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Trophoblastic Giant Cells of the Mouse Placenta as the Site of Proliferin Synthesis*

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ABSTRACT. Proliferin (PLF) is a PRL-related glycoprotein secreted by a number of mouse cell lines and by minced mouse placenta. To further investigate the hormone-like characteristics of PLF, we have determined the site of PLF synthesis and storage in the placenta and its concentration in blood serum during pregnancy. By immunohistochemical staining and *in situ* hybridization PLF protein and messenger RNA (mRNA) were localized to the trophoblastic giant cells. Individual cells contained both PLF and placental lactogen II. Trophoblastic giant cells appear to secrete PLF into the circulation since PLF was found at levels up to 5 μg/ml in the serum of pregnant mice by RIA and at somewhat lower concentrations in the amniotic fluid. Moreover, the serum concentration of PLF during pregnancy varied directly with the level of PLF mRNA in the placenta and with the number of placentas per animal. These findings are consistent with the hypothesis that PLF is a placental hormone; its function is not known. (*Endocrinology* **122:** 1761–1768, 1988)

PROLIFERIN (PLF) is a PRL-related glycoprotein secreted by a number of proliferating mouse cell lines and by mouse placenta during mid and late gestation (1–3). We have hypothesized that PLF may be an autocrine growth factor for cells in culture and a placental hormone that may regulate the growth or differentiation of fetal or maternal tissues during pregnancy. Here we report two additional hormone-like characteristics of placental PLF. First, by immunohistochemical staining of PLF protein and *in situ* hybridization to PLF messenger RNA (mRNA), the site of PLF synthesis and storage has been localized to the trophoblastic giant cells of the placenta (4, 5); and second, by RIA, PLF has been found in the serum of pregnant mice and in amniotic fluid. Murine trophoblastic giant cells are known to contain another PRL-related hormone, murine placental lactogen II (mPL II) (6). We find that individual giant cells contain both mPL II and PLF.

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Materials and Methods

Analysis of proteins

PLF was purified from the conditioned medium of a constructed Chinese hamster ovary (CHO) cell line carrying amplified copies of mouse PLF complementary DNA (cDNA) (7). Except for the immunohistochemical experiments (see below), the anti-PLF serum used was prepared against recombinant PLF expressed in bacteria as described previously (3). Mouse (m)PRL, GH, and PL II were purified as described previously (8–10). Proteins were electrophoresed in 12% sodium dodecyl sulfate-polyacrylamide gels (11) and either stained with silver (12) or transferred to nitrocellulose and bound to antibody and [^{125}I]iodoprotein A (New England Nuclear, Boston, MA) as described (13).

Immunohistochemistry

BALB/c female mice (Charles River Breeding Laboratories, Wilmington, MA) were checked for the presence of a vaginal plug (designated as day 0) to obtain timed pregnancies. Placentas at day 10, 12, and 14 of gestation were removed, fixed in Carnoy's solution (4 C) for 24 h, and embedded in paraffin. Five micron sections were prepared and mounted on chrome alum gelatin-coated slides. Sections were deparaffinized and stained using the Vectastain ABC kit (Vector, Burlingame, CA)

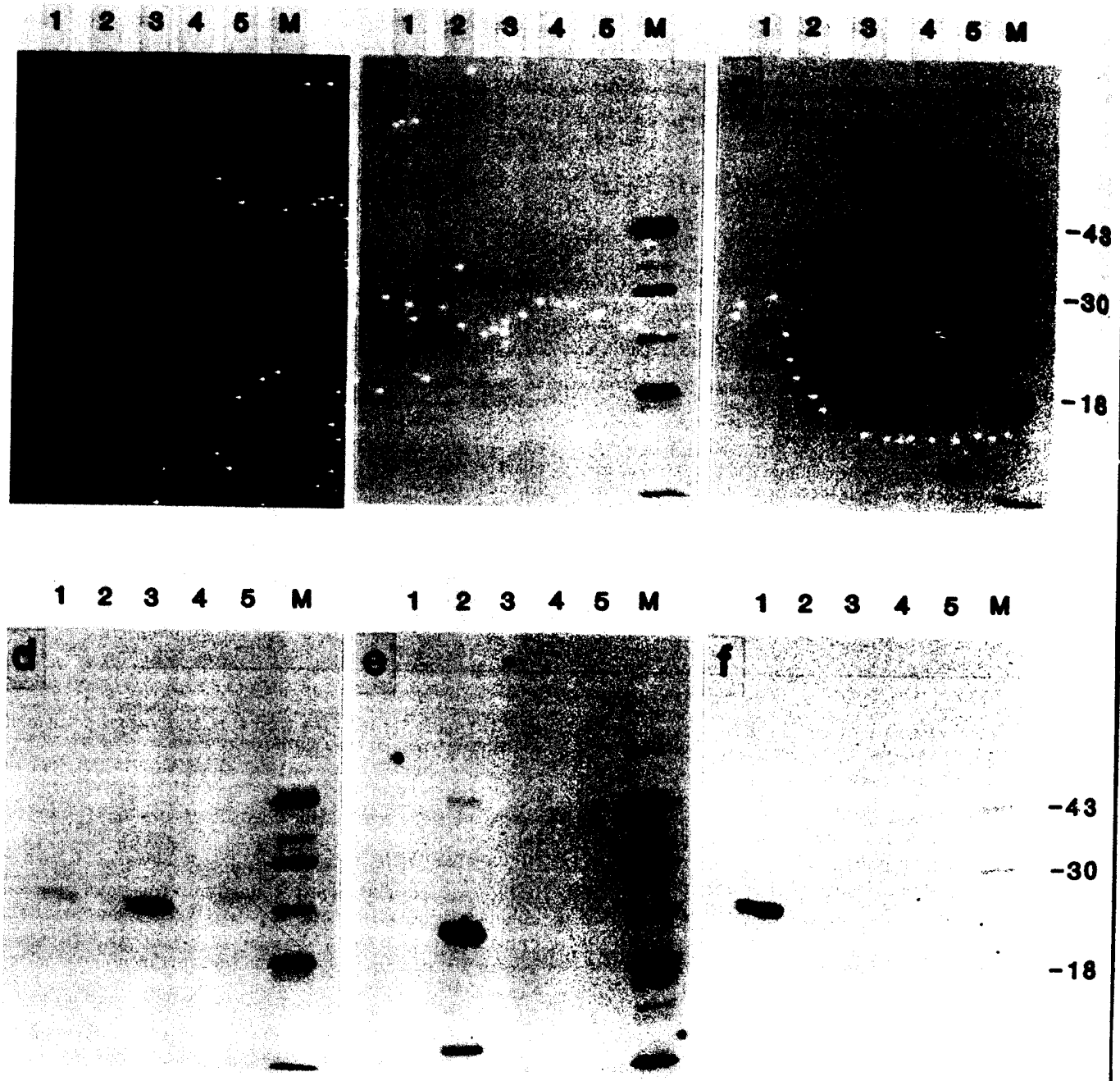


FIG. 1. Immunological characterization of PLF. Purified mPL II (lanes 1), mGH (lanes 2), mPRL (lanes 3), nonglycosylated PLF from tunicamycin-treated cells (lanes 4), and glycosylated PLF (lanes 5) were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels and silver-stained (a) or transferred to nitrocellulose and bound to nonimmune (b), anti-PLF (c), anti-mPRL (d), anti-mGH (e), or anti-mPL II (f) serum and [^{125}I] iodoprotein A. M, Molecular weight standards of 43 K (ovalbumin), 30 K (carbonic anhydrase), and 18 K (lactoglobulin). In panel f, the positions of the molecular weight standards were obtained from a longer exposure of the filter.

and diaminobenzidine tetrahydrochloride (Polysciences, Warrington, PA) according to the directions provided by the manufacturer. Primary antisera were used at 1:500 dilution; for the experiments in which antisera were preabsorbed with antigen, purified PLF, mPRL, and mPL II were used at 1 μg/ml. For these localization experiments, we used an anti-PLF serum prepared by immunizing rabbits with PLF purified from the overproducing CHO cell line (7). PLF was deglycosylated with trifluoromethanesulfonic acid (7) before immunization to ensure that antibodies would not be directed against carbohydrates.

In situ hybridization

PLF cDNA was cloned in both orientations into the Bam H site of pGEM-2 (Promega Biotec, Madison, WI). The plasmids

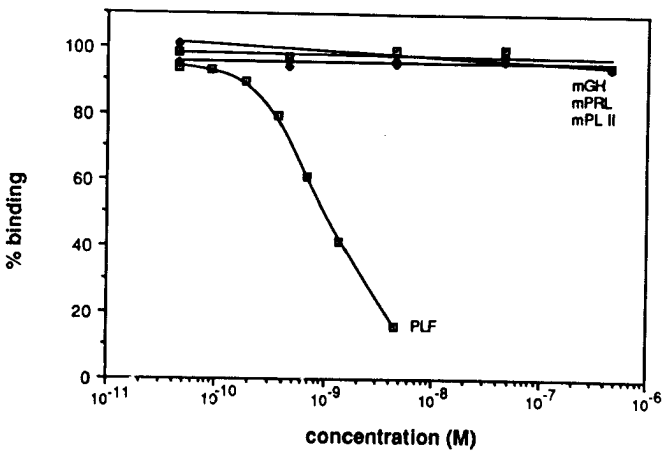
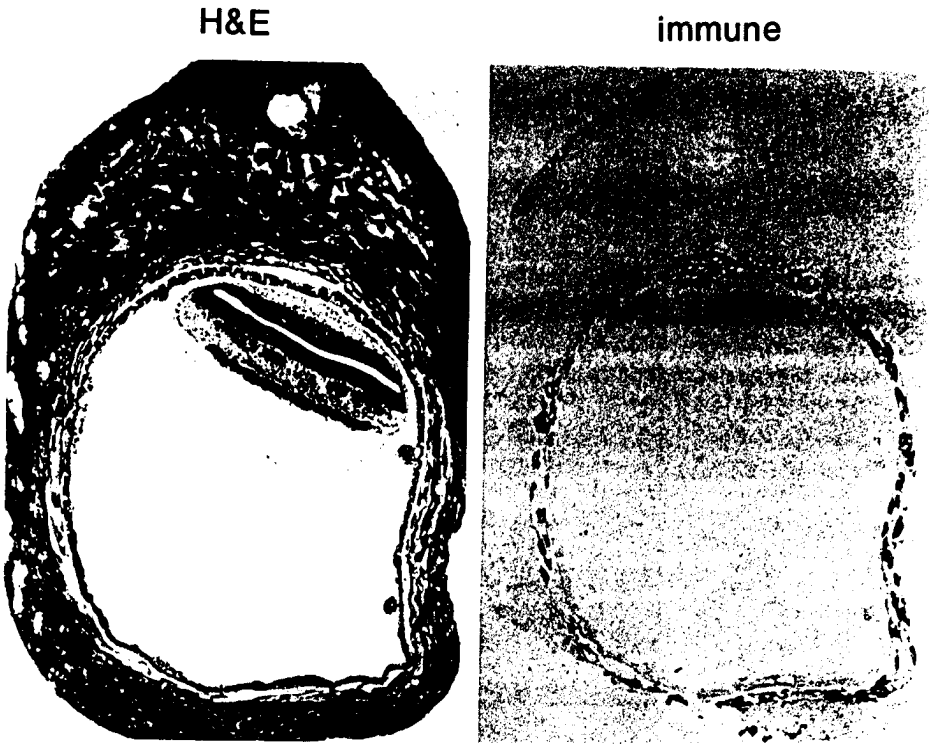


FIG. 2. RIA for PLF. [^{125}I]PLF was incubated with anti-PLF serum in the presence of various concentrations of PLF, mGH, mPRL, or mPL II, and the amount of [^{125}I]PLF bound was determined, as described in *Materials and Methods*. The amount bound in the absence of unlabeled protein was taken as 100%. Each point represents the average of duplicate measurements.



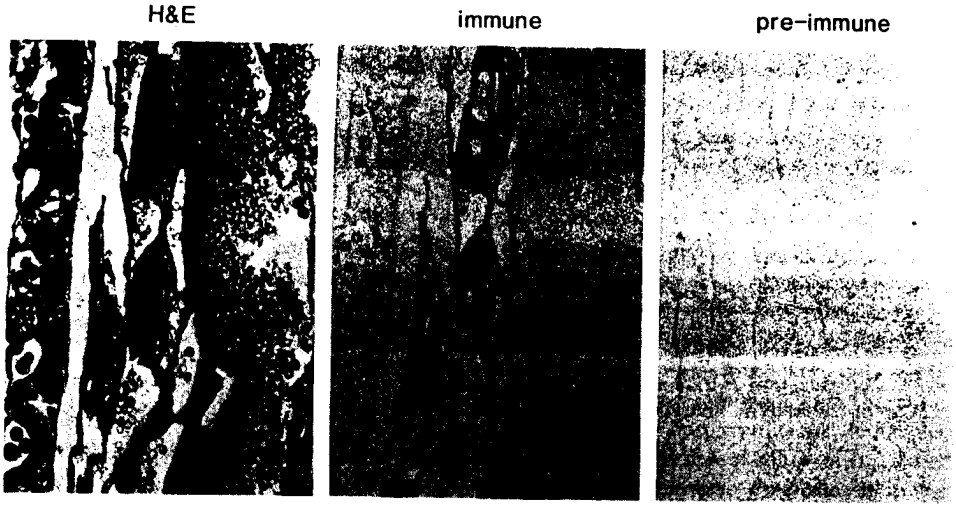


FIG. 3. Immunohistochemical staining of PLF in placenta. Adjacent sections of a 10 day placenta were stained with hematoxylin and eosin (H and E) or bound to preimmune or anti-PLF (immune) serum. Bound antibody was detected using the Vectastain ABC method. *Top*, Low power; *bottom*, high power.

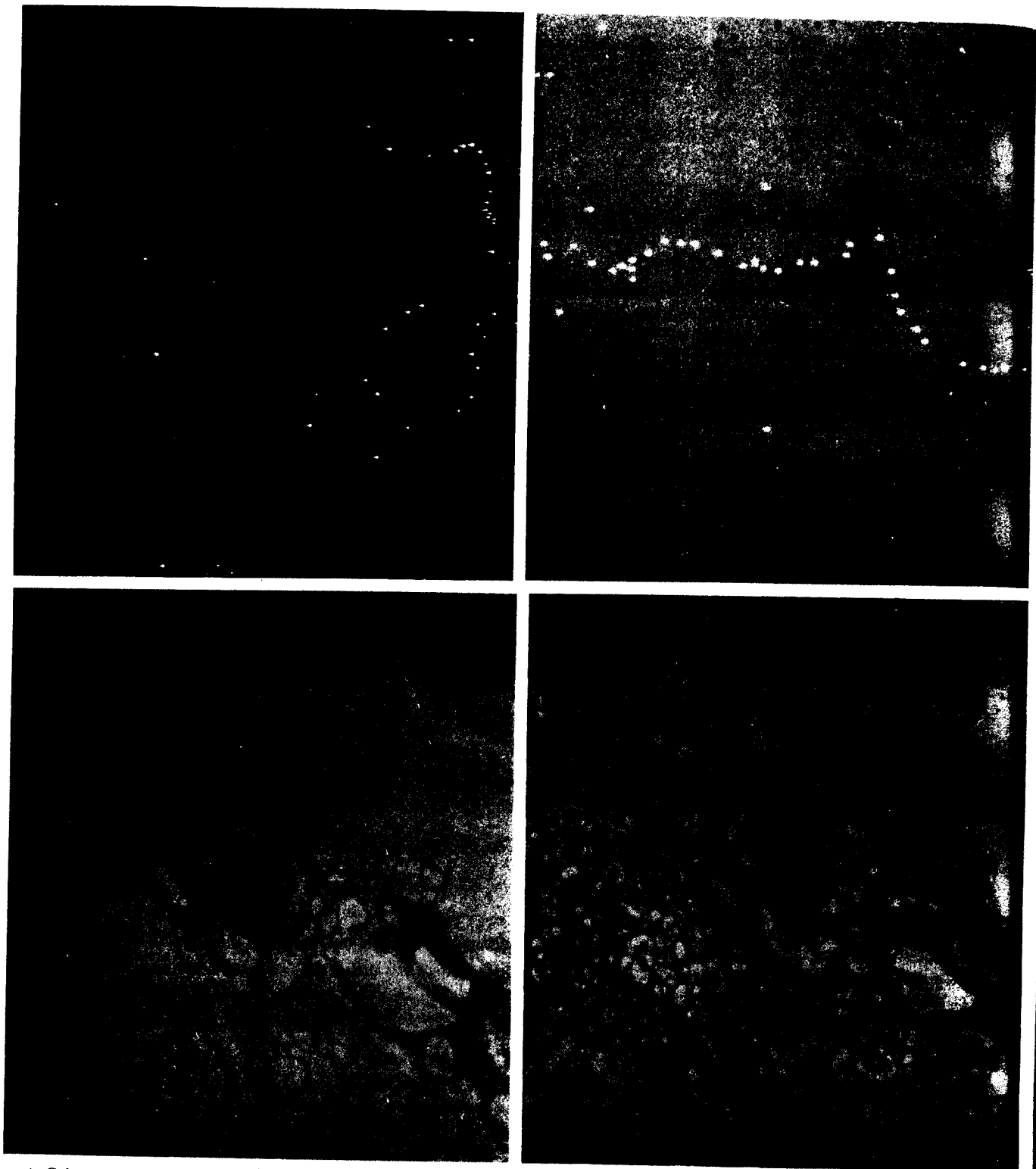


FIG. 4. Colocalization of PLF and mPL II to trophoblastic giant cells. Adjacent sections of a 12 day placenta were stained with hematoxylin and eosin (a) or bound to nonimmune (b), anti-PLF (c), or anti-mPL II (d) serum. Bound antibody was detected using the Vectastain ABC method.

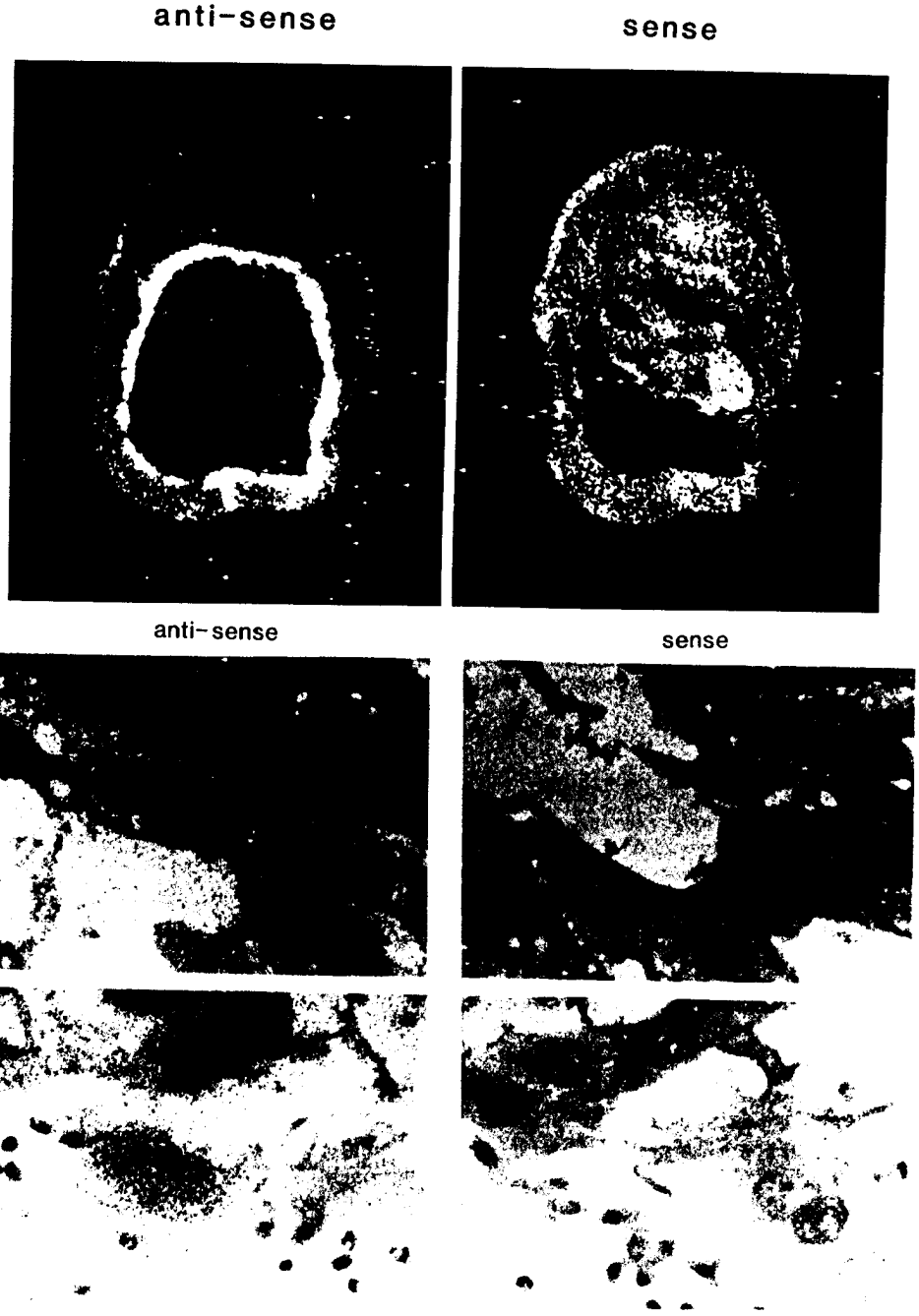


FIG. 5. *In situ* hybridization to PLF mRNA in placenta. Sections of a 10 day placenta were hybridized to either an antisense or sense PLF probe and (*top*) exposed to Amersham β max hyperfilm, which was then used as a negative to obtain the low power images shown, or (*bottom*) exposed to NTB-2 Kodak emulsion to obtain high power images. In the high power images, two areas from one slide hybridized with an antisense probe are shown on the *left*, and two comparable areas from one slide hybridized with a control sense probe are shown on the *right*.

toxylin and
method.

were linearized with Eco RI, and run-off transcripts were prepared using T7 RNA polymerase and α [^{35}S]thio-UTP (New England Nuclear) according to the directions provided by Promega Biotec. *In situ* hybridization using these probes was carried out as described (14) on placental sections prepared as above.

RIA

Purified PLF (7) was radioiodinated using the iodogen reagent (Pierce Chemical Co., Rockford, IL) to 50–150 μCi/μg. [^{125}I]Iodo-PLF (15,000 cpm) was incubated with nonimmune rabbit immunoglobulin G (2 μl of an ammonium sulfate fraction) and anti-PLF serum (1:3000 dilution) in 100 μl PBS containing 0.1% Triton X-100 and 1 mg/ml BSA at room temperature for 24 h. Goat antirabbit immunoglobulin G (Research Products International, Mt. Prospect, IL) was added (1:37.5 dilution) to a final reaction volume of 150 μl, and the mixture was incubated for an additional 30 min. The mixture was diluted to 1.5 ml, the immune complexes were pelleted by centrifugation, the supernatant was removed, and the pellet was counted in a γ-counter. Under these conditions, 3000–4000 cpm were recovered in the pellet in the absence of unlabeled competitor, and less than 400 cpm were precipitated in the

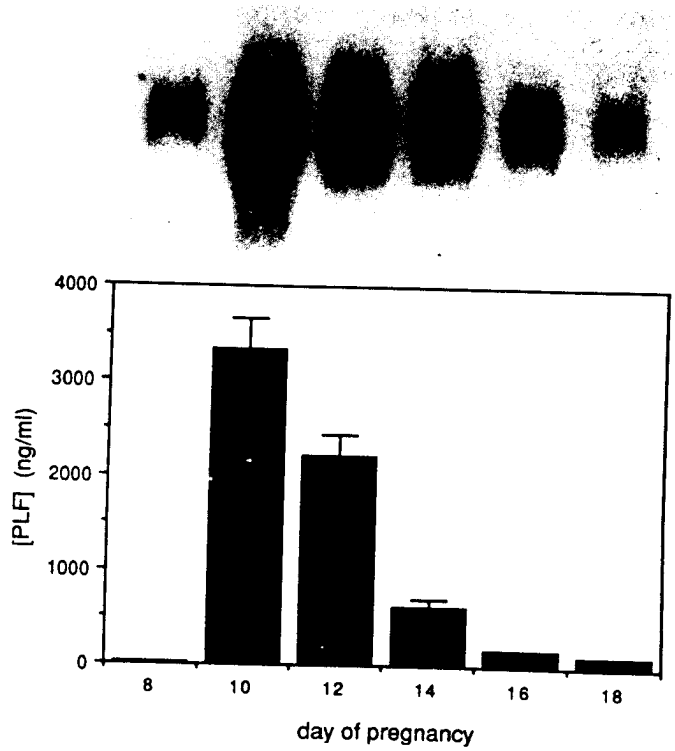


FIG. 6. Correlation between levels of serum PLF and placental PLF mRNA during pregnancy. Serum PLF concentrations were measured at various days of gestation by RIA. For a given time point, serum samples from each of two mice were assayed in duplicate. The *solid bar* represents the average value for the four samples. The small bar indicates the maximal deviation from the average. All measurements were obtained from a single set of RIAs (that is, with respect to a single standard curve). *Above the bar graph* are the results of electrophoresing 20 μg total RNA from the placentas of the same animals used for PLF assay, followed by hybridization with nick-translated PLF cDNA and autoradiography (19).

presence of 10 ng PLF. (With excess anti-PLF serum, ~50% of the input radioactivity was precipitated.) Each sample was measured in duplicate with a maximal deviation from the mean of 7%, and a standard curve was determined on each assay using purified PLF from 0.1–10 ng. PLF concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA) as described (15).

RNA purification and filter hybridization

Total RNA, prepared from guanidinium thiocyanate lysates of tissues, was electrophoresed on denaturing formaldehyde agarose gels, transferred to nitrocellulose, and hybridized, as described (16).

Results

Immunocytochemical localization of PLF

We first demonstrated that anti-PLF serum does not react with the structurally homologous hormones mPRL, mGH, and mPL I and II (2, 17, 18). It was shown previously that mPL I and II secreted by placental minces did not react with antiserum raised against nonglycosylated PLF (3). Similarly, anti-PLF serum did not react by Western blotting with purified mPRL, mGH, or mPL II; nor did anti-mPRL, anti-mGH, or anti-mPL II react with PLF (Fig. 1). Additionally, RIA using [^{125}I] PLF and anti-PLF serum was specific for PLF (Fig. 2).

To determine which cells of the mouse placenta contain PLF, placental sections were incubated with anti-PLF serum, and the sites of antibody binding were visualized as described in *Materials and Methods*. The predominant site of antibody localization was in the cytoplasm of trophoblastic giant cells (Fig. 3). No staining was seen with preimmune serum, nor with immune serum preincubated with purified PLF. However, preincubation of anti-PLF serum with comparable amounts of mPRL or mPL II had no effect on the detection of PLF. PLF was present in giant cells from day 10, 12, and 14 placentas, corresponding to the times at which placental PLF mRNA was most abundant (19).

Giant cells of the mouse placenta have been shown to produce mPL II (6). To find out whether the same cells produce both PLF and mPL II, adjacent sections of a day 12 placenta were stained after reacting with either anti-PLF or anti-mPL II. As seen in Fig. 4, individual giant cells contained both proteins.

Localization of PLF mRNA

To determine whether PLF was synthesized by trophoblastic giant cells, the distribution of placental PLF mRNA was analyzed by *in situ* hybridization with synthetic PLF antisense RNA, or (as a control) with sense RNA. As seen in Fig. 5, the mRNA probe was localized to a ring of cells corresponding to those found to contain PLF. At higher power these cells are seen to be trophoblastic giant cells.

PLF in serum and amniotic fluid

To learn whether PLF secreted by trophoblastic giant cells is found in the maternal circulation, sera from pregnant animals were analyzed by RIA at different times during pregnancy. PLF was readily detected in the serum of pregnant animals; the maximal concentration was found at day 10, coinciding with the peak of PLF mRNA in the same animals (Fig. 6). No PLF was found in the serum of male or nonpregnant female mice. In addition, the serum level of PLF correlated directly with the number of placentas in individual mice (Fig. 7), an observation consistent with a placental source for circulating PLF (20). PLF was also detected in amniotic fluid during fetal development (Fig. 8); its concentration was lower than that in maternal serum but followed a similar time course. Western blot analysis of serum indicated that circulating PLF has an electrophoretic mobility

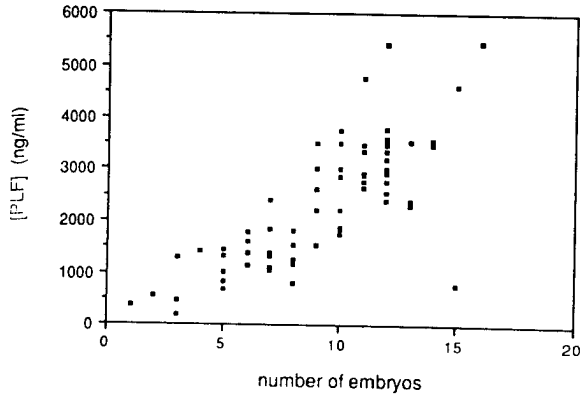


FIG. 7. Correlation between levels of serum PLF and litter size. PLF levels were measured by RIA in serum samples isolated from day 10 pregnant mice. Each *point* represents the average of duplicate measurements from the serum of 1 mouse. These data represent a composite of 2 experiments with 39 samples measured in 1 experiment and 29 samples measured in another experiment.

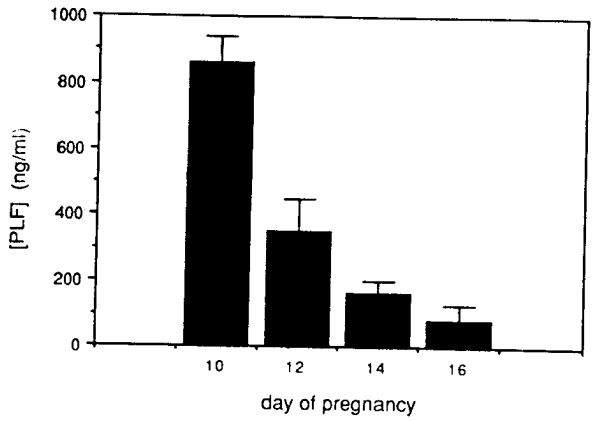


FIG. 8. Changes in PLF concentration in amniotic fluid during pregnancy. PLF levels in amniotic fluid isolated from two mice at each time point were measured by RIA and the data plotted as in Fig. 6. All measurements were obtained from a single set of RIAs.

similar to that of glycosylated PLF secreted by minced placenta (data not shown).

Discussion

Based on the relationship between PLF and PRL, the presence of PLF mRNA in mouse placenta, and secretion of PLF by minced placenta, it has been hypothesized that PLF is a placental hormone (2, 3, 19). Here we report two additional hormone-like characteristics of PLF: first, PLF is synthesized and stored in trophoblastic giant cells of the placenta, which are known to produce both steroids (21) and mPL II (6); and second, PLF is present in the serum of pregnant animals and in amniotic fluid. The peak serum concentration of PLF is substantially higher than that reported for mPRL, mGH, and mPL II (22, 23), but similar to that found for mPL I (24).

The trophoblastic giant cells of the placenta appear to be the source of circulating PLF. These cells are directly apposed to the maternal circulation (4, 5), and they are the only cells of the placenta in which PLF and PLF mRNA have been detected. Moreover, there is a correlation during pregnancy between the serum concentration of PLF and the level of PLF mRNA in the placenta. The serum concentration of PLF also correlates with the number of placentas in individual mice.

As noted earlier, another PRL-like hormone (mPL II) has been localized to trophoblastic giant cells (6). By assaying adjacent placental tissue sections for PLF or mPL II, we have shown that individual cells contain both proteins. Even though mPL II mRNA has not yet been localized, we take this finding to indicate that at least some giant cells can synthesize both hormones. Since other PRL-related proteins have recently been identified by analysis of cDNA clones from rodent placental mRNA (25, 26), it will be interesting to determine whether there is a single type of trophoblastic giant cell capable of synthesizing all the members of this hormone family.

Acknowledgments

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A one day post-graduate workshop will be held on Computers in Diabetes Management at the Delta Chelsea Inn, Toronto, Canada, on September 24/25, 1988. This hands-on workshop is for physicians and allied health professionals who are involved in diabetes care and are interested in updating themselves in computer assisted diabetes management.

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