence and to derive the amino acid sequence of the isolated, sequenced cDNA. Nucleotide and amino acid sequences of the isolated cDNA were compared with GeneEMBL and Swis Pro, respectively.

Radiolabeling of cDNA

(Appendices I and J)

All cDNA probes were radioactively labeled by the random prime labeling methodology of Feinberg and Vogelstein (1982). This methodology employs Klenow fragments (DNA Polymerase I; Gibco BRL; Gaithersburg, MD) to synthesize probes using cDNA as a template for hexamer oligonucleotide primers and the four deoxynucleoside triphosphates. Complimentary DNA probes for Northern and slot-blot RNA analyses were radioactively labeled by incorporation of [α-^{32}P]-dATP. Excess hexamer primers and

nucleosides were separated from the radiolabeled-cDNA by utilization of Sephadex G-50 Nick Columns (Pharmacia LKB Biotechnology; Uppsala, Sweden).

Screening of Placental cDNA Library

(Appendices K, L, M, N, O, P, Q, and R)

The cDNA library used in this part of the study was kindly provided by Dr. Frank Talamantes (University of California; Santa Cruz). Briefly, poly (A)+ RNA extracted from Day 16 hamster placentae was reverse transcribed to cDNA. The resulting cDNA products were ligated and packaged into lambda ZAP II bacteriophage (Lambda ZAP II; Stratagene Cloning Systems; La Jolla, CA). Screening of the library involved transforming XL-1 Blue strain of E. coli (Stratagene Cloning Systems; La Jolla, CA) with dilute recombinant bacteriophage, plating the transformants on agarose plates to form a bacterial lawn, and incubating the plates until plaques were formed The recombinant phage were transferred to nitrocellulose membranes which were screened for the presence of PL-I like and PRP-like clones by hybridization with [α-^{32}P]-labeled probes. Positive clones were isolated by properly aligning autoradiography films with the nitrocellulose membranes and the original plates. The corresponding plaques were plugged; recombinant phage contained within the agar were released into SM broth, titered, and replated on fresh XL-1 Blue cells. Plaque purification was conducted until the recombinant phage of interest were purified. The linear lambda ZAP II bacteriophage genome with the cloned insert were converted to circular single stranded DNA by

cotransfecting XL-1 Blue cells with the selected purified recombinant phage and a helper phage (ExAssist/SOLR System; Stratagene Cloning Systems; La Jolla, CA). Protein products encoded within the helper phage genome initiated transcription of the linear lambda ZAP II bacteriophage genome and the cloned insert sequences. This process generates circular single stranded DNA phagemids. Additional helper phage proteins induced secretion of these circular phagemids from the XL-1 Blue cells. These pBluescript phagemid were then used to transform a SOLR strain of E. coli cells in order to generate double stranded DNA. Isolation of this double stranded DNA was accomplished by utilization of an alkaline lysis methodology (Appendices E and F). Phagemid (pBluescript) and cloned insert sequences were obtained by chain termination procedures (Appendices G and H).

Gene Expression during Pregnancy

(Appendices S and T)

The ha-PRP cDNA was used as a radiolabeled probe in Northern Analysis of total placental RNA recovered throughout the latter half of pregnancy. Ten micrograms of total placental RNA from Days 8, 9, 10, 11, 12, 14, and 15 of gestation were size fractionated by electrophoresis in a 1.2% agarose gel containing 2.2M formaldehyde. Following electrophoresis, the RNA was transferred by capillary action onto a nylon membrane (Hybond; Amersham International plc; Amersham, UK). Prehybridization and hybridization of RNA bound to the membrane was carried out at 42°C in solutions

containing 50% formamide and 1 x 10^6 cpm [α-^{32}P]-labeled cDNA per ml. Hybridization was visualized by autoradiography. Total RNA from hamster liver was run adjacent to placental RNA to serve as a control for hybridization specificity. Size markers were also run simultaneously with samples to identify the size of the RNA species detected by the probe.

Quantitation of Total RNA

(Appendix U)

Total RNA was analyzed by slot blot methodology. Two lanes containing seven micrograms of total placental RNA from each of the designated days of gestation was applied directly to a nylon membrane (Hybond; Amersham International plc; Amersham, UK). One lane of RNA was then hybridized to the [α–^{32}P]-labeled ha-PRP cDNA and the other to the [α-^{32}P]-labeled ß actin cDNA as a loading control. The β-actin cDNA was kindly provided by Dr. Phil Pekala (East Carolina School of Medicine; Greenville, NC). Hybridizations were visualized by autoradiography using Kodak X-Omat film (Eastman Kodak; Rochester, NY) to provide a photographic record of the results. Relative levels of haPRP were quantified using a model 425E phosphorimager (Molecular Dynamics; Sunnyvale, CA) to measure radiolabelled probe hybridized to membrane-bound RNA. Proliferin-related protein values were divided by β-actin values for the duplicate sample to adjust for sample differences in membrane loading.

Adjusted proliferin-related protein mRNA values were subjected to one-way ANOVA using the NCSS statistical package (Jerry Hintze; Kaysville, Utah). Differences among days of gestation were analyzed using Duncan's multiple range test (Duncan, 1955).

Immunocytochemistry

(Appendices V and W)

Placental tissues were fixed in Bouin's solution, dehydrated using a graded ethanol series, and embedded in paraffin. Section were cut at 7 μm and mounted on gelatin-coated slides. Rabbit antiserum to mPRP (kindly provided by Dr. Daniel Linzer; Northwestern University, Evanston, IL) was used to localized PRP antigens in hamster placental tissue. Hamster PRP was localized using the avidin-biotin peroxidase complex method (Vectastain ABC Elite kit; Vector Labs; Burlingame, CA) as previously described by Jones and Renegar (1994). Control sections were incubated with normal rabbit serum in place of mPRP antiserum.

RESULTS

Initial attempts to identify and characterize the putative hamster PL-I began with Northern blot analysis using an mPL-I cDNA and total hamster placental RNA from Days 12 and 14 of gestation. All efforts were unsuccessful. In addition, immunocytochemistry studies demonstrated that antisera to mPL-I does not crossreact with antigens in hamster placental tissue.

Subsequently, oligonucleotide primers were chosen from regions of sequence homology shared by mPL-I and rPL-I. These oligonucleotides and total hamster placental RNA from Days 9, 10, and 11 were used in 3' RACE methodology to yield specific cDNA products. When primer "C" and total RNA from Days 9 and 10 were used in 3' RACE methodology, several products were generated (Figure 1). Subsequently, Southern blot analysis using mPL-I cDNA as a radiolabeled probe indicated that several PCR products from Day 9 possessed sequence similarity with mPL-I (Figure 2). From the various sized cDNA products identified, one of approximately 400 base pairs was selected. This size selection was based on the oligonucleotide primer position within mPL-I and rPL-I sequences. The selected cDNA fragment (Figure 2, arrow) was ligated into a plasmid (pCR II) vector (Invitrogen; San Diego, CA) and sequenced using chain termination methodology. This fragment was 444 base pairs in length and contained considerable nucleotide and deduced amino acid sequence similarity with members of the PRL-GH gene family. Contrary to expectations, this cDNA fragment had only 58% nucleotide

sequence homology with mouse or rat PL-I. Maximum sequence homology was with mouse proliferin-related protein (78%). The other primers selected did not generate PCR products or the generated products did not have sequence homology with members of the PRL-GH gene family, and therefore were not investigated.

In order to obtain the remaining 5' sequence of the PRP-like molecule, two gene specific primers were selected from the available 3' cDNA sequence. These primers and total hamster placental RNA from Days 9, 10, and 11 were utilized unsuccessfully in 5' RACE methodology. Subsequently, a Day 16 hamster placental bacteriophage cDNA library (kindly provided by Dr. Frank Talamantes) was screened using the previously identified 444 base pair cDNA fragment as a probe. A mPL-I cDNA was also used as a probe to evaluate the presence of a PL-I-like molecule. Screening of 200,000 recombinant bacteriophage plaques identified three positive clones; two clones hybridized to the hamster cDNA probe and one clone hybridized to the mPL-I cDNA probe. Clones hybridizing to the hamster cDNA probe were isolated by two rounds of plaque purification; however, the clone hybridizing to the mPL-I probe was not detected after the first round of plaque purification. Isolation of a PL-I containing clone was not further pursued. Bacteriophage were recovered and transformed in E. coli (ExAssist/SOLR System; Stratagene; La Jolla, CA). Restriction endonuclease (Eco RI) digestion of pBluescript SK(+) phagemids corresponding to the two identified clones revealed inserts of approximately 870 and 2000 base pairs. Since the expected size for a member of the PRL-GH gene family is in the range of 800 to 1000 base pairs, the clone containing an

insert of approximately 870 base pairs was selected for sequence analysis (Figure 3). This cDNA molecule possessed a 3' cDNA sequence identical to the previously described 444 base pair cDNA fragment. Complete sequence information was generated by "primer walking" of both the sense and antisense strands of the phagemid cDNA insert. Nucleotide sequence analysis indicated that the insert was 870 bp in length with an open reading frame of 704 bp (Figure 4). This molecule had 76% nucleotide sequence identity with mouse proliferin-related protein (mPRP) and somewhat less homology (~ 60%) with hamster, mouse, and rat prolactin or placental lactogen (Table 1). Interestingly, the 21 bp oligonucleotide primer "C" used in 3' RACE was highly conserved in mPRP as well as mPL-I and rPL-I. There was no homology with growth hormone. Nucleotides 1-70 encompass the 5' untranslated region followed by a start codon at positions 71-73 (Figure 4). The open reading frame spans from nucleotide 71 to the termination codon at 775. A poly(A) addition signal (A-A-T-A-A-A) is located at 831-836, and therefore, the 3' untranslated region spans from 776-870. The deduced amino acid sequence of this molecule contains a 15 residue signal sequence and a 219 residue peptide with a calculated molecular weight of 25,477 daltons (Figure 5). The peptide shares 58% amino acid sequence identity with mPRP (Table 1). Based on sequence data, this molecule has been tentatively identified as hamster proliferin-related protein (haPRP). Alignment of the deduced haPRP peptide sequence with mPRP indicated residues which are conserved in numerous members of the PRL-GH gene family including four cysteines at positions 86, 202, 219, and 227 and two tryptophans at 119 and 178 (the numbers correspond to

positions within the proposed haPRP peptide). Several previously characterized members of the PRL-GH gene family also contain possible N-linked glycoslyation sites and proteolytic cleavage sites. Five possible N-linked glycosylation sites (Asn-Ile-Ser at 19-21, Asn-Ala-Thr at 36-38, Asn-Ile-Thr at 43-45, Asn-Phe-Ser at 59-61, and Asn-Ile-Thr at 110-112) as well as one basic dipeptide (Arg-Lys at 100-101) were present. Even though the consensus sequence for glycosylation, Asn-X-Ser/Thr, and proteolytic cleavage (basic dipeptides) were present, additional analysis is required to determine if glycosylation and/or cleavage actually occurs at these sites.

Northern analysis was used to determine the number and size of haPRP mRNA transcripts expressed in the placenta during gestation (Figure 6). A single transcript of approximately 1 kb was detected on Days 9 to 14. This transcript was similar in size to that described for other members of the PRL-GH gene family. At Day 15, the 1 kb mRNA species was undetectable yet a faint transcript slightly greater than 1 kb was present (Figure 6). Since a haPRP mRNA transcript was detected on Day 11 of gestation but a product was not generated from using Day 11 total placental RNA in the 3' RACE methodology RNase contamination may have occurred during extraction.

Analysis of total hamster placental RNA by slot blot hybridization was used to quantify the relative amounts of haPRP mRNA present during the latter half of gestation (Figure 7 and 8). In contrast to results obtained using film autoradiography for detection of hybridized haPRP probe following Northern blotting, phosphorimage analysis of the slot blot detected low levels of haPRP mRNA in several samples on Day 8. Levels

increased ($p \leq 0.05$) between Days 9 and 11. Transcript levels then decreased ($p \leq 0.05$) to Day 14.

Specific staining for PRP was localized within cytotrophoblasts of the trophospongium layer (Figure 9, C). Histologically these cells appeared heavily vacuolated and were scattered among the numerous giant trophoblast cells. Positive staining was also localized within cytotrophoblast cells surrounding the placental artery as it coursed through the trophospongium. Giant trophoblast cells were conspicuously devoid of positive staining product.

Due to the unavailability of the mPRP peptide used in generation of the mPRP antisera, it was not possible to determine specificity of the mPRP antisera in hamster placental tissues. However, immunocytochemical results were consistent with preliminary *in situ* hybridization studies conducted in this laboratory using a haPRP specific oligonucleotide probe. Background immunostaining due to non-specific binding of the antibody to tissue components was assessed by substitution of normal rabbit serum for the mPRP antiserum (Figure 9, D).

Figure 1. Recovered PCR fragments from 3' RACE methodology using oligonucleotide primer "C" chosen from homologous sequences shared by mPL-I and rPL-I. Fragments were generated from 35 cycles of amplification and size fractionated by electrophoresis using a 1.5% agarose gel run at 25 volts. Lane 1 contains size markers. Lanes 2, 3, and 4 represent PCR samples generated using total hamster placental RNA from Days 9, 10, and 11, respectively. Lane 5 is a negative control containing no RNA, only primers. Note the prominent band at the anticipated size of ~400 bp in lanes 2 and 3 (arrow).

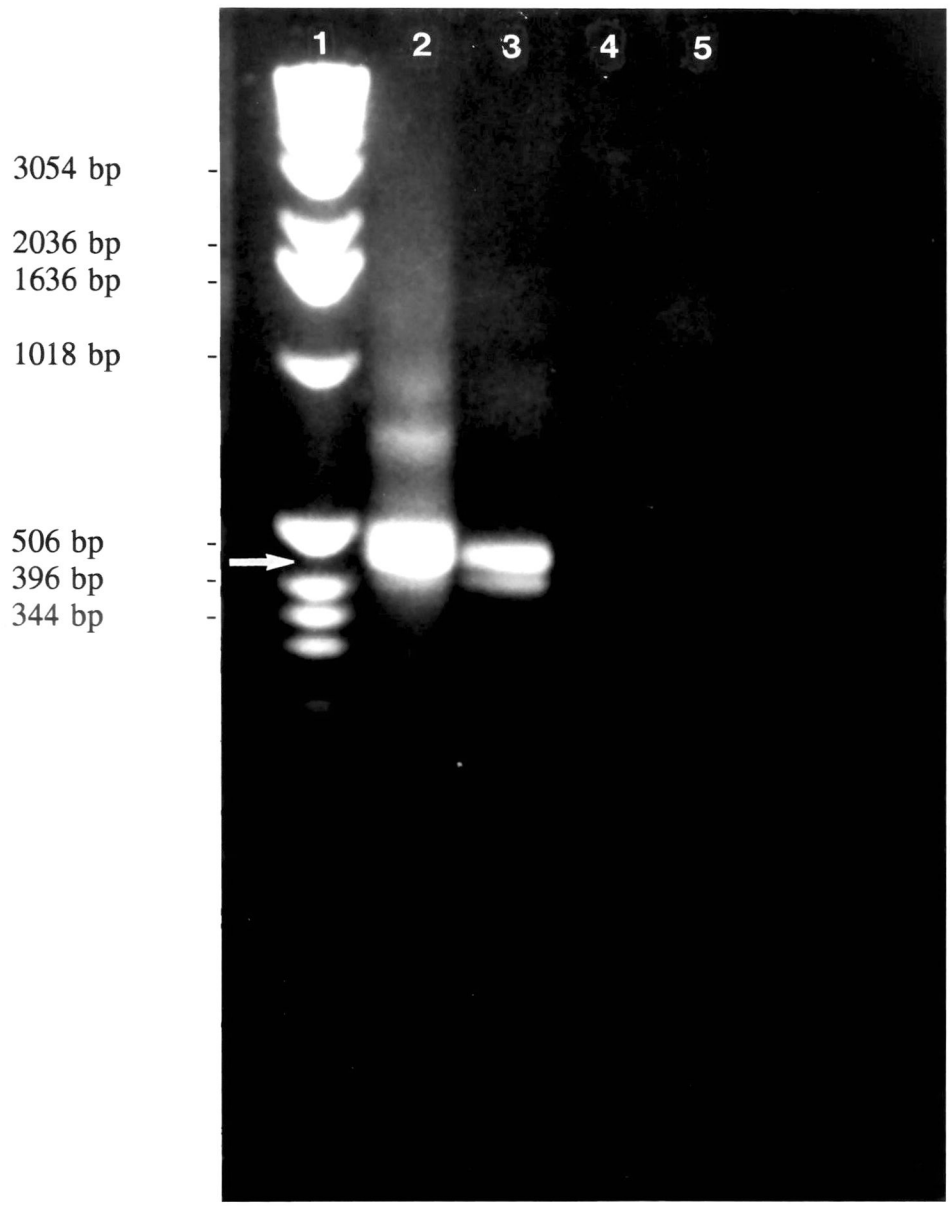

1
2
3
4
5
3054 bp
2036 bp
1636 bp
1018 bp
506 bp
396 bp
344 bp

Figure 2. Southern hybridization analysis of recovered PCR fragments. Radiolabeled mPL-I cDNA was used as a probe to identify PL-I-like cDNA fragment(s). Hybridization revealed several fragments in Lane 2 (arrow) which correspond to the ethidium bromide-stained bands indicated in Lane 2 of Figure 1.

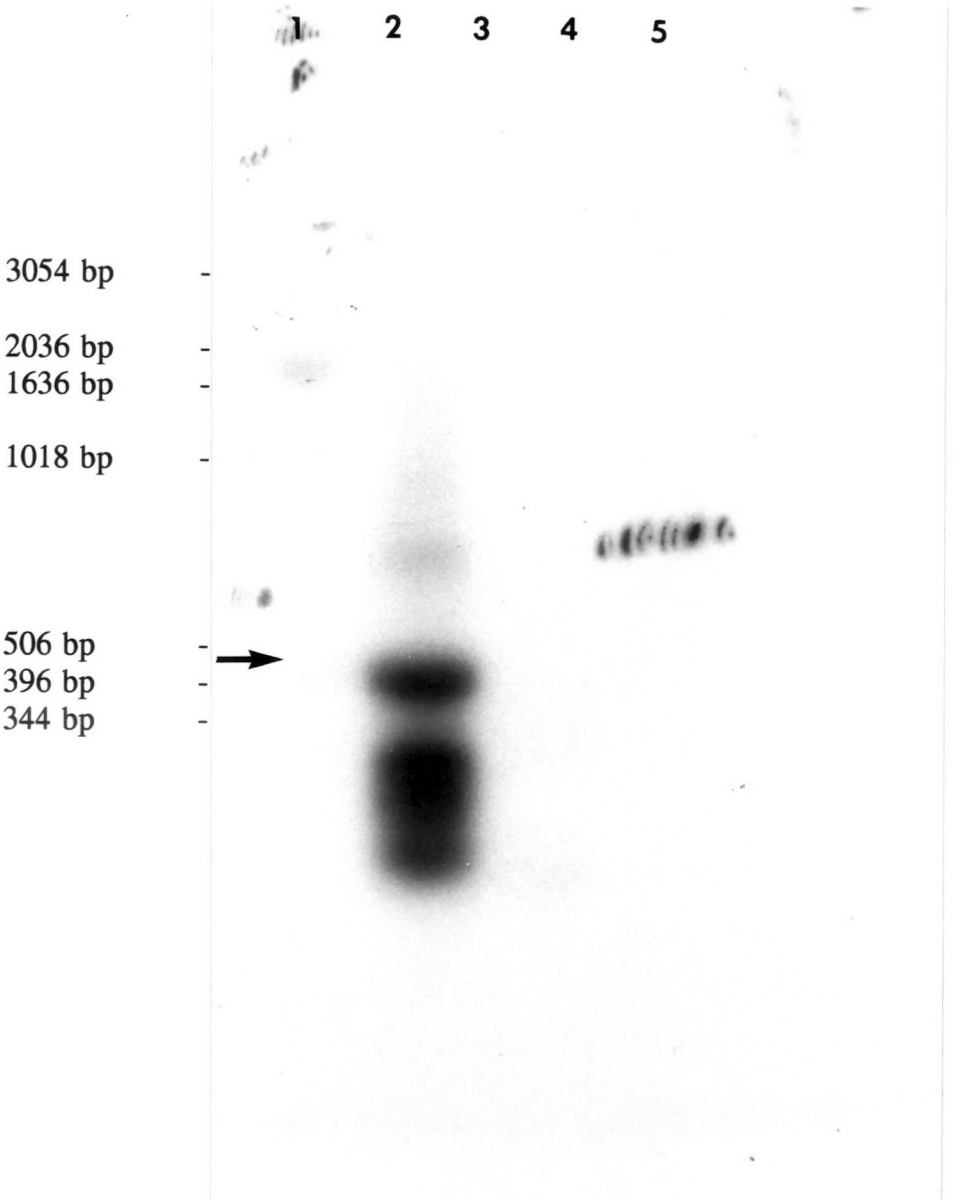

1
2
3
4
5
3054 bp
2036 bp
1636 bp
1018 bp
506 bp
396 bp
344 bp

Figure 3. Vector construction of pBluescript SK(+) II containing the hamster proliferin-related protein (870 bp) cDNA insert.

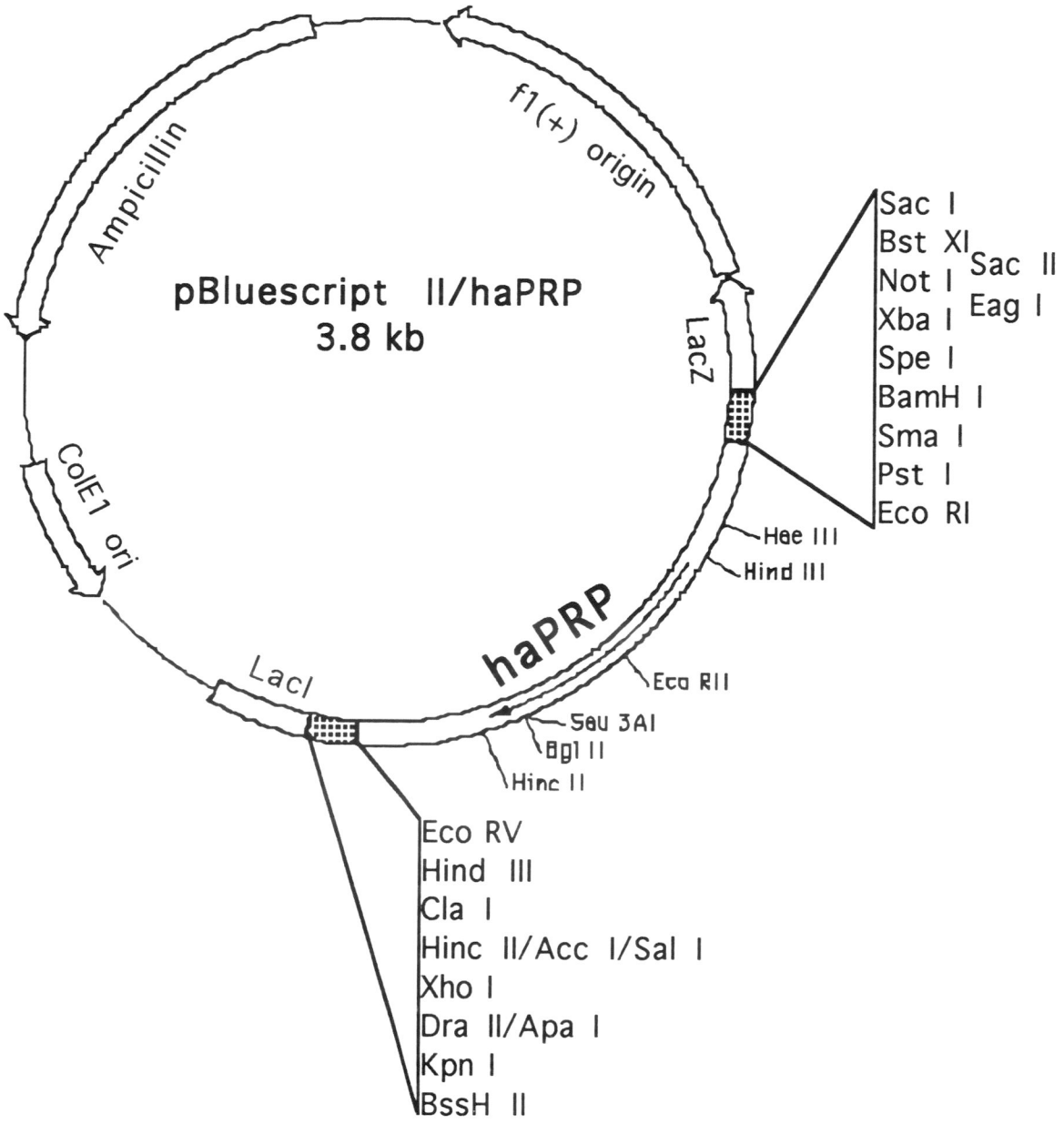
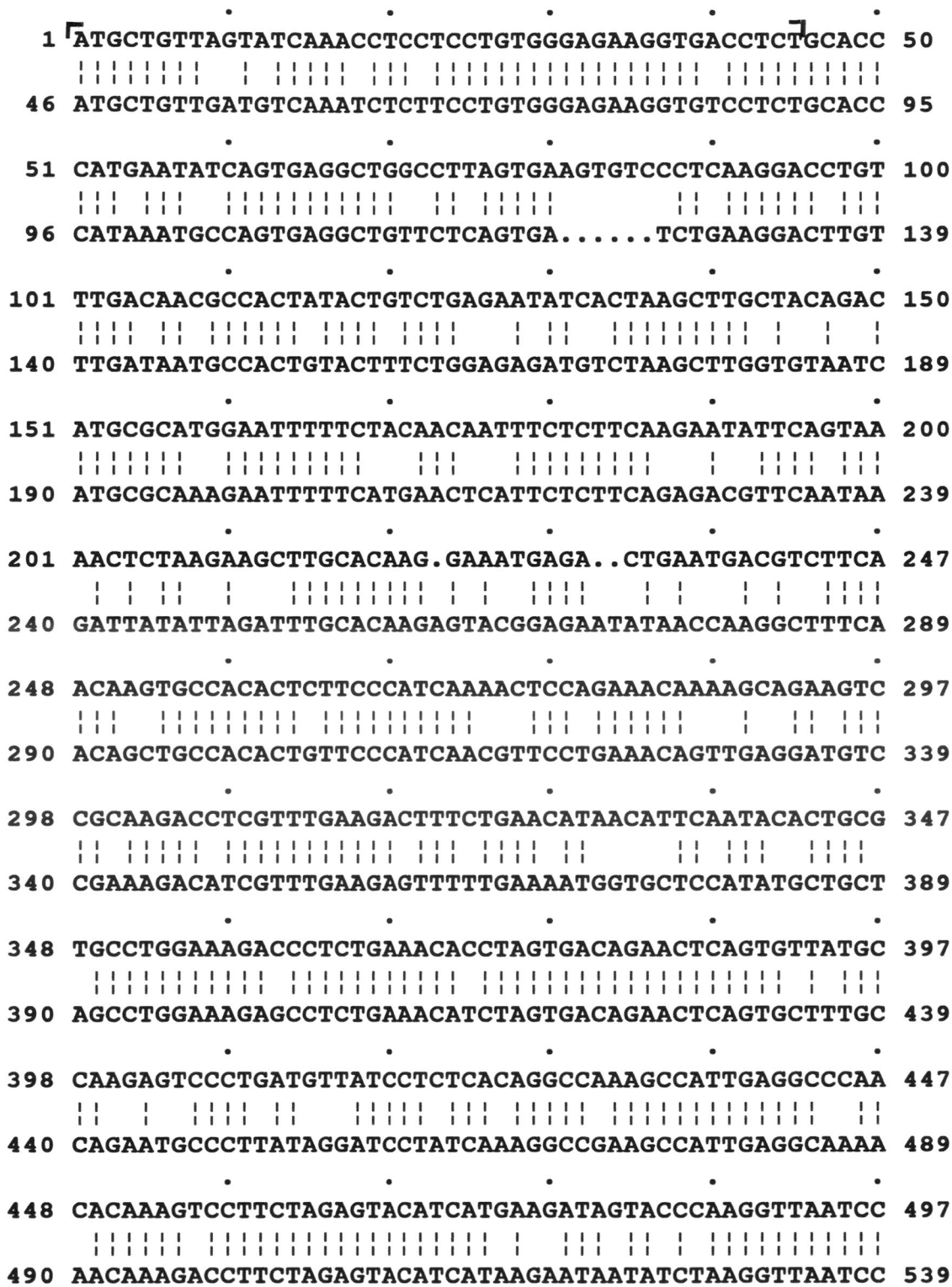

pBluescript II/haPRP
3.8 kb
Ampicillin
f1(+) origin
LacZ
ColE1 ori
LacI
haPRP
Sac I
Bst XI
Not I
Xba I
Spe I
BamH I
Sma I
Pst I
Eco RI
Sac II
Eag I
Hae III
Hind III
Eco RII
Sau 3AI
Bgl II
Hinc II
Eco RV
Hind III
Cla I
Hinc II/Acc I/Sal I
Xho I
Dra II/Apa I
Kpn I
BssH II

Figure 4. Comparison of haPRP (upper) and mPRP (lower) cDNA sequences. Nucleotides coding for the signal peptide are enclosed by brackets.



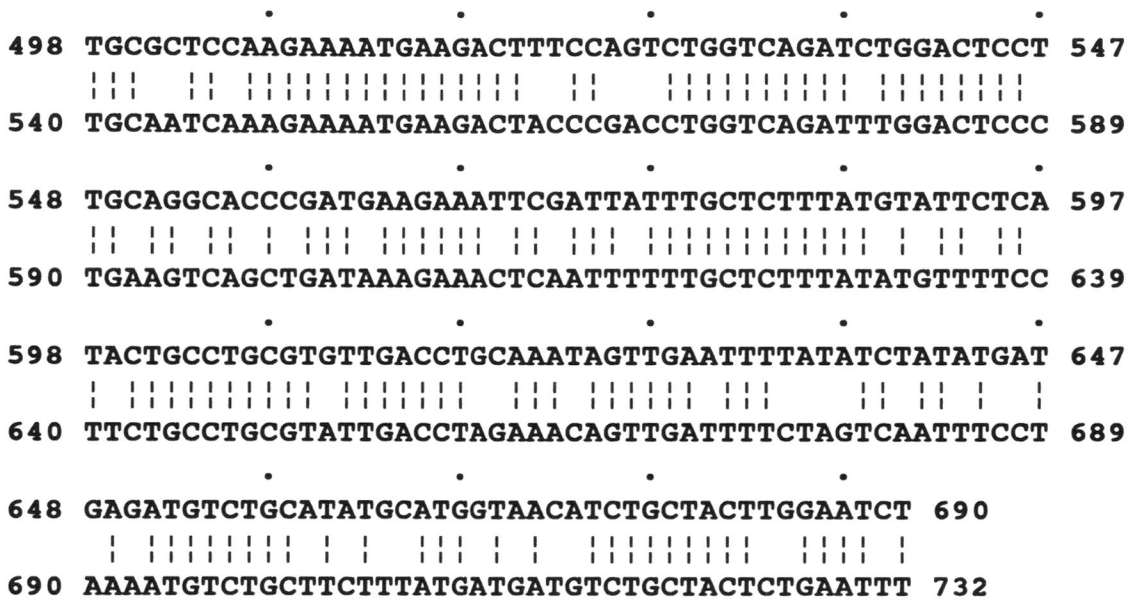

498 TGCGCTCCAAGAAAATGAAGACTTTCCAGTCTGGTCAGATCTGGACTCCT 547
540 TGCAATCAAAGAAAATGAAGACTACCCGACCTGGTCAGATTTGGACTCCC 589
548 TGCAGGCACCCGATGAAGAAATTCGATTATTTGCTCTTTATGTATTCTCA 597
590 TGAAGTCAGCTGATAAAGAAACTCAATTTTTTGCTCTTTATATGTTTTCC 639
598 TACTGCCTGCGTGTTGACCTGCAAATAGTTGAATTTTATATCTATATGAT 647
640 TTCTGCCTGCGTATTGACCTAGAAACAGTTGATTTTCTAGTCAATTTCCT 689
648 GAGATGTCTGCATATGCATGGTAACATCTGCTACTTGGAATCT 690
690 AAAATGTCTGCTTCTTTATGATGATGTCTGCTACTCTGAATTT 732

Table 1. Comparison of nucleotide and deduced amino acid sequences of haPRP with other rodent members of the PRL-GH gene family. Maximum homology was found with mouse proliferin-related protein (mPRP). There was no homology with GH.

HaPRP Compared with PRL-GH Gene Family	Identical nucleotides	Identical amino acids
Hamster PRL	61.0%	36.5%
PL-II	60.4%	33.7%
GH	-----	-----
Mouse PRL	59.2%	37.6%
PL-I	58.4%	35.6%
PL-II	59.8%	33.3%
Proliferin (PLF)	59.8%	29.4%
*PLF-related protein	75.1%	57.5%
GH	-----	-----
Rat PRL	59.2%	38.6%
PL-I	56.6%	31.3%
PL-II	58.9%	30.4%
PLP-A	57.2%	27.4%
PLP-B	58.0%	30.8%
PLP-C	55.7%	31.5%
GH	-----	-----

Figure 5. Comparison of the deduced amino acid sequence for haPRP (upper) and mPRP (lower). Residues coding for the signal peptide are enclosed by brackets. Cysteine (C) and tryptophan (W) residues highly conserved among members of the PRL-GH gene family are indicated with an asterick (*). Potential glycosylation sites are indicated by underlining and potential proteolytic cleavage site is indicated with a star.

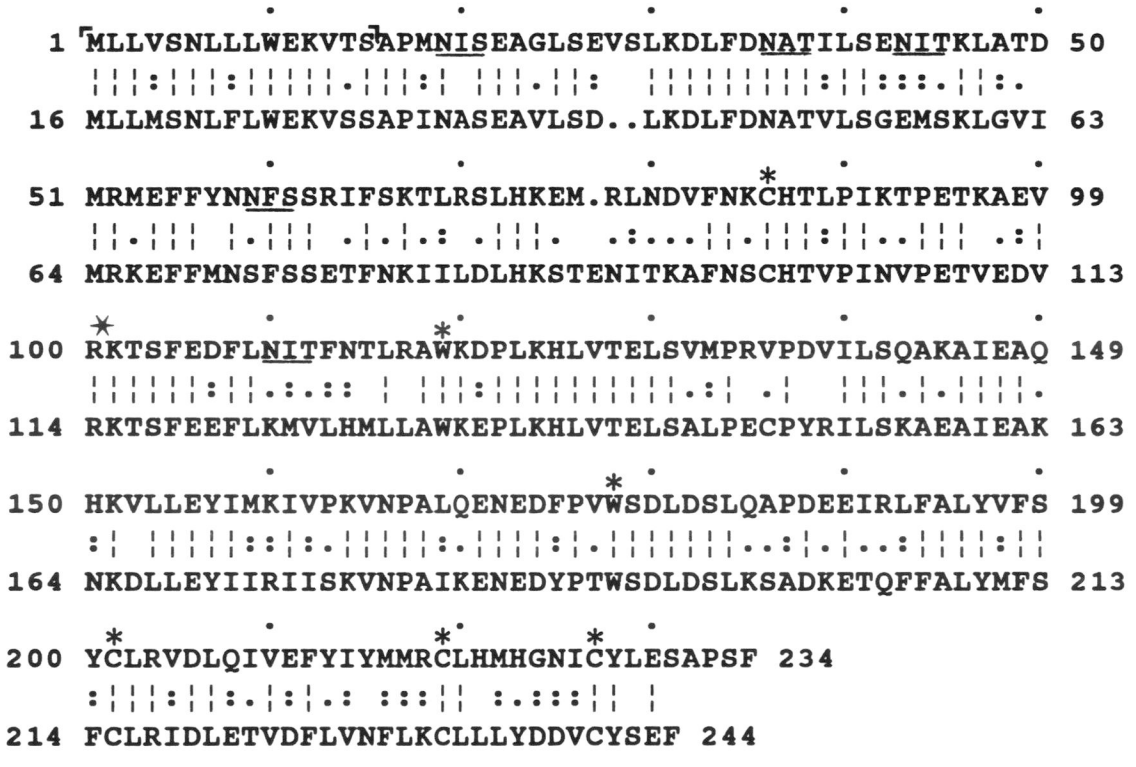


Figure 6. Northern blot analysis of 10 μg total hamster placental RNA. The radiolabeled 444 bp cDNA fragment generated by 3' RACE methodology identified a single transcript of approximately 1 kb in samples (n=3) from Days 9 to 14 of gestation. A slightly larger transcript (~1.2 kb) appeared to be present in samples (n=3) from Day 15 of gestation. Hamster PRP mRNA was visualized by autoradiography (exposure time = 120 hours).

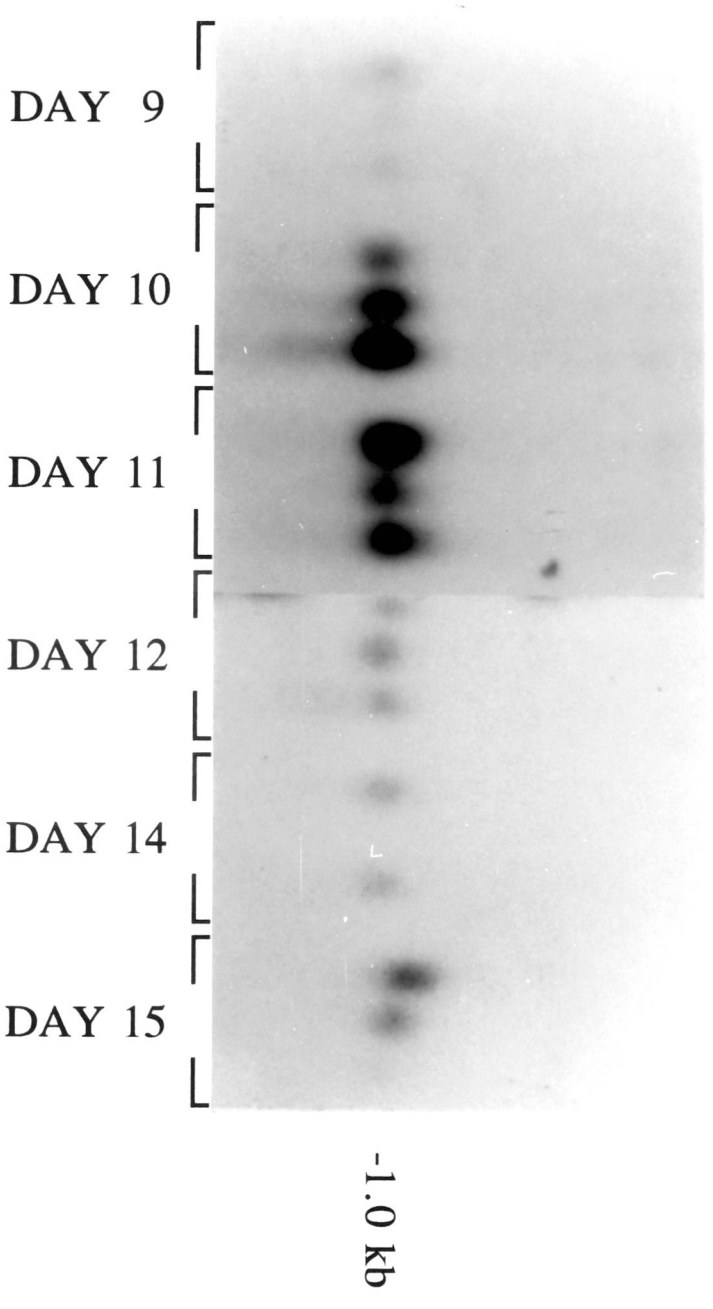

DAY 9
DAY 10
DAY 11
DAY 12
DAY 14
DAY 15
-1.0 kb

Figure 7. Slot blot analysis of haPRP mRNA expression during gestation. Duplicate lanes of total hamster placental RNA (7 μg) were hybridized with a cDNA probe for haPRP or β-actin. Hybridization was detected by phosphorimaging the membrane and the computer generated image is shown.

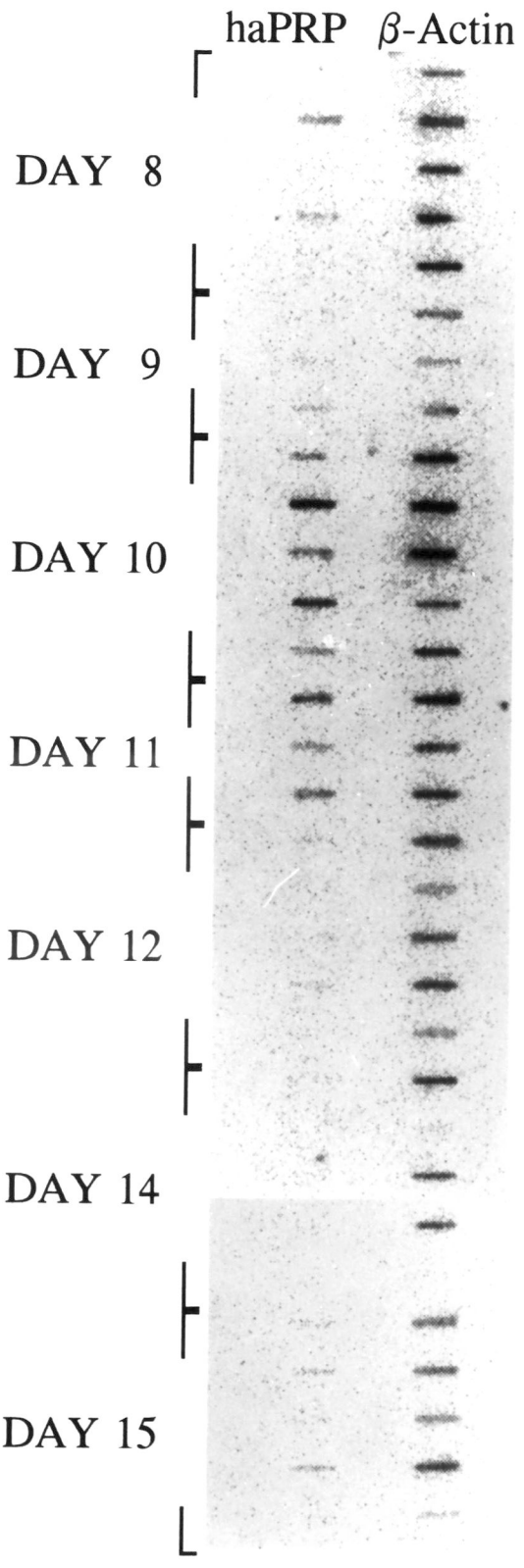

haPRP
β-Actin
DAY 8
DAY 9
DAY 10
DAY 11
DAY 12
DAY 14
DAY 15

Figure 8. Bar graph of haPRP mRNA expression during gestation. Hybridization values were determined from the slot blot shown in Figure 7. Hamster PRP mRNA values were standardized for loading differences using β-actin mRNA values. Data were analyzed by one-way ANOVA and means compared by Duncan's multiple range test. Values with different superscripts differ ($p \leq 0.05$). Numbers in parentheses indicate the number of samples analyzed.

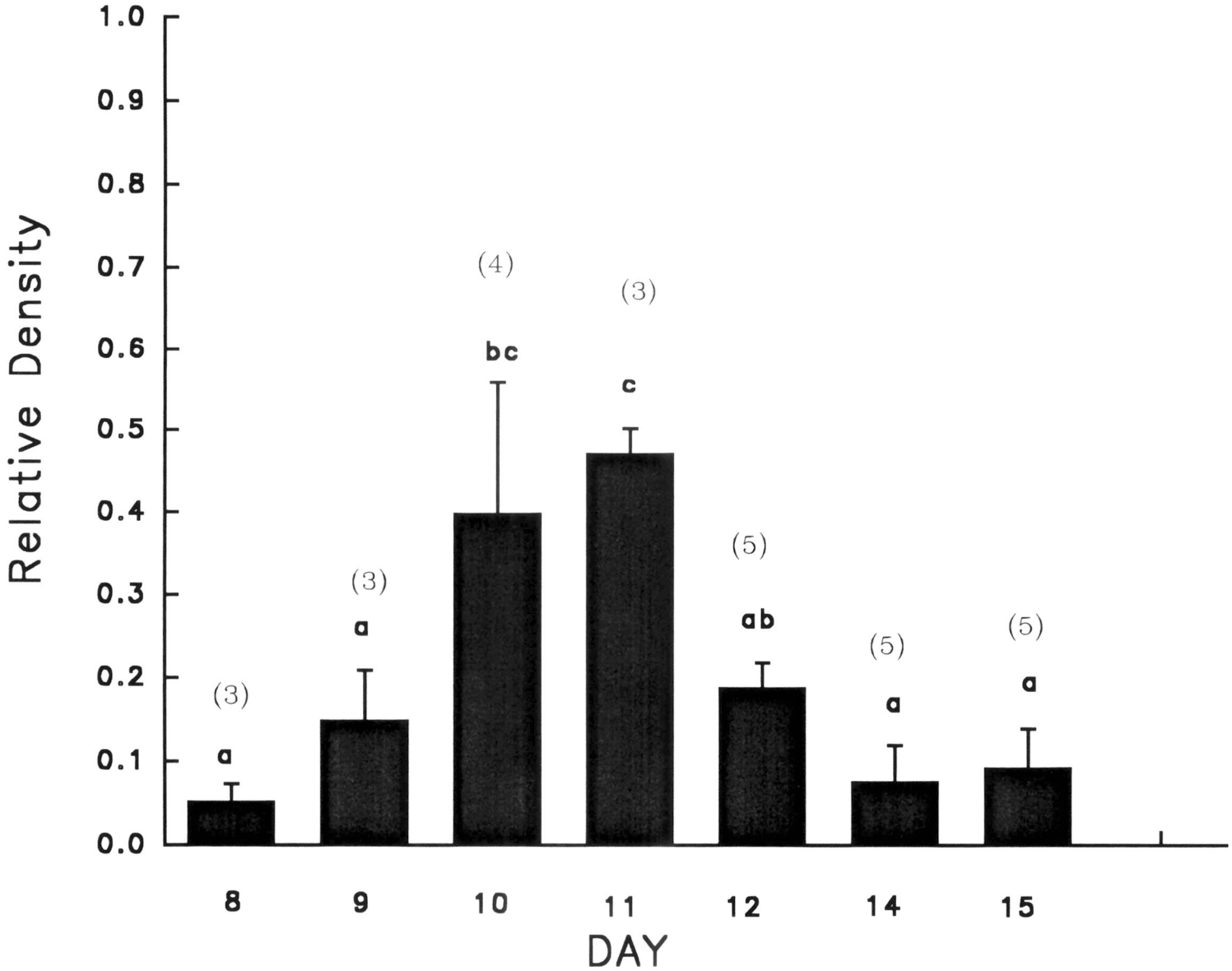
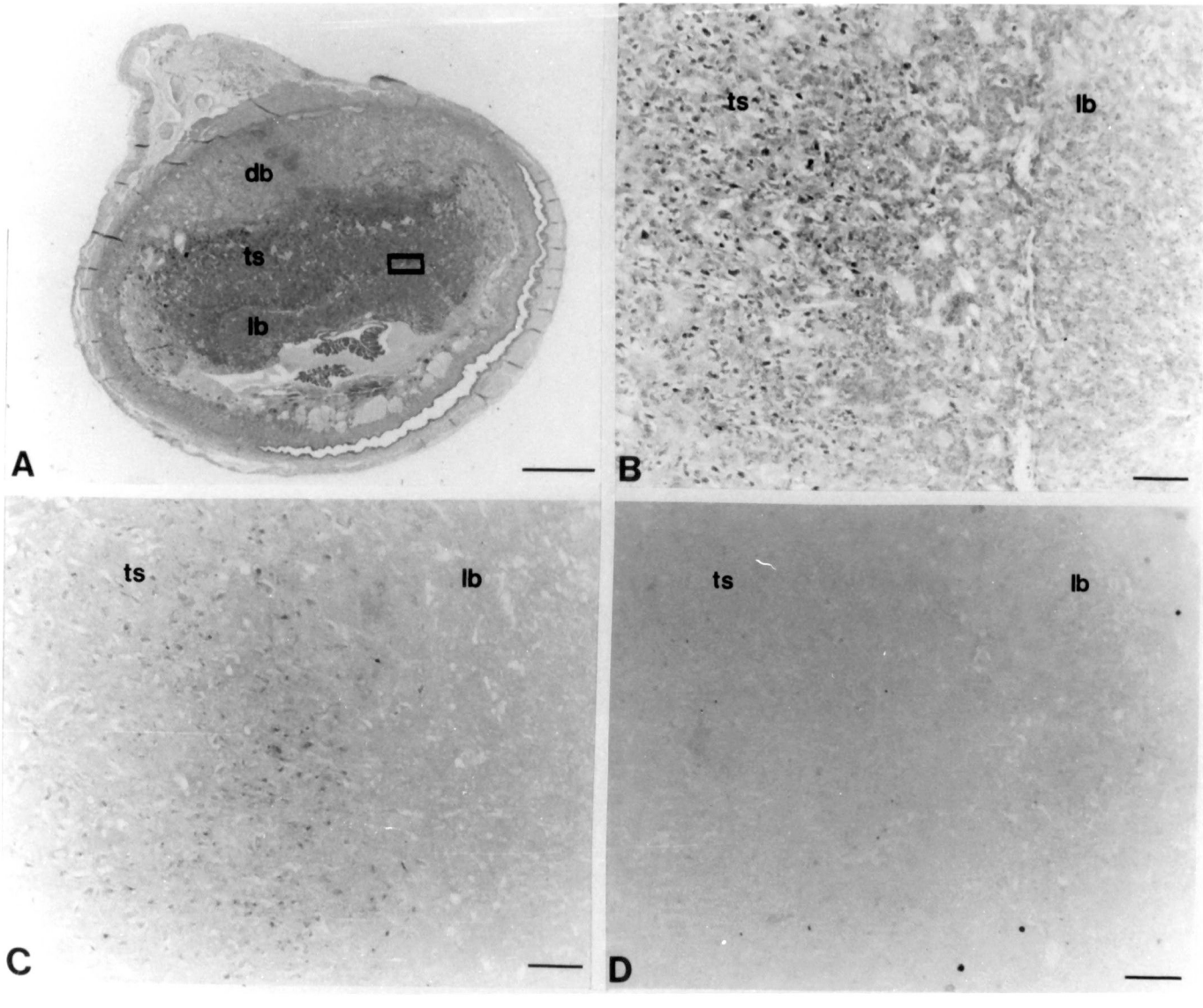

Relative Density
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
(3)
a
(3)
a
(4)
bc
(3)
c
(5)
ab
(5)
a
(5)
a
8
9
10
11
12
14
15
DAY

Figure 9. Immunocytochemical localization of haPRP in hamster placental tissue from Day 11 of gestation. Protein was immunolocalized using the avidin-biotin-peroxidase method for rabbit IgG and rabbit antisera to mPRP at a 1:5,000 dilution. A) Implantation site from Day 11 of gestation; decidua basalis (db), trophospongium (ts), and labyrinth (lb). Hematoxylin stained. Bar = 250 μm. B) High magnification micrograph of labyrinth and trophospongium interface. Hematoxylin stained. C) Placental section treated with rabbit antisera to mPRP. Immunostaining is localized within cytotrophoblast cells of the trophospongium (ts); no staining is detected in the labyrinth (lb). D) Placental section in which normal rabbit serum at a 1:5,000 dilution was substituted for the specific mPRP antiserum. Note the lack of positive immunostaining. Bar = 100 μm.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DISCUSSION

Placentas from vivaparous species are able to synthesize and secrete polypeptides which are structural and/or functional analogs of the pituitary hormones, PRL and GH. The mouse and rat placentas produce two distinct lactogenic hormones, PL-I and PL-II, as well as several non-lactogenic PRL-like proteins. As for the hamster placenta, a midgestation lactogenic polypeptide (PL-I) has been proposed and a late gestation lactogenic polypeptide (PL-II) has been characterized extensively. This study reveals a new member of the PRL-GH gene family which is synthesized in the hamster placenta. Comparison of members of the PRL-GH gene family with the identified cDNA sequence indicates this hamster placental mRNA has the most extensive nucleotide sequence identity (76%) with mouse proliferin-related protein (mPRP), and therefore it will be tentatively referred to as hamster proliferin-related protein (haPRP).

The nucleotide sequence encodes a single open reading frame containing 234 amino acids. Significant amino acid sequence identity (59%) is also shared by hamster and mouse PRPs. Analysis of the proposed amino acid sequence suggests haPRP peptide may undergo several posttranslation processes. Fifteen amino acids located at the amino-terminus may serve as a signal peptide for a 219 amino acid secreted peptide. This region of fifteen amino acids satisfies all criteria for a signal peptide; the length is 15 to 30 residues, it is rich in hydrophobic residues, and the proposed cleavage site is marked by serine, an amino acid with a small neutral side chain (Docherty and Steiner, 1982; Von

Heijne, 1983). Interestingly, this is the shortest signal peptide described for any rodent placental PRL-like protein; until now, the usual signal peptide length was 29-31 amino acids (reviewed by Southard and Talamantes, 1991).

The consensus signal for N-linked glycosylation, Asn-X-Ser/Thr (Bahl and Shah, 1977), appears at five locations within the secreted peptide of haPRP. Even though most mammalian PRLs are secreted as non-glycosylated polypeptides, several placental PRL-like proteins contain one or more potential N-linked glycosylation sites. Two of the five glycosylation sites in haPRP pair with two of three sites in mPRP; neither haPL-II or haPRL contain glycosylation sites. Comparison of available amino acids sequences for the placental PRL-like proteins demonstrates variability in the positioning of these N-linked glycosylation sites, except for mPL-I and rPL-I which share two sites.

Further modification of haPRP peptide may occur at the basic dipeptide, the proposed consensus sequence for proteolytic cleavage of prohormones (Docherty and Steiner, 1982). Although the presence of basic dipeptides has been observed in other placental PRL-like proteins, including mPLF and mPRP, proteolytic cleavage at these residues has not been demonstrated.

Other noteworthy structural features of haPRP include the presence of four cysteine and two tryptophan residues within the peptide. The number and location of the cysteine residues is a structural feature which differentiates the placental proteins from their pituitary relatives (reviewed by Southard and Talamantes, 1991). Placental PRL-like proteins contain four, five, six, and nine cysteines at various locations while mammalian

PRLs and GHs contain six and four, respectively, at conserved locations. Interestingly, the four cysteines in haPRP pair with four of six cysteines in haPRL and haPL-II. The four cysteines in haPRP also pair with four of the five cysteines in mPRP. These conserved cysteine residues are believed to participate in disulfide interactions which yield tertiary structures similar to that of PRL/GH (Abdel-Meguid et al., 1987; Cunningham et al., 1990), but currently, tertiary structures have not been assessed for placental PRL-like proteins. The presence of tertiary structures similar to those of PRL/GH may predict whether the placental polypeptide is able to interact with and/or bind PRL/GH receptors. Placental non-lactogenic PRL-like polypeptides seem to lack the ability to interact with and/or bind PRL receptors, and as a result lack PRL-like activities. If primary structural similarities are indicative of tertiary structural similarities, haPRP, like its homologue mPRP, may be considered a non-lactogenic PRL-like protein. Determination of the presence or absence of lactogenic activity will also determine whether haPRP is the proposed PL-I that was previously detected in pregnant hamster serum (Southard et al., 1987) or whether it is a novel placental PRL-like polypeptide. The two tryptophan residues within the haPRP peptide pair with the two in haPRL and mPRP and with two of three in haPL-II. The role(s) of these amino acid residues remains unclear; however, to date all PRLs (except mPRL) contain two tryptophan residues and all GHs contain one. Conservation of these residues throughout evolution implies some structural/functional significance.

A single haPRP mRNA transcript of approximately 1 kb in size was detected on Days 9-14. On Day 15, a slightly larger transcript (~ 1.2 kb) was detected. A transcript of approximately 1 kb has been detected for a majority of the PRL-GH gene family members including haPL-II, mPL-I, mPL-II, rPL-I, rPL-II, and mPLF. In contrast, the mRNA transcript for mPRP was estimated to be 1.1 kb (Linzer and Nathans, 1985). The larger mRNA transcript detected in Day 15 hamster placentas might be due to alternative post-transcriptional processing of a haPRP gene or expression of an additional haPRP-like gene. There may also be some correlation between this larger mRNA transcript and the 2000 bp cDNA molecule obtained from screening the Day 16 hamster placental bacteriophage cDNA library; further analysis of this 2000 bp cDNA molecule should be pursued. Additional studies are also required to determine the number of PRP genes in the hamster genome and the presence of additional placental PRL-like genes.

Hamster PRP is the only polypeptide reported to be synthesized by cytotrophoblast cells of the hamster placenta. Previous studies demonstrated that relaxin (Johns and Renegar, 1990) and haPL-II (Renegar et al., 1990) are synthesized by giant trophoblast cells within the trophospongium. Similar to haPRP, mPRP is also localized to cytotrophoblast cells but not giant trophoblast cells of the mouse placenta (Colosi et al., 1988). Considering the significant sequence identity shared by mPRP and haPRP as well as the similar sites of synthesis, the similarity of physiological activities seems likely. Mouse PRP has anti-angiogenic properties (personal communication, Linzer); therefore, it may be involved in regulating invasion of fetal vasculature into maternal tissue. Synthesis

of haPRP within cells of the trophospongium is consistent with the hypothesis that this molecule regulates migration of fetal vessels into the trophospongium.

In summary, this study revealed the existance of a molecule with structural features similar to mouse proliferin-related protein. Hamster PRP mRNA was first detected on Day 8 with maximal expression on Day 11, which coincides with the previously determined serum profile for lactogenic activity in the pregnant hamster (Southard et al., 1987). Hamster PRP was localized to cytotrophoblast cells of the trophospongium which is consistent with localization of mPRP. Further analysis of haPRP is necessary in order to determine whether this placental protein is the previously proposed lactogenic hormone, PL-I, or whether it is a novel hamster placental protein with functional properties similar to those of mPRP.

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APPENDIX A

REAGENTS FOR RNA EXTRACTIONS

1. Denaturing solution
   4 M guanidium hydrochloride
   25 mM sodium citrate
   0.5% sarkosyl
   0.1 M ß-mercaptoethanol
   DEPC-treated water

2. 3 M sodium acetate, pH 5.2

3. $T_{10}E_1$-saturated phenol

4. CIA
   chloroform : isoamyl alcohol (24 parts : 1 part)

5. isopropanol

6. 75% ethanol

7. 100% ethanol

8. 0.5% sodium dodecyl sulfate (SDS)

APPENDIX B

RNA EXTRACTION PROTOCOL

Day 1

1. Add 0.5 gm of tissue to a sterile glass tube containing 5 ml of denaturing solution.
2. Prevent thawing of tissue by quickly homogenizing for approximately 30 seconds with a polytron.
3. Add: 5 ml phenol
   1 ml CIA
   0.333 ml 3 M sodium acetate, pH 5.2
4. Cap tube and invert gently for 10 seconds. Incubate tube on ice for 15 minutes.
5. Centrifuge tube at 9,000 rpm in an SS34 rotor (Sorvall; Wilmington, DE) for 30 minutes at 4°C.
6. Transfer the upper aqueous layer to a new sterile tube and add 1 volume of isopropanol. Incubate tube at -20°C for at least one hour to precipitate RNA.
7. Centrifuge tube as in step 5.
8. Pour off supernatant and resuspend pellet in 0.5 ml denaturing solution. Transfer solution to a sterile 1.5 ml microfuge tube.
9. Add 2 volumes of cold 100% ethanol and incubate at -20°C overnight.

Day 2

1. Centrifuge tube in a microcentrifuge at 14,000 rpm for 20 minutes at 4°C.
2. Wash the pellet with 1.0 ml 75% ethanol. Repeat centrifugation.
3. Pour off supernatant and allow pellet to air dry briefly.

4. Resuspend the pellet in 50-100 µl 0.5% SDS. Quantitate RNA by reading at A_{260}.

APPENDIX C

REAGENTS FOR 3' RACE

1. RNase H at 2U/µl (Gibco BRL; Gaithersburg, MD)

2. Taq DNA Polymerase at 5U/µl (Stratagene Cloning Systems; La Jolla, CA)

3. 10X Taq reaction buffer (Stratagene Cloning Systems; La Jolla, CA)
   100 mM Tris-HCl, pH 8.3
   500 mM KCl
   15 mM $MgCl_2$
   0.1% (w/v) gelatin and other stabilizers

4. Superscript Reverse Transcriptase at 200U/µl (Gibco BRL; Gaithersburg, MD)

5. dNTPs (10 mM each nucleotide)

6. 25 mM $MgCl_2$

7. DEPC-treated distilled water

8. 0.1 M dithiothreitol (DTT)

9. oligo(dT) primers (400 ng/µl)

10. custom oligonucleotide primer (400 ng/µl)

11. sterile distilled water

APPENDIX D

3' RACE PROTOCOL

All incubations were conducted in a programmable (Model PTC-150) thermal controller (MJ Research INC.; Watertown, MA).

First Strand cDNA Synthesis

1. In a sterile microfuge tube combine: 5.0 µg total RNA, 5.0 µl oligo(dT), and 52.22 µl DEPC-treated distilled water
2. Incubate tube at 70°C for 10 minutes to denature RNA, and then place on ice for 1 minute.
3. Briefly centrifuge and add:
   9.5 µl 10X Taq reaction buffer
   2.0 µl DTT
   16.0 µl dNTPs
4. Incubate tube at 42°C for 2 minutes and add 2.28 µl $MgCl_2$ and 1.0 µl Superscript Reverse Transcriptase.
5. Continue incubating the tube at 42°C for an additional 30 minutes followed by 5 minutes at 55°C. Hold at 55°C to allow for the addition of 1.0 µl RNase H. Maintain 55°C for an additional 10 minutes and then place tubes on ice.

Polymerase Chain Reaction (PCR)

1. The sample from the cDNA synthesis reaction now contains 100 µl. To this tube add 5.0 µl custom oligonucleotide primer and overlay with 90.0 µl mineral oil.
2. Make enough diluted Taq solution for one reaction by combining:
   0.5 µl 10X Taq reaction buffer
   0.75 µl Taq DNA Polymerase I
   3.75 µl sterile distilled water
3. Incubate tube at 94°C for 8 minutes, reduce temperature to 80°C and add 5.0 µl diluted Taq solution below the mineral oil overlay.

4. Incubate the tube at 94°C for 45 seconds, 55°C for 1 minute, and 70°C for 1 minute. Repeat this cycle 34 more times.
5. A final incubation at 72°C for 10 minutes will maximize strand elongation and inactivate any remaining enzyme.
6. PCR products are analyzed by electrophoresing 10 µl of each reaction in a 1.5% agarose gel.

APPENDIX E

ALKALINE LYSIS REAGENTS

1. TSE
   25 mM Tris-HCl
   10 mM EDTA
   15% sucrose

2. lysozyme (10 mg/ml) in TSE

3. 5 M potassium acetate, pH 4.2

4. 100% ethanol

5. PCI
   phenol, chloroform, isoamyl alcohol (25 parts : 24 parts : 1 part)

6. NaOH, SDS
   0.2 N sodium hydroxide, 1% sodium dodecyl sulfate

7. CIA
   chloroform, isoamyl alcohol (24 parts : 1 part)

8. 70% ethanol

9. $T_{10}E_1$, pH 8.0 with RNase
   10 mM Tris-HCl, pH 8.0
   1 mM EDTA
   50 μg/ml RNase

APPENDIX F

ALKALINE LYSIS PROTOCOL

1. Pour 1.5 ml of overnight E. coli broth culture into a microfuge tube and centrifuge at 14,000 rpm for 1 minute.
2. Remove supernatent and resuspend bacterial pellet in 80 µl of cold TSE and 20 µl lysozyme solution.
3. Incubate tube at room temperature for 5 minutes.
4. Add 200 µl NaOH, SDS and invert rapidly for 10 seconds. Place tube on ice for 5 minutes.
5. Add 150 µl cold potassium acetate, and then vortex for 10 seconds. Place tube on ice for 5 minutes. Centrifuge at 14,000 rpm for 5 minutes at 4°C.
6. Transfer the supernatant to a sterile microfuge tube and add an equal volume of PCI. Vortex gently. Centrifuge at 14,000 rpm for 2 minutes at 4°C. Remove aqueous phase and place in sterile microfuge tube.
7. Repeat step 6 using CIA instead of PCI.
8. Add two volumes of room temperature 100% ethanol. Vortex gently and incubate at room temperature for 10 minutes.
9. Centrifuge at 14,000 rpm for 15 minutes. Remove supernatent and drain excess fluid away from plasmid pellet.
10. Wash pellet in 1.0 ml 70% ethanol, vortex, and centrifuge again.
11. Discard supernatent and dry pellet in speed vacuum.
12. Resuspend the purified plasmid pellet in 50 µl TE. Store at 4°C.

APPENDIX G

REAGENTS FOR SEQUENCING

1. 10 N sodium hydroxide
2. 100 mM EDTA
3. 3 M sodium acetate, pH 4.5-5.5
4. 95% ethanol
5. 70% ethanol
6. forward and reverse primers at 0.5-1.0 pmol
7. sterile distilled water
8. [α-^{35}S]-dATP at 1000-1500 Ci/mmol
9. Sequenase Version 2.0 DNA Sequencing Kit:
   5X Sequenase buffer
   Labeling mix (dGTP)
   ddG termination mix
   ddA termination mix
   ddT termination mix
   ddC termination mix
   Sequenase Version 2.0 enzyme
   enzyme dilution buffer
   0.1 M dithiothreitol (DTT)
   Stop Solution
10. Rainx (Unelko Corporation; Scottsdale, AZ)
11. 100% ethanol
12. 10% acetic acid
13. Bind-silane (Pharmacia LKB; Uppsala, Sweden)
14. Urea
15. 10X TBE
    1 M Tris-HCl
    0.83 M Boric acid
    10 mM EDTA, pH 7.5

16. acrylamide-bis solution
    38% acrylamide, 2% N, N'-bismethylene acrylamide
17. 10% (w/v) ammonium persulphate
18. TEMED
19. methanol
20. glacial acetic acid
21. Ultrapure water

APPENDIX H

SEQUENCING PROTOCOL

Day 1: Sequencing reactions

1. In two sterile microfuge tubes combine 3-5 μg of plasmid DNA and sterile water to yield a final volume of 18 μl.

2. Prepare an alkaline denaturing solution (0.2 N NaOH, 2.0 mM EDTA) by adding:
   200 μl 10 N NaOH
   20 μl 100 mM EDTA
   780 μl sterile distilled water

3. Add 2 μl of this denaturing solution to each 18 μl of plasmid DNA and incubate at 37°C for 30 minutes.

4. After denaturing the double stranded plasmid, neutralize the reaction with 2 μl 3 M sodium acetate, and then add 80 μl cold 95% ethanol to precipitate DNA. Incubate tubes at -70°C for at least 15 minutes.

5. Centrifuge the tubes at 14,000 rpm for 15 minutes at 4°C. Wash the pellet with 80 μl of 70% ethanol and repeat centrifugation.

6. Allow pellets to air dry for approximately 1 hour then resuspend each in 7 μl sterile distilled water.

7. Into each tube containing denatured plasmid (template) DNA add 2 μl 5X Sequenase buffer and 1 μl of the designated primer. Anneal the primers by incubating at 37°C for 30 minutes then cool to room temperature.

8. While annealing, thaw all radioactive materials.

9. Dilute enough labeling mix (dGTP) for two reactions by combining:
   0.9 μl mix
   3.6 μl sterile distilled water

10. Dilute enough Sequenase Version 2.0 enzyme for two reactions by combining:
    0.56 μl enzyme
    3.94 μl cold enzyme dilution buffer

11. Prepare termination tubes by aliquoting 2.5 µl of each termination mix into its designated tube. An entire set of termination tubes is required for each sequencing reaction. Prewarm these tubes at 37°C for at least 1 minute.

12. While working behind the radioactivity shield combine the following labeling reaction mixture on ice:
10.0 µl template DNA with annealed primer (from step 7)
1.0 µl dithiothreital
2.0 µl diluted labeling mix (dGTP)
0.5 µl [α-^{35}S]-dATP
2.0 µl diluted Sequenase Version 2.0 enzyme

13. Mix gently, centrifuge briefly, and incubate at room temperature for 2-5 minutes.

14. Remove 3.5 µl from the labeling reaction mixture and transfer to a pre-warmed termination tube. Repeat for all four termination mixes. Mix gently, centrifuge briefly, and incubate tubes at 37°C for 3-5 minutes.

15. Terminate all enzymatic activity by adding 4 µl Stop Solution to each tube. Store tubes at -20°C.

Day 1: Acrylamide Gel Preparation

1. Clean the gel plates and coat the thermostatic plate with Rainx. After Rainx dries, buff and apply a second coat. After drying of the second coat, remove excess Rainx by buffing with 95% ethanol.

2. Prepare the Bind-silane mixture by combining:
12.5 ml 100% ethanol
375 µl 10% acetic acid
40 µl Bind-silane

Apply one coat of this mixture to the notched plate, allow it to dry and then buff with 95% ethanol.

3. Assemble the gel apparatus by positioning the plates, spacers, and clips on the gel stage.

4. Prepare a 6% DNA sequencing gel by combining:
   21.0 gm urea
   5.0 ml 10X TBE
   20.0 ml Ultrapure water
   7.5 ml acrylamide-bis solution.

   Bring this mixture to a final volume of 50.0 ml with ultrapure water. Stir to dissolve all urea and filter to remove any particulate material.

5. Add 0.4 ml 10% ammonium persulphate and 40.0 µl TEMED to the gel solution, swirl and pour. Position the comb, clamp, and bullclips. Allow the gel to polymerize overnight.

Day 2

1. Position the gel assembly in the electrophoresis unit (Pharmacia LKB; Uppsala, Sweden) and fill the buffer chambers with 1X TBE. Remove the comb and pre-electrophorese at 2,000 V for 15 minutes at 55°C.

2. While pre-electrophoresing, denature the samples by incubating at 80°C for 5 minutes. Quench tubes on ice and centrifuge briefly.

3. Flush all wells with 1X TBE to remove any excess urea then load 2 µl of each sample into its designated well. Electrophorese at 55°C for 3-4 hours at 2,100 V for long runs. Stop electrophoresis, load 2 µl of each sample into additional designated wells and electrophorese at 55°C for 1-2 hours at 2,100 V for short runs.

4. Disassemble the electrophoresis unit and separate the gel plates so the gel adheres to the knotched plate and the thermostatic plate is removed. Wash the gel in 10% methanol, 10% glacial acetic acid for 45 minutes to remove urea. Dry

the gel for approximately 1 hour and place in X-ray cassette with film. Allow cassette to remain at room temperature overnight.

Day 3

1. Remove film from cassette and process in autoprocessor.

APPENDIX I

REAGENTS FOR RADIOLABELING

1. DTM
   0.1 mM dCTP, dGTP, and dTTP
   0.25 M Tris-HCl, pH 8.0
   25 mM $MgCl_2$
   50 mM dithiothreitol

2. OL
   1 mM Tris-HCl
   1 mM ethylenediaminetetraacetate, pH 7.5 (EDTA)
   90 OD units of hexamer oligonucleotide primers (Pharmacia; Piscataway, NJ)

3. LS
   0.278 M HEPES, pH 6.6
   0.56 mg/ml Bovine Serum Albumin (acetylated/nuclease-free)
   7.8% OL solution
   27.8% DTM solution

4. [α-^{32}P]-dATP at 3000 Ci/mmol (DuPont NEN; Wilmington, DE)

5. $T_{10}E_1$, pH 8.0
   10 mM Tris-HCl
   1 mM ethylenediaminetetraacetate

6. Klenow fragments at 10U/µl (DNA Polymerase I; Gibco BRL; Gaithersburg, MD)

7. scintillation cocktail (Scinti Verse II; Fisher Scientific Company; Fair Lawn, NJ)

8. sterile distilled water

APPENDIX J

RADIOLABELING PROTOCOL

Day 1

1. Thaw LS solution and radioactive materials.
2. In a sterile microfuge tube combine 200 ng cDNA and sterile water to yield a final volume of 6 µl.
3. Denature cDNA by boiling for 10 minutes. Quench denatured cDNA on ice for approximately 5 minutes.
4. Briefly centrifuge to collect all contents at the bottom of the tube.
5. Add the following:
   10 µl [α-^{32}P]-dATP
   18 µl LS solution
   2 µl Klenow fragments
6. Mix gently and centrifuge briefly.
7. Incubate overnight at room temperature.

Day 2

1. Rinse a Sephadex G-50 Nick column (Pharmacia LKB Biotechnology; Uppsala, Sweden) with 1.0 ml TE buffer then equilibrate the column with 3.0 ml TE buffer.
2. Add the radiolabeled sample to the column and allow solution to enter the gel bed.
3. Elute the sample with 400 µl TE buffer. Repeat three times.
4. Determine the counts per minute by liquid scintillation counting. Combine 2 µl label, 300 µl sterile water, and 3.0 ml scintillation cocktail and count for one minute.

APPENDIX K

REAGENTS FOR TRANSFORMATION, PLATING, AND SCREENING OF XL-1 BLUE CELLS WITH λZAP II PHAGE

1. Luria broth (1 liter)
   10 gm tryptone
   5.0 gm yeast extract
   5.0 gm sodium chloride
   1.0 ml 1 N sodium hydroxide

2. TB broth (1 liter)
   10 gm tryptone
   5 gm sodium chloride

3. 2.0% maltose

4. $MgSO_4$
   1 M magnesium sulfate

5. LB agar with tetracycline (1 liter)
   Luria broth
   15 gm agar
   12.0 μg/ml tetracycline

6. NZY broth (1 liter)
   5 gm sodium chloride
   2 gm $MgSO_4$-H_2O
   5 gm yeast extract
   10 gm NZ Amine

7. NZY agar (1 liter)
   NZY broth
   1.5% agar

8. NZY top agar (1 liter)
   NZY broth
   0.7% agarose

APPENDIX L

BACTERIAL TRANSFORMATION AND PLATING PROTOCOL

Day 1

1. Streak XL-1 Blue strain of E. coli from frozen glycerol stocks on an LB agar plate containing tetracycline.
2. Incubate plates at 37°C for 12-16 hours.

Day 2

1. Remove plate from incubator and select a well isolated colony to inoculate 3.0 ml TB broth supplemented with 0.02% maltose and 10 mM $MgSO_4$.
2. Shake vigorously overnight at 37°C.

Day 3

1. Centrifuge cells at 1,000 x g for 5 minutes.
2. Gently resuspend the bacterial pellet in 1.5 ml 10 mM $MgSO_4$.
3. Mix 100 µl cells with 900 µl sterile water and read A_{600} to determine the cell concentration and then calculate the dilution necessary to give a bacterial stock of $A_{600} = 0.5$ cells.
4. Label tubes and perform 10-fold dilutions of bacteriophage library sufficient to allow counting of individual plaques (<1000 plaque forming units (pfu)/plate). Assuming there are ~10^{12} pfu/ml of stock.
5. Combined 200 µl of diluted cells with 20 µl of each lambda bacteriophage library dilution in a sterile 100 x 17 disposable tube. Two tubes were used for each bacteriophage dilution (1:100 to 1:10,000).
6. Shake cells and phage at 37°C for 15 minutes.

7. Add 3.5 ml 50°C NZY top agar to each tube, quickly swirl and pour onto the designated dry NZY agar plate (82 mm).
8. Place plates upright with the lids opened in 37°C for 30 minutes then close the lids and invert the plates.
9. Incubate at 37°C until plaques are visible. Place at 4°C.

Day 4

1. Count plaques and calculate bacteriophage titer.
2. Grow a fresh overnight 3.0 ml liquid culture of XL-1 Blue strain of E. coli as described previously.

Day 5

1. Obtain enough bacterial stock with $A_{600} = 0.5$ cells to have 600 µl per plate (150mm).
2. In a 100 x 17 disposable tube, combine 600 µl of diluted cells with 20 µl of lambda bacteriophage library. The plaque forming units (pfu) titer of the lambda bacteriophage library previously determined is used to determine the proper dilution to give ~10,000 pfu/20 µl.
3. Shake cells and phage at 37°C for 15 minutes.
4. Add 6.5 ml 50°C NZY top agar to each tube, quickly swirl and pour onto the designated dry NZY agar plate (150 mm).
5. Place plates upright with the lids opened in 37°C for 30 minutes then close the lids and invert the plates.
6. Incubate at 37°C until plaques are visible. Place at 4°C.

APPENDIX M

REAGENTS FOR PLAQUE LIFTS

1. 2X SSC
   0.3 M sodium chloride
   0.03 M sodium citrate, pH 7.4

2. Lysis buffer
   0.2 N sodium hydroxide
   1.5 N sodium chloride

3. Neutralization buffer
   0.4 M Tris-HCl, pH 7.6
   2X SSC

4. nitrocellulose membranes (Schleicher and Schuell; Keene, NH)
   82mm and 137 mm

5. 3MM filter paper (Whatman; Maidstone, England)

APPENDIX N

PLAQUE LIFT AND LYSIS PROTOCOL

1. Label two sets of nitrocellulose membranes for each plate.
2. After holding the plates at 4°C for at least 2 hours, slowly apply the first set of membranes to the surface of each plate and allow them set for 2 minutes.
3. To facilitate realignment at a later time, pierce the nitrocellulose membrane and agar three times in an asymmetric pattern with a needle containing India ink.
4. Gently lift the membrane from the plate and place on 3MM filter paper to air dry for 10 minutes.
5. Apply the second set of membranes to the plates and allow these to set for 4 minutes. Again, mark membranes and agar with ink.
6. Prepare for bacteriophage lysis by placing 3 sheets of 3MM filter paper on the bench top (covered) and saturating them with lysis buffer, neutralization buffer, and 2X SSC. After all the membranes have dried, place each membrane with the phage side facing up on the lysis buffer. Incubate for 2 minutes.
7. Promptly remove membranes from lysis buffer and place on the neutralization buffer for 2 minutes.
8. Finally, place neutralized membranes on the 2X SSC in order to assure complete neutralization of the nitrocellulose membranes.
9. Layer membranes between paper towels and bake at 80°C for 2 hours.

APPENDIX O

REAGENTS FOR HYBRIDIZATION OF PLAQUE LIFTS

1. prehybridization buffer
   50% formamide
   5X Denhardt's solution
   5X SSC
   100 μg/ml salmon testes DNA

2. low stringency solution
   2X SSC
   0.1% SDS

3. high stringency solution
   0.2X SSC
   0.1% SDS

APPENDIX P

PLAQUE LIFT HYBRIDIZATION PROTOCOL

Day 1

1. Place nitrocellulose membranes and prehybridization buffer into plastic bag and seal.
2. Denature salmon testes DNA by incubating in a boiling water bath for 10 minutes following by 5 minutes on ice.
3. Add denatured salmon testes DNA to the prehybridization buffer.
4. Prehybridize the membranes by shaking in a 42°C waterbath for at least 1 hour.
5. Denature the radiolabeled probe as in step 2 and add to the membranes.
6. Hybridize the membranes by shaking overnight in the 42°C waterbath.

Day 2

1. Remove hybridization solution from the bag and place membranes in a plastic container.
2. Add 500 mls low stringency solution and shake for 15 minutes at room temperature. Repeat twice.
3. Pre-heat high stringency solution to 65°C.
4. Separate the membranes with forceps and add 500 ml pre-heated high stringency solution. Shake at 65°C for 5 minutes.
5. Wash again with high stringency solution at 65°C for 20 minutes.
6. Remove excess solution from membranes by blotting on 3MM filter paper. Individually wrap membranes in plastic.
7. In the darkroom, tape membranes to film and puncture holes through both. Position the film and membranes between intensity screens in the cassette and place the cassette at -70°C for 3 days.

Day 5

1. Remove cassettes from freezer and allow them to thaw for 20 minutes. Develop film in an autoprocessor.

APPENDIX Q

REAGENTS FOR ISOLATING SELECTED BACTERIOPHAGE

1. chloroform

2. SM buffer (1 liter)
   5.8 gm sodium chloride
   2.0 gm $MgSO_4$
   50.0 ml 1M Tris-HCl
   5.0 ml 2% gelatin

3. NZY agar
   refer to Appendix K

4. LB agar with 50 µg/ml ampicillin
   refer to Appendix K

5. Luria broth
   refer to Appendix K

APPENDIX R

ISOLATION OF SELECTED PHAGE

Day 1

1. Once a positive clone has been identified on the original plates, core the plaque and place in a sterile microfuge tube.
2. To this tube add 500 µl SM buffer and 20 µl chloroform.
3. Vortex the tube and incubate at 4°C overnight to release the phage from the agar . This solution will contain the lambda bacteriophage stock.
4. Also prepare 3.0 ml XL-1 Blue liquid cultures as described in Appendix L.

Day 2

1. Determine XL-1 Blue cell concentration and adjust to yield a bacterial stock of A_{600} = 0.5 cells. Perform several 10-fold dilutions of the lambda bacteriophage stock (1:10 to 1:1000).
2. Label microfuge tubes. Combine 200 µl bacterial stock with 20 µl diluted phage. Shake at 37°C for 15 minutes.
3. Add 3.5 ml NZY top agar to each tube, swirl and pour on the surface of dry LB agar plate (82 mm).
4. Incubate plates in upright position with lids ajar at 37°C for 30 minutes then close lids and invert plates. Incubate an additional 8-16 hours until plaques are visible.
5. Count plaques and calculated the bacteriophage titer.
6. If necessary, repeat steps 1-5 to obtain ~50 pfu/plate.
7. Refer to appendices M, N, O, and P in order to conduct plaque lifts and lysis as well as hybridization of the phage membranes; these procedures will conclude the primary screening of the selected bacteriophage clone. Secondary and any

additional screenings of this bacteriophage clone will require repeating steps 1-6 of this protocol as well as the protocols provided in appendices M, N, O, and P.

8. Once the bacteriophage clone of interest has been isolated, prepare liquid cultures of XL-1 Blue and SOLR strains of E. coli by inoculating 3.0 ml Luria broth supplemented with 0.2% maltose and 10 mM MgSO4. Shake at 30°C overnight.

Day 3

1. Determine bacterial cell concentrations by reading A_{600} and prepare bacterial stock solutions for XL-1 Blue of $A_{600} = 0.5$ cells and SOLR of $A_{600} = 1.0$ cells.
2. In order to obtain a purified stock of the clone of interest conduct the *in vivo* excision protocol (ExAssist/SOLR System; Stratagene Cloning Systems; La Jolla, CA).. This procedure will recover the bacteriophage clones as corresponding pBluescript phagemids.

APPENDIX S

REAGENTS FOR RNA ANALYSIS

1. 10X formaldehyde gel running buffer(pH7.0)
   0.2 M 3,N-morpholinopropanesulfonic acid (MOPS)
   50 mM sodium acetate
   10 mM EDTA

2. sample buffer in DEPC-treated water
   1X formaldehyde gel-running buffer
   50% formamide
   2.2 M formaldehyde
   0.2% ethidium bromide

3. loading buffer in DEPC-treated water
   1 mM EDTA, pH 8.0
   0.25% bromophenol blue
   0.25% xylene cyanol
   50% glycerol

4. agarose gel in DEPC-treated water
   1.2% agarose (electrophoresis grade)
   1X formaldehyde gel running buffer
   2.2 M formaldehyde

5. 20X SSC
   3 M sodium chloride
   0.3 M sodium citrate, pH 7.4

6. 10% (w/v) sodium dodecyl sulfate (SDS) in DEPC-treated water

7. nylon membrane (Hybond; Amersham International plc; Amersham, UK)

8. RNA size markers (Gibco BRL; Gaithersburg, MD)

9. 50X Denhardt's solution
   25 μM Ficol
   0.25 mM Polyvinylpyrrolidone
   10 mg/ml Bovine Serum Albumin (Acetylase/Nuclease-free)

10. prehybridization buffer
    5X SSC
    5X Denhardt's solution
    0.5% SDS

50% formamide
0.5 mg salmon testes DNA (Sigma Molecular Biology; St. Louis, MO)

11. hybridization buffer
prehybridization buffer
[α-^{32}P]-labeled probe

12. 2X SSC, 0.1% SDS

13. 1XSSC, 0.1% SDS

14. 0.1X SSC, 0.1% SDS

15. slot blot denaturing solution
5 parts formamide : 1.63 parts formaldehyde : 1 part formaldehyde gel running buffer

16. 3MM filter paper (Whatman; Maidstone, England)

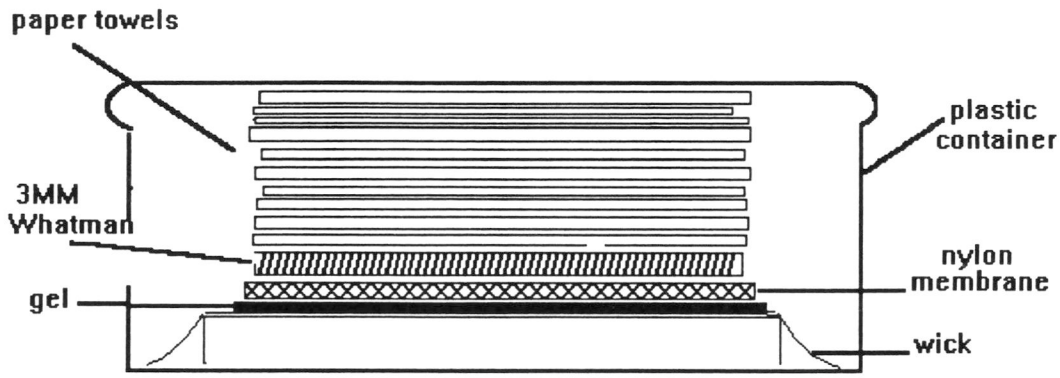
APPENDIX T

NORTHERN ANALYSIS PROTOCOL

Day 1

1. Prepare the gel by microwaving agarose and water on defrost setting until all agarose melts. Allow the agarose solution to cool for approximately 5 minutes.
2. In the fume hood add the remaining ingredients, mix by swirling, and pour into the casting tray. Insert the comb and allow gel to harden for 1 hour.
3. Prepare the RNA samples by combining 10 μg of total RNA with 3.4 volumes sample buffer in a microfuge tube. Mix gently and incubate at 55°C for 30 minutes.
4. While denaturing the RNA samples, remove gel from the casting tray and fill electrophoresis unit with 1X formaldehyde gel running buffer. Submerge gel in buffer and remove comb.
5. Quench denatured RNA on ice for 5 minutes. Add enough loading buffer to equal 1/5 the total volume, mix, centrifuge briefly, and load samples into assigned wells.
6. Promptly close the electrophoresis unit and run at 25 V until bromophenol blue has migrated approximately 3/4 the length of the gel.
7. Remove gel from electrophoresis unit. Soak in copious amounts of DEPC-treated water followed by soaking in 10X SSC for 45 minutes to remove excess formaldehyde and ethidium bromide.
8. Place gel on surface of UV light box, align fluorescent ruler, and photograph.
9. Cut one sheet of nylon membrane to the dimensions of the gel and soak in 20X SSC for 10 minutes. Meanwhile, cut three sheets of 3MM filter paper and enough paper towels to yield a three inch stack; dampen one of these 3MM filter paper strips in 20X SSC. Set up blotting apparatus as in diagram below, add enough

20X SSC to generously cover the bottom of the container, and blot overnight. Be sure to remove any air bubbles as this will interfere with proper transfer of nucleic acids.



Day 2

1. Disassemble the blotting apparatus a few layers at a time. Keeping the nylon membrane and gel together mark the locations of the wells with a pencil. Separate the nylon membrane from the gel and crosslink the RNA to the membrane by exposing to UV light (UV Stratalinker 1800; Stratagene; La Jolla, CA).
2. Preheat the hybridization oven (American Synthesis Inc.; Pleasanton, CA) and prehybridization buffer to 42°C. Meanwhile, cut a piece of mesh slightly larger than the nylon membrane and dampen both with 2X SSC. Place both inside the hybridization bottle.
3. Add 0.50 ml denatured salmon testes DNA and 15 ml prewarmed prehybridization buffer to the bottle containing the membrane and mesh. Incubate at 42°C for at least 1 hour.

4. After prehybridizing, denature the radiolabeled probe by incubating in boiling water for 10 minutes, and then placing on ice for 5 minutes. To the prehybridization buffer, add enough probe to yield 1.5×10^7 cpm. Hybridize overnight at 42°C.

Day 3

1. In the morning remove the hybridization buffer from bottle. Wash the membrane with 2X SSC, 0.1% SDS at room temperature for 15 minutes. Remove washing solution and repeat.
2. Wash with 1X SSC, 0.1% SDS at 65°C for 15 minutes.
3. Wash with 0.1X SSC, 0.1% SDS at 65°C for 10 minutes.
4. Place membrane on sheet of 3MM filter paper to dry briefly. Wrap membrane in plastic.
5. In darkroom make the following sandwich in an X-ray cassette: intensity screen, wrapped membrane, film, and intensity screen. Close cassette and place in -70°C.

Day 4

1. Remove cassette from freezer and allow it to thaw for 20 minutes. Process film in autoprocessor

APPENDIX U

SLOT BLOT ANALYSIS OF TOTAL RNA

1. Combine 7 μg total RNA and enough water to equal 50 μl in a microfuge tube.
2. Add 150 μl of slot blot denaturing solution and mix well.
3. Place tubes containing samples in a 65°C waterbath for 5 minutes.
4. Briefly centrifuge tubes to bring down all condensation, add 200 μl cold 20X SSC, and place on ice.
5. Set up slot blot apparatus according to manufacturer's instructions and label wells to be used.
6. Load each sample into the assigned well and watch for liquid to be drawn through the nylon membrane.
7. Load 200 μl 6X SSC into each sample well.
8. After liquid has been removed from all sample wells, take apparatus apart, label and remove nylon membrane.
9. Crosslink RNA to membrane (UV Stratalinker 1800; Stratagene; La Jolla, CA).
10. Hybridize membrane as for Northern analysis (Appendix T).

APPENDIX V

REAGENTS FOR IMMUNOCYTOCHEMISTRY

1. 1X phosphate-buffered saline (PBS)
   3 mM sodium phosphate, pH 7.4
   0.72% saline

2. normal blocking serum
   150 µl normal goat serum
   10 ml 1X PBS

3. biotinylated antibody solution
   150 µl normal goat serum
   10 ml 1X PBS
   50 µl biotinylated antibody stock (Vector Labs; Burlingame, CA)

4. antibody diluent
   100 µl normal goat serum
   10 ml 1X PBS

5. primary antibody (mPRP)
   mPRP antisera diluted 1:5000 in diluent

6. normal rabbit serum (control)
   normal rabbit serum diluted 1:5000 in diluent

7. ABC reagent (Vector Labs; Burlingame, CA)
   100 µl solution A
   100 µl solution B
   10 ml 1X PBS

8. substrate
   Combine 20 ml each of 0.2% hydrogen peroxide and 0.1% diaminobenzidine tetrachloride (Sigma; St. Louis, MO) in 0.1M Tris, pH 7.2

9. histology reagents
   Hemo-de
   100% ethanol
   95% ethanol
   70% ethanol
   50% ethanol

APPENDIX W

IMMUNOCYTOCHEMISTRY PROTOCOL

Day 1

1. Deparaffinize and hydrate sections by incubation of slides in the following solutions:

Hemo-de	2X 5 min.
100% ethanol	3 min.
95% ethanol	3 min.
70% ethanol	3 min.
50% ethanol	3 min.
water	5 min.

2. Transfer slides to a coplin jar containing water. Incubate for 6 min. changing the water once.
3. To eliminate endogenous peroxidase activity incubate sections for 30 min. in 0.3% hydrogen peroxide in methanol.
4. Rinse slides 3X 3 min. in PBS in coplin jar.
5. Treat sections dropwise for 20 min. with normal blocking serum.
6. Incubate sections dropwise overnight with primary antibody at 4C. Control sections are incubated with normal rabbit serum.

Day 2

7. Wash slides 3X 3 min. in PBS in coplin jar.
8. Incubate sections dropwise for 30 min. in biotinylated antibody solution.
9. Prepare ABC reagent and allow to stand for 30 min. before use.
10. Wash slides in PBS 3X 3 min. in coplin jar.
11. Treat sections dropwise for 30 min. with ABC reagent.
12. Rinse slides 3X 3 min. in PBS in coplin jar.
13. Treat slides in coplin jar with freshly made substrate for 8 min..

14. Rinse slides in water 2X 3 min. in coplin jar.

15. To prepare slides for mounting, immerse in the following solutions:

50% ethanol	dip
70% ethanol	dip
95% ethanol	dip
100% ethanol	2X 2 min.
Hemo-de	2X 2 min.

16. Coverslip slides with Permount (Fischer; Fair Lawn, NJ).